2′,5′-Anhydro-8α-histidylflavins: Their Formation and Structural Elucidation†

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ABSTRACT: Acid treatment of synthetic or naturally occurring 8α-substituted N^3- or N^1-histidylriboflavins results in the formation of histidylflavins with chromatographic properties differing from those of the respective parent compounds. Similarly, acid treatment converts riboflavin or flavin mononucleotide to an acid-modified flavin distinguishable from riboflavin. This compound is identical with the flavin produced by reductive Zn cleavage of acid-modified 8α-(N^3- or N^1-histidyl)riboflavin. Acid treatment modifies the flavin in the ribityl side chain, not in the isoalloxazine ring system, since riboflavin and acid-modified riboflavin have identical absorption, fluorescence, and electron paramagnetic resonance spectra and since both yield lumiflavin on alkaline irradiation. Riboflavin and its 8α-substituted adducts consume 3 mol of periodate with liberation of 1 mol of formaldehyde, whereas their acid-modified forms consume only 1 mol of periodate with no liberation of formaldehyde. Proton magnetic resonance and chemical ionization mass spectrometry spectral data provide confirming evidence that acid treatment of either synthetic or naturally occurring 8α-histidylflavins, riboflavin, or flavin mononucleotide results in the cyclization of the ribityl side chain to form the respective 2′,5′-anhydroflavin analogues. These results provide experimental evidence for the suggestion (Baddiley, J., Buchanan, J. G., and Carss, B. (1957), *J. Chem. Soc.*, 4058–4063) that acid treatment of riboflavin might cause formation of 2′,5′-anhydroriboflavin.

In the course of the structural elucidation of the covalently bound flavin of succinate dehydrogenase as 8α-(N^3-histidyl)-FAD (Walker et al., 1972), it was found that acid hydrolysis of both the naturally occurring flavin peptide and of synthetic 8α-(N^3-histidyl)riboflavin results in the conversion to a new histidylflavin the structure of which was assumed to be 8α-(N^1-histidyl)riboflavin. In other words, acid was suggested to cause a migration of riboflavin from the N^3 to the N^1 position of the imidazole ring. 8α-(N^1-Histidyl)FAD was subsequently shown to be present in thiamine dehydrogenase (Kenney et al., 1976a), β-cyclopiazonate oxidocyclase (Kenney et al., 1976c), and L-gulono-γ-lactone oxidase (Kenney et al., 1976b). 8α-(N^1-Histidyl)riboflavin isolated from these enzymes differed in several respects from the acid-modification product of 8α-(N^3-histidyl)riboflavin (Edmondson et al., 1976), showing that the structure assumed for the latter compound is incorrect. The same conclusion could be reached from the observation that exposure to acids modifies both the 8α-(N^3- and N^1-histidyl)riboflavin isomers without interconversion to one another. It was concluded that acid modification affects the ribityl side chain because on alkaline irradiation of the flavins obtained by reductive cleavage of either 8α-(N^1- or N^3-histidyl)riboflavin, or of their respective acid modification products, lumiflavin was obtained. The exact nature of the change in the ribityl chain still remained to be established, however.

As documented in this paper, acid treatment of riboflavin and FMN as well as their 8α-substituted derivatives results in the cyclization of the ribityl chain with accompanying dehydration to form 2′,5′-anhydroflavins. This structure was, in fact, predicted by Baddiley et al. (1957) many years ago, based on analogy with the effect of acids on ribitol and ribitol phosphates.

Experimental Section

8α-Histidylflavin Analogues. 8α-(N^3-Histidyl)riboflavin, 8α-(N^1-histidyl)riboflavin, and their respective acid-modified forms were synthesized and purified as previously described (Walker et al., 1972; Edmondson et al., 1976). The histidine moiety of 8α-histidylflavins was removed as in previous work (Edmondson et al., 1976) based on the original procedure of Walker et al. (1972). Cellulose impurities originating from electrophoresis filter paper were removed from 8α-histidylflavin preparations by repeated precipitation by diethyl ether from water–methanol (1:1 v/v) solutions.

Formation and Purification of Acid-Modified Riboflavin. The flavin (riboflavin or FMN, 150 mg) was dissolved in 15 mL of 3 N HCl and refluxed gently in the dark for 24 h. After removal of HCl in vacuo at 40 °C, the acid-modified riboflavin was separated from riboflavin and other hydrolytic products either by preparative descending paper chromatography on Whatman no. 3 filter paper (15 mg of flavin per 40 × 50 cm sheet; 1-butanol–acetic acid–water, 4:1:5 v/v, upper phase as solvent) or by column chromatography on silicic acid (3 × 32.5 cm column, chloroform–acetic acid–methanol, 18:1:1 v/v, as developing solvent). In both procedures, the acid-modified riboflavin migrates faster than does riboflavin. The material eluted was dried in vacuo at 40 °C, dissolved in a minimum volume of 3:1 v/v methanol–water, precipitated with 3 vol of diethyl ether at 0 °C, washed with ether, and dried in air. The yield of acid-modified riboflavin was around 15% starting with FMN and was only 3–5% with riboflavin. The yield of riboflavin recovered from the dephosphorylation of FMN or as unreacted riboflavin was 20–25% using either flavin as starting material.

Methods

Absorption spectra were recorded with a Cary 14 spectrophotometer, fluorescence spectra with a Hitachi MPF-3

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spectrofluorometer, and CD[1] and ORD spectra with a Jasco UV-5 (Sproul SS-10 modification) instrument and a Cary 60 spectropolarimeter, respectively. ^{1}H NMR spectra were measured with a Perkin-Elmer R-12B instrument, using 1:1 v/v ^2H_2O-[^{2}H]trifluoroacetic acid as solvent. EPR spectra were recorded with a Varian E-4 instrument under the conditions previously used (Edmondson et al., 1976). Chemical ionization mass spectrometry data were obtained with an AEI MS-9 instrument.

Riboflavin and its acid-modified form were trimethylsilylated by mixing the flavin (1 mg) with 1.0 mL of tetrahydrofuran, 0.2 mL of hexamethyldisilazine, 0.02 mL of trimethylchlorosilane, and 0.02 mL of pyridine (all freshly distilled and anhydrous). The reaction mixture was incubated 16 h in the dark at room temperature. After removal of precipitated NH_4Cl by centrifugation, the reaction mixture was washed four times with equal volumes of water, the combined water washes were extracted twice with equal volumes of chloroform, and the combined chloroform and tetrahydrofuran fractions were taken to dryness. TLC (system A, see below) showed a single spot, migrating with a much higher mobility than the respective starting materials.

Primary vicinal hydroxyl groups were quantitated by formaldehyde analysis after periodate treatment. The procedure used is a modification of that published by Frisell and Mackenzie (1958). To 0.4 mL of a flavin solution (0.125–0.25 mM), 0.1 mL of 0.1 M periodate was added and, after a 5-min incubation, the reaction was quenched by the addition of 0.1 mL of a 10% solution of sodium bisulfite. After 10 min the reaction mixture was diluted to 2.0 mL with water, a 0.4-mL aliquot was removed and mixed with 5.0 mL of chromotropic acid reagent. The color was developed by heating 30 min in a boiling water bath and the absorbance at 570 nm read after cooling. Serine and sorbitol solutions were used as standards. Commercial preparations of riboflavin used without purification gave 1.3–1.4 mol of formaldehyde per mol of flavin. After purification by benzyl alcohol extraction and reextraction into distilled water, following the addition of three vol of diethyl ether to the benzyl alcohol solution, the yield of formaldehyde was 1.0–1.1 mol per mol of flavin.

Periodate titers were determined as follows. A series of micro test tubes, containing identical quantities of flavin (17–20 nmol in 10 μL), were incubated with 10 μL of 0.01 M standardized periodate (Dyer, 1956) in the dark at 0 °C. The contents of individual tubes were diluted at periodic intervals to 3.0 mL and the absorbance read at 220 nm. Periodate consumption was calculated from the decrease in absorption at 220 nm.

TLC was carried out either in system A (silica gel plate, chloroform–acetic acid–methanol, 18:1:1 v/v) or in system B (cellulose plate, 1-butanol–acetic acid–water, 4:1:5 v/v, upper phase).

Results and Discussion

Evidence That Acid-Modified Flavins Are Altered Only in the Ribityl Side Chain. Our suggestion (Edmondson et al., 1976) that acid treatment modifies the ribityl side chain and not the isoalloxazine ring of 8α-histidylriboflavin was based on the following observations. Reductive cleavage of the N^1 and N^3 isomers of 8α-histidylriboflavin by Zn yields riboflavin, whereas the same treatment applied to their respective acid-modified forms yields a new flavin with the same absorption spectrum and fluorescence as riboflavin but differing from the latter in TLC. Alkaline irradiation of both this unknown flavin and of riboflavin yields lumiflavin, suggesting that the difference between the two compounds was in the ribityl side chain. No evidence has been published until now, however, demonstrating that acid modifies the side chain of riboflavin itself or of one of its nucleotides.

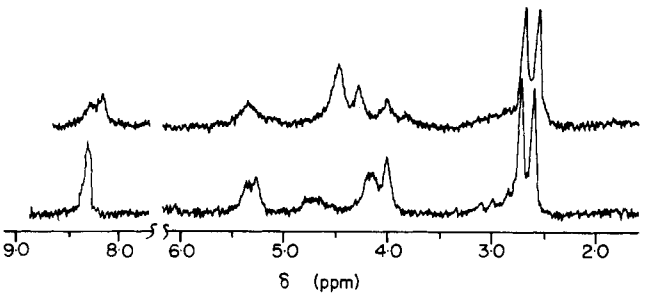

FIGURE 1: ^{1}H NMR spectrum of riboflavin (bottom spectrum) and of acid-modified riboflavin (top spectrum). The spectra were measured at 60 MHz at a flavin concentration of 25 mg/mL. Sodium 4,4-dimethyl-4-silapentanesulfonate was used as an internal standard.

Acid treatment of FMN or riboflavin results in the formation of acid-modified riboflavin with identical TLC properties as the flavin product from Zn cleavage of the acid-modified N^1 or N^3 isomers of 8α-histidylriboflavin (R_f = 0.44, system A; 0.58, system B). As expected, acid-modified riboflavin and riboflavin itself give identical absorption and EPR spectra, have the same fluorescence properties, and both yield lumiflavin but differ significantly in mobility in two TLC systems with respective R_f values of 0.44 and 0.14 (system A) and 0.58 and 0.48 (system B).

The foregoing observations are compatible with the hypothesis that the differences are in the ribityl side chain but does not unequivocally prove it. Direct evidence for this hypothesis comes from ^{1}H NMR data. Resonances due to the 7-CH_3 and 8-CH_3 protons are observed at the same positions as in the spectrum of riboflavin (Figure 1). A slight difference is found in the C^6 and C^9 protons between these two compounds: in the spectrum of riboflavin, both occur at 8.30 ppm whereas they are partially resolved in the spectrum of acid-modified riboflavin with chemical shifts of 8.30 and 8.19 ppm, respectively. The most significant observation bearing on acid modification in the spectral region of ribityl proton resonance (3–6 ppm) is markedly different for the two compounds (Figure 1), although integration showed that seven nonexchangeable ribityl protons are present in each compound. It may be concluded that acid treatment causes structural alteration of the ribityl side chain without affecting the number of *nonexchangeable* protons. This would, of course, preclude the breakage of carbon–carbon bonds as the mechanism of acid modification.

Periodate as a Probe for the Site of Acid Modification. The data above show that in two relatively apolar solvent systems used for TLC, the acid-modified flavin moved faster than riboflavin and that this increase in mobility was inversely related to the degree of solvent polarity (Experimental Section). This would suggest that acid-modified riboflavin is less polar than riboflavin. Dehydration of the ribityl side chain to a cyclic structure would be compatible with these observations as well as the ^{1}H NMR data showing that no loss of nonexchangeable protons occurs on acid treatment.

Evidence for this structure was sought from determination of the number of vicinal hydroxyl groups by periodate titration (Table I). Riboflavin reacted smoothly with 3 mol equiv of periodate in 60 min with no additional uptake up to 120 min.

[1] Abbreviations used are: CD, circular dichroism; ORD, optical rotatory dispersion; ^{1}H NMR, proton magnetic resonance; EPR, electron paramagnetic resonance; TLC, thin-layer chromatography; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

TABLE I: Stoichiometry and Formaldehyde Analysis in Periodate Reaction with Various Flavin Analogues.

Flavin	Mol of IO_4^-/mol flavin	No. of vicinal hydroxyl groups	Mol of HCHO/mol flavin	No. of primary hydroxyl groups
Riboflavin	3.02	4	1.07	1
Acid-modified riboflavin	1.02	2	0	0
8α-(N^3-Histidyl)riboflavin	2.62[a]	4	1.1	1
Acid-modified 8α-(N^3-histidyl)riboflavin	1.09	2	0	0
8α-(N^1-Histidyl)riboflavin	ND		1.03	1
Acid-modified 8α-(N^1-histidyl)riboflavin	ND		<0.1	0
Acid-modified 8α-(N^3-histidyl)riboflavin after reductive Zn cleavage	ND		0	0

[a] Due to the slow rate, the reaction may not have reached completion.

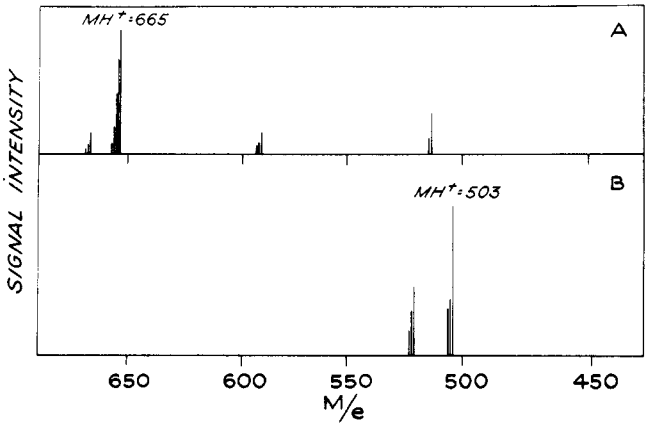

FIGURE 2: Chemical ionization mass spectra of trimethylsilylated derivatives of (A) riboflavin and (B) acid-modified riboflavin.

8α-(N^3-Histidyl)riboflavin reacted rapidly with 2 mol of periodate (15–20 min) and then more slowly with ~90% of the theoretical amount of periodate consumed after 160 min. Acid-modified 8α-(N^3-histidyl)riboflavin reacted rapidly with 1 mol of periodate (10 min) with no further consumption up to 50–60 min. A small, slow further uptake of 0.3–0.5 mol took place by 270 min with no further uptake up to 340 min. This secondary reaction is thought to be a nonspecific oxidation because of its slowness, as compared with the primary reaction. Acid-modified riboflavin reacted smoothly and rapidly (15 min) with 1 mol equiv of periodate and no further periodate consumption.

The uptake of approximately 3 mol of periodate by riboflavin and by 8α-(N^3-histidyl)riboflavin is as expected for 4 vicinal hydroxyl groups. In contrast, acid-modified riboflavin or 8α-(N^3-histidyl)riboflavin consumed 1 mol equiv of periodate showing the presence of 2 vicinal hydroxyl groups.

Analysis for formaldehyde arising from the oxidation of primary hydroxyl groups by periodate is in accord with the above data (Table I). Riboflavin and the N^1 and N^3 isomers of 8α-histidylriboflavin gave 1 mol of formaldehyde, whereas the acid-modified forms of these compounds as well as the flavin arising from reductive Zn cleavage of acid-modified 8α-(N^3-histidyl)riboflavin produced no formaldehyde and thus lacked a primary hydroxyl group. It may be concluded that

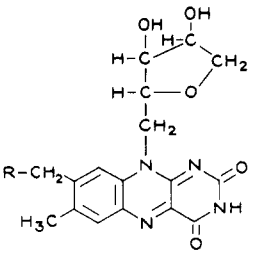

FIGURE 3: Structure of 2′,5′-anhydroriboflavin. R denotes either H or histidine (N^3 or N^1 isomer).

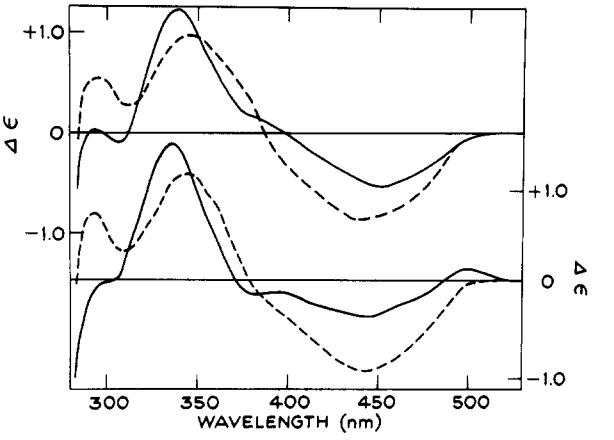

FIGURE 4: Circular dichroism spectra of flavin analogues. Top: (—) riboflavin and (- - -) 2′,5′-anhydroriboflavin in H_2O. Bottom: (—) 8α-(N^3-Histidyl)riboflavin and (- - -) 2′,5′-anhydro-8α-(N^3-histidyl)riboflavin in 1 mM sodium phosphate, pH 7.0.

dilute acid causes cyclization in the ribityl side chain of flavins between the 5′ primary hydroxyl group and a secondary ribityl hydroxyl. Since acid-modified riboflavin does not hydrolyze to form riboflavin under acidic or basic conditions and is not converted to formylmethylflavin on periodate treatment (as judged by ^1H NMR and TLC data), the 4′,5′-cyclic epoxide structure can be ruled out. Consideration of other possible structures leads to the conclusion that the secondary hydroxyl must be in the 2′ position in order to explain the observed periodate data.

Evidence from Mass Spectrometry. Trimethylsilylation of riboflavin results in the uptake of 6 mol of trimethylsilyl compound, 4 of which are on the ribityl side chain and are stable to hydrolysis and 2 on positions 2 and 4 of the isoalloxazine ring which are hydrolyzed during purification in aqueous media (Jorns et al., 1975). The molecular weight of the trimethylsilyl derivative of riboflavin containing 4 trimethylsilyl groups should be 664. The expected protonated peak (M + 1) at *m/e* 665 was indeed found in the chemical ionization mass spectrum (Figure 2). The expected value of M + 1 for trimethylsilylated acid-modified riboflavin is 503 if its structure is as discussed above. The data in Figure 2 verify this expectation and provide further evidence that acid-modified riboflavin is 2′,5′-anhydroriboflavin (Figure 3). This assignment verifies the prediction of Baddiley et al. (1957) and is in accord with their demonstration that acid dehydrates ribitol and ribitol phosphate to the 1,4-anhydro form.

CD and ORD Studies. Since the 2′ position of riboflavin is asymmetric, it was of interest to determine whether the cyclization occurred in a stereospecific or racemic manner. Baddiley et al. (1957) concluded from their studies on 1,4-anhydroribitol formation that the product was racemic. The same conclusion (i.e., that cyclization results in a racemic

mixture) was reached in the present study as regards riboflavin since ultraviolet ORD spectra of riboflavin and 2′,5′-anhydroriboflavin were essentially identical.

Interesting differences were noted, however, between the CD spectra of riboflavin and of 2′,5′-anhydroriboflavin and between those of 8α-(N^3-histidyl)riboflavin and of its 2′,5′-anhydro form (Figure 4). The upper part of Figure 4 shows a twofold increase in intensity of the negative dichroic band in the 450-nm region, a red shift in the positive band at 340 nm, and a substantial increase in positive intensity of the band at 295 nm upon cyclization of the ribityl side chain of riboflavin. Very similar differences are shown in the lower part of Figure 4 in the visible CD spectra of 8α-(N^3-histidyl)riboflavin and its 2′,5′-anhydro form. These data show that the side chains of riboflavin and 2′,5′-anhydroriboflavin interact with the isoalloxazine ring system in a different manner.

This difference in side chain interaction with the isoalloxazine ring is also observed in published ^{1}H NMR spectra of 8α-(N^3-histidyl)riboflavin and its 2′,5′-anhydro form (Walker et al., 1972)[2] and of 8α-(N^1-histidyl)riboflavin and its 2′,5′-anhydro form (Edmondson et al., 1976). In both studies, the position of the C^6 proton resonance is unaffected by cyclization. The position of the C^9 proton resonance is shifted downfield 0.17 ppm for the N^3 isomer and 0.37 ppm for the N^1 isomer upon side chain cyclization. This is as expected since the C^9 proton is on the same side of the isoalloxazine ring system as the ribityl chain whereas the C^6 proton is on the opposite side.

Although the mechanism of acid-catalyzed anhydroflavin formation from riboflavin or FMN remains to be established, some of the observations described here may provide a basis for future studies. Acid treatment of FMN or 8α-histidyl-FMN analogues results in a greater yield of 2′,5′-anhydroflavin than their respective riboflavin derivatives. Baddiley et al. (1957) have observed that anhydroribitol formation occurs more rapidly with ribitol phosphate than with ribitol itself; they however, did not compare the relative yields of 1,4-anhydroribitol from the two compounds. Taken together, these observations suggest either that dephosphorylation does not occur prior to the cyclization reaction or that the presence of inorganic phosphate (arising from hydrolysis of the phosphate ester) facilitates the reaction. Further work is required to distinguish which of these alternatives is correct.

Until now, it seems to have been widely assumed that riboflavin and its analogues are stable to treatment with acids, even at elevated temperatures. Procedures for the synthesis and analysis of flavins freely utilize acid treatments. It seems likely, at least in some instances, that such procedures led to the formation of the corresponding anhydro derivatives which was undetected because anhydroflavins have the same spectral and fluorescence properties as normal flavins. Since acid treatment under hydrolytic conditions has now been shown to cause dehydration and cyclization of the side chain of flavins, caution is indicated in interpreting data in the literature involving the use of such conditions during isolation or synthesis of flavins.

[2] The legend to Figure 6 in the paper of Walker et al. (1972) incorrectly assigns the two ^{1}H NMR spectra as judged from the results given in Table II of their manuscript and from comparison with ^{1}H NMR data obtained by the author. The upper spectrum in Figure 6 of their paper is that of 8α-(N^3-histidyl)riboflavin while the lower spectrum is that of 2′,5′-anhydro-8α-(N^3-histidyl)riboflavin.

Acknowledgments

The author acknowledges the excellent technical assistance of Mr. G. Trousdale and wishes to thank Dr. T. P. Singer for his critical reading of the manuscript. Special thanks are extended to Dr. J. T. Yang for the use of his CD and ORD instruments.

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