SYNTHESIS AND PROPERTIES OF 8α-SUBSTITUTED RIBOFLAVINS OF BIOLOGICAL IMPORTANCE *

William C. KENNEY and Wolfram H. WALKER
Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121
and
Department of Biochemistry and Biophysics, University of California, San Francisco, California 94122, U.S.A.

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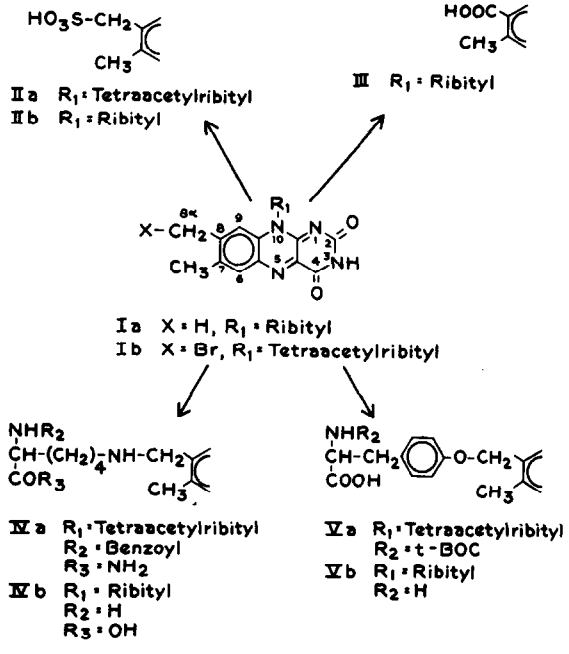
1. Introduction

Within the last 3 yr the structures of 2 types of flavin prosthetic groups covalently linked to proteins have been elucidated. In each case the co-enzyme is linked through the 8α-position of flavin (cf. scheme 1) to functional groups of amino acids. In the respiratory enzyme, succinate dehydrogenase, FAD is covalently linked to the imidazole N-3 nitrogen of histidine as determined by degradative studies and by chemical synthesis [1–4]. The amino acid sequence around the histidyl-8α-flavin has also been determined [5]. The prosthetic group of mitochondrial monoamine oxidase has been identified as flavin in thioether linkage to a cysteinyl peptide [6–9]. 8α-Cysteinyl-riboflavin was chemically synthesized [10]. Recently, a third novel covalently bound flavin prosthetic group was reported which was isolated from Chromatium flavocytochrome *c*-552 enzyme [11]. Hendriks and Cronin [12] showed that this flavin is probably FAD linked through the 8α-position of the flavin. This assignment was definitely established from the ESR hyperfine structure of the cation radical and by oxidation of the flavin with performic acid to 8-carboxy-riboflavin and evidence was obtained for the probable presence of sulfur in the immediate vicinity of the 8α-position [13, 14].



In view of the varieties of covalently bound flavins so far discovered, other types of flavins linked to functional groups other than in histidine and in cysteine may exist in nature. Their characteristics and properties are of considerable importance.

In this paper, the chemical syntheses and properties of 8α-(ε-*N*-lysyl)-riboflavin and 8α-(*O*-tyrosyl)-riboflavin (compounds IVb and Vb, respectively, scheme 1) are described and a comparison with previously characterized 8α-substituted flavins is made.

* Abbreviations: t–BOC = t-butyloxycarbonyl; Rfl = riboflavin; TA = tetraacetyl; FAD = flavin-adenine-dinucleotide; ESR = electron spin resonance.

Both the ε-amino group of lysine and the phenolic oxygen of tyrosine are possible sites for covalent linkage of flavins to proteins. This paper also describes the chemical synthesis of 8α-sulfonyl-riboflavin, a flavin derivative that may arise from performic acid oxidation of proteins containing flavin in disulfide linkage.

2. Materials and methods

Electrophoresis at pH 1. 6 (8% formic acid) and pH 3.5 (10% acetic acid/1% pyridine) was run at 50 V/cm. α-*N*-benzoyl-L-lysineamide acetate and *N*-t-BOC-L-tyrosine were purchased from Cyclo Chemical. 8α-Bromo-tetraacetyl-riboflavin (8α-Br-TARf1) [4] was synthesized according to a procedure kindly provided us by Drs. Ghisla and Hemmerich, University of Konstanz.

2.1. *8α-sulfonyl-riboflavin (IIb)*

3.3 mg (32 μmoles) sodium bisulfite and 10 mg (16 μmoles) 8α-Br-TARf1 (Ib) were incubated in 0.1 ml dimethylformamide under N_2 for 16 hr at 40°. The reaction mixture was evaporated at 60° in vacuo and separated by preparative electrophoresis at pH 1.6. The single anionic band was eluted to give 8α-sulfonyl-TARf1 (IIa, 3 μmoles, 19% yield). 8α-Sulfonyl-riboflavin (IIb) was obtained in quantitative yield by hydrolysis in 2 N HCl for 15 hr at 20°.

2.2. *8α-(ε-N-lysyl)-riboflavin (IVb)*

9.2 mg α-*N*-benzoyl-L-lysine amide acetate (30 μmoles) were heated in a sealed tube together with 10 mg (16 μmoles) 8α-Br-TARf1 (Ib) and 0.1 ml dimethylformamide for 15 hr at 80°. The reaction mixture was evaporated in vacuo at 60° and purified by preparative electrophoresis at pH 1.6. The cationic band was eluted with water: 1.4 μmoles of 8α-(α-*N*-benzoyl-ε-*N*-lysyl-amide)-TARf1 (IVa), 9% yield. 8α-(ε-*N*-lysyl)-RF1 (IVb) was obtained in high yield by hydrolysis of IVa in 6 N HCl at 80° for 15 hr.

2.3. *8α-(O-tyrosyl)-riboflavin (Vb)*

9.3 mg (32 μmoles) *N*-t-BOC-L-tyrosine were heated in a sealed tube together with 10 mg (16 μmoles) of 8α-Br-TARf1 (Ib) and 0.1 ml dimethylformamide for 15 hr at 80°. The reaction mixture was evaporated in vacuo and the t-BOC protective group eliminated with trifluoracetic acid. 8α-(*O*-tyrosyl)-TARf1 was isolated by preparative electrophoresis at pH 1.6. The cationic band yielded 0.8 μmoles (5% yield) flavin. 8α-(*O*-tyrosyl)-Rf1 (Vb) was obtained by hydrolysis in 2 N HCl for 15 hr at 20°.

3. Results and discussion

3.1. *8α-sulfonyl-riboflavin*

This compound (IIb, scheme1) was synthesized using the classical reaction of alkyl halides with sodium bisulfite. Some properties of this material are given in table 1. From the electrophoretic properties of IIb (table 1) it can be concluded that the pK_a of the sulfonic acid (ca. 0.5) is lower than that of cysteic acid. The flavin moiety appears to influence the pK_a of the 8α-substituted sulfonic acid, as it does for the ε-amino group of lysine in 8α-(ε-*N*-lysyl)-riboflavin (see below).

The second optical maximum of 8α-sulfonyl-riboflavin is at 364 nm (table 1). The position of this maximum may be compared with values of 367 nm for 8α-cysteinyl-riboflavin and 354 nm for the corresponding sulfone derivative (table 1 and [7, 10]). The expected hypsochromic shift upon oxidation of 8α-thio-riboflavin to the 8α-sulfonyl-riboflavin appears to be masked by a bathochromic shift as a result of increased electron density due to the negatively charg ed sulfonate group. As expected, the fluorescence of 8α-sulfonyl-riboflavin is not quenched with respect to riboflavin.

3.2. *8α-(ε-N-lysyl)-riboflavin (IVb)*

This compound has cationic properties at pH 3.4 and 1.6 (table 1) as expected for a secondary amine. A pK_a of 7.2 for the ε-amino group of IVb is determined by flavin fluorescence as a function of pH (fig. 1). As for 8α-histidyl-riboflavin, the fluorescence of flavin is quenched upon deprotonation of the basic function at the 8α-flavin position [1–4]. The pK_a of the ε-amino function is shifted to a more acidic value relative to lysine, which is also found on comparing 8α-histidyl-riboflavin with histidine [1–4] Fig. 2 presents the absorption spectrum of 8α-(α-*N*-benzoyl-ε-*N*-lysyl amide)-tetraacetyl-riboflavin (IVa). Protonation of the substituted ε-amino group results in a shift of the second λ_{max} from 355 nm (pH 9.2)

Table 1
Properties of 8α-substituted riboflavins.

8α-substituent	pH	λ max (nm)	Fluorescence (%)[b]	Electrophoretic mobility [a]	
				pH 1.6	pH 3.4
α-*N*-benzoyl-	5.0	445, 345, 268, 220	95	− 1.1	− 0.7
ε-*N*-lysylamide (IVa)	9.2	448, 355, 268, 220	11	–	–
ε-*N*-lysyl (IVb)	4.0	444, 343, 266, – –	100	− 2.2	− 0.9
	9.0	445, 357, 264, – –	10	–	–
O-tyrosyl (Vb)	6.0	449, 346, 268, 221	20	− 0.8	0
sulfonyl (IIb)	6.0	448, 364, 268, 222	100	+ 1.6	+ 1.1
carboxy (III)	6.0	446, 367, 267, 222	84	+ 0.2	+ 1.1
N-3-histidyl [c]	3.0	445, 345, 268, – –	100	− 1.3	–
	7.0	445, 355, 268, – –	10	–	–
S-cysteinyl [e]	7.0	448, 367, – –, – –	10	− 0.7	–
S-cysteinyl sulfone	7.0	448, 354, – –, – –	80 [e]	–	–
S-glutathione	7.0	448, 365, – –, – –	15	−0.8	–

a) Migration of flavin mononucleotide relative to riboflavin = + 1.0.
b) Relative to riboflavin = 100%.
c) From [3].
d) From [10].
e) From [7].

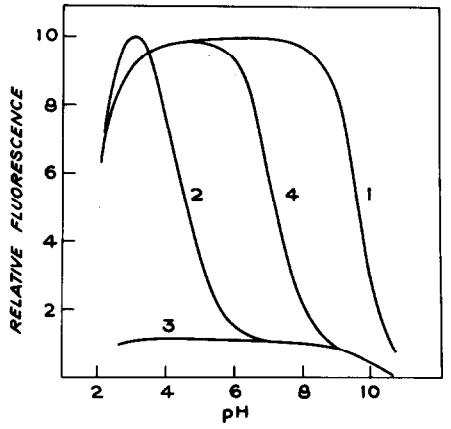


Fig. 1. pH dependence of the fluorescence of various flavins. 1) riboflavin; 2) 8α-histidyl-riboflavin [4]; 3) cysteine-peptide from monoamine oxidase [7]; 4) 8α-(α-*N*-benzoyl-ε-*N*-lysyl amide)-tetraacetyl-riboflavin (IVa).

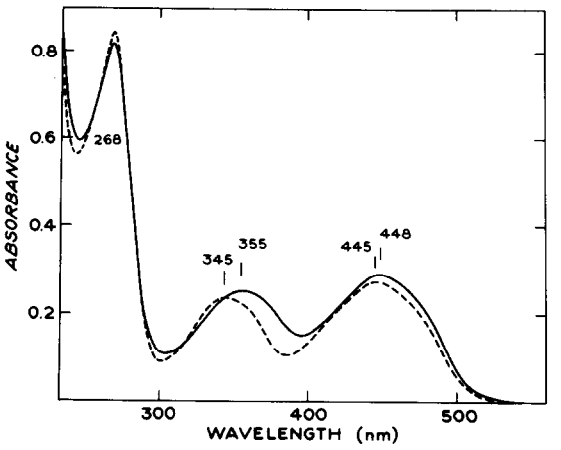


Fig. 2. Optical spectra of 8α-(α-*N*-benzoyl-ε-*N*-lysyl amide)-tetraacetyl-riboflavin (IVa), unbuffered solutions. (——): pH 9.2; (– –): pH 5.0. Both solutions contained the same concentration of flavin.

to 345 nm (pH 5.0). A similar optical shift of the second λ_{max} has been observed for 8α-histidyl-riboflavin [1–4]. A slight hypsochromic shift is also observed in the first λ_{max} (448 to 445 nm) together with a 5% decrease of the extinction coefficient at the respective λ_{max}.

Upon hydrolysis in 6 N HCl 8α-(ε-*N*-lysyl)-riboflavin (IVb) is obtained. As expected, the electrophoretic mobility at pH 1.6 increases after unblocking (table 1) and the material has the expected ninhydrin reaction typical for α-amino acids. Since the linkage of the flavin 8α-group to the ε-amino group of lysine appears to be more stable than a peptide bond, it would be possible to isolate 8α-(ε-*N*-lysyl)-riboflavin from crude peptide or protein mixtures by controlled acid hydrolysis. The properties described should facilitate the identification of this structure, if it should occur, in biological material.

8α-(ε-*N*-lysyl)-riboflavin is also satisfactorily synthesized from α-*N*-t-BOC-lysine. Reaction conditions are as described in Methods for the benzoylamide derivative; however, much milder unblocking conditions may be used (trifluoroacetic acid at room temp).

3.3. *8α-(O-tyrosyl)-riboflavin (Vb)*

The structure of this compound is established from its electrophoretic mobility (table 1), positive ninhydrin reaction, and fluorescence properties. 8α-(*O*-tyrosyl)-riboflavin has only 20% of the fluorescence of riboflavin (table 1). This is another example of a π electron donor in the 8α-position quenching the flavin fluorescence [3, 4]. As expected, no pH dependence of fluorescence is observed, and a pH-fluorescence curve similar to 8α-substituted flavin thioether (fig. 1) is obtained; i.e., IVb has a plateau from pH 3 to 9. In addition to the λ_{max} given in table 1, a shoulder is observed at 261 nm which arises from the tyrosine absorption. Unfortunately this material was not found to be stable under standard conditions of protein hydrolysis (6 N HCl, 110°, 15 hr). Deacetylation already results in some cleavage of the flavin tyrosine bond, as indicated by the appearance of tyrosine and of a neutral flavin compound on electrophoresis at pH 1.6 which has intense fluorescence. Therefore an *O*-tyrosyl-flavin, if present, may only be isolated from proteins by mild enzymatic hydrolysis of peptide bonds. A flavin peptide containing 8α-*O*-tyrosine-riboflavin should show one mole of tyrosine formation on hydrolysis but should not result in the formation of *O*-dansyl derivative upon mild reaction with 1-dimethylaminonaphthlene-5-sulfonyl-chloride.

3.4. *8-carboxy-riboflavin (III)*

This compound, previously characterized by McCormick [15] was obtained in modest yield by direct performic acid oxidation of 8α-Br-TARf1 (Ib). Some properties are given in table 1. A pK_a of the 8-carboxy group of 2.5 was determined. A characteristic shift in the second excitation maximum from 367 nm (pH > 3.5) to 345 nm (pH < 1.0) occurs.

The ESR spectra of various substituted flavins is given in fig. 3. Both carboxyflavin (III) and *O*-tyrosyl flavin (Vb) give similar hyperfine patterns. The same pattern (17 lines, 2.3 G spacing) is found for 8α-(S-cysteinyl)-riboflavin [6, 8, 10]. The spectrum (not presented) of sulfonyl-flavin (IIa) showed similarities with the above spectra, but was strongly asymmetric and indicated the presence of more than

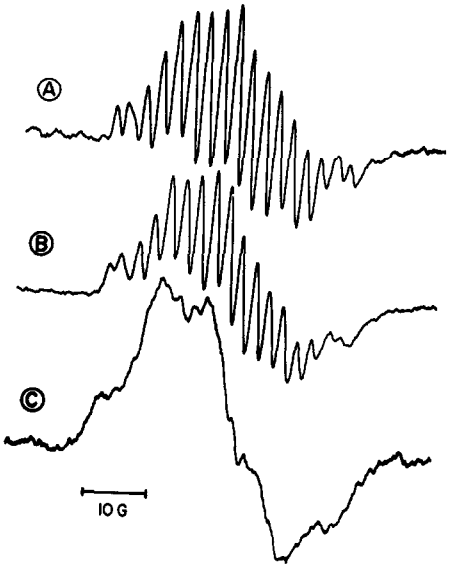


Fig. 3. ESR spectra of cation radicals of (A): 8-carboxy-riboflavin (III); (B): 8α-(*O*-tyrosyl)-riboflavin (Vb), (C): 8α-(α-*N*-benzoyl-ε-*N*-lysyl amide)-tetraacetyl-riboflavin (IVa). The flavins (1 mM) in 6 N HCl were reduced with $TiCl_3$ and measured at room temperature with a Varian E-3 spectrometer. Power: 0.5 mW, 1.25 G modulation amplitude, 100 KHz modulation frequency, 1 sec time constant, 8 min scanning time.

17 lines. On the other hand, ε-*N*-lysyl-flavin (IVa) does not show well resolved hyperfine lines and resembles the pattern of 8α-histidyl flavin [2, 3]. This appears to be a property of an ionizable nitrogen function in the 8α-position.

3.5. *8α-(S-glutathione)-riboflavin*

This compound was synthesized following the procedure for synthesis of 8α-(S-cysteinyl)-riboflavin of Ghisla and Hemmerich [10]. Characterization of structure was established by liberation of expected amino acids upon acid hydrolysis and by comparison of physical properties with 8α-(S-cysteinyl)-riboflavin and monoamine oxidase flavin peptides [8–10]. Some of these properties are given in table 1.

Acknowledgments

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