Biochemistry

Volume 15, Number 14 July 13, 1976

Structural Elucidation and Properties of 8α-(N^1-Histidyl)riboflavin: The Flavin Component of Thiamine Dehydrogenase and β-Cyclopiazonate Oxidocyclase†

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ABSTRACT: In addition to 8α-(N^3-histidyl)riboflavin, 8α-(N^1-histidyl)riboflavin is also formed during the reaction of N^{α}-blocked histidine with 8α-bromotetraacetylriboflavin in a yield of 20–25% of the total histidylflavin fraction. The properties of 8α-(N^1-histidyl]riboflavin are identical with those of the histidylflavin isolated from thiamine dehydrogenase and β-cyclopiazonate oxidocyclase but differ from those of 8α-(N^3-histidyl)riboflavin. These properties include pK_a of fluorescence quenching, electrophoretic mobility at pH 5.0, stability to storage, and reduction by $NaBH_4$. Proof for 8α substitution is shown by the electron paramagnetic resonance and electron–nuclear double resonance spectra of the cationic semiquinone form, as well as by the proton magnetic resonance spectrum of the oxidized form. The site of histidine substitution by the 8α-methylene of the flavin moiety was shown by methylation of the imidazole ring with methyl iodide, cleavage of the methylhistidine–flavin bond by acid hydrolysis at 150 °C, and identification of the methylhistidine isomer by electrophoresis. 3-Methylhistidine is the product from the N^1-histidylflavin isomer, while 1-methylhistidine is produced from the N^3 isomer. The flavin product from reductive Zn cleavage of either isomer has been identified as riboflavin. The compound obtained on acid treatment of 8α-(N^3-histidyl)riboflavin (previously thought to be the N^1 isomer) differs from the parent compound only in the ribityl side chain, since chemical degradation studies show 1-methylhistidine as a product and a flavin product which differs from riboflavin only in mobility in thin-layer chromatography, but not in absorption, fluorescence, and electron paramagnetic resonance spectral properties. Proof that acid modification involves only the ribityl chain has come from the observations that alkaline irradiation of this flavin yields lumiflavin, that the proton magnetic resonance spectrum of the compound differs from that of riboflavin in the region of the ribityl proton resonance, and that its periodate titer is lower than that of authentic riboflavin. The identity of 8α-(N^1-histidyl)riboflavin with the histidylflavin from thiamine dehydrogenase and β-cyclopiazonate oxidocyclase shows that both isomeric forms of 8α-histidylflavin occur in nature.

Previous studies in this laboratory (Kenney et al., 1974a,b) have shown that the covalently bound flavin moieties of thiamine dehydrogenase and β-cyclopiazonate oxidocyclase differ in properties from all three known classes of covalently bound flavins and thus constitute a fourth type of covalent flavin structure (for recent reviews of covalently bound flavins see Singer and Edmondson, 1974 and Singer and Kenney, 1974). Flavin peptides derived from either enzyme may be degraded by acid hydrolysis to a histidylflavin that differs from 8α-(N^3-histidyl)riboflavin (Walker et al., 1972) in being considerably more labile and in having an imidazole pK_a of about 5.2, 0.5 pH unit higher than the known compound. This new type of histidylflavin also differed in pK_a and in stability from a model compound that was then thought to be 8α-(N^1-histidyl)riboflavin and that had been described earlier (Walker et al., 1972) as an acid modification product of the N^3 isomer.

In order to elucidate the structure of this new type of histidylflavin, it was desirable to obtain milligram quantities of material, more than could be obtained by isolation from enzymes. When a synthetic preparation of 8α-(N^3-histidyl)riboflavin was subjected to high-voltage electrophoresis at pH 5.0, a histidylflavin was isolated, which amounted to 20–25% of the total histidylflavin fraction. This material, available in the desired amounts, has been found to be identical in every respect with the histidylflavin component of thiamine dehydrogenase and β-cyclopiazonate oxidocyclase. As documented in this paper, the structure of this flavin is 8α-(N^1-histidyl)-riboflavin. The structure of the acid modification product of 8α-(N^3-histidyl)riboflavin, previously thought to be the N^1

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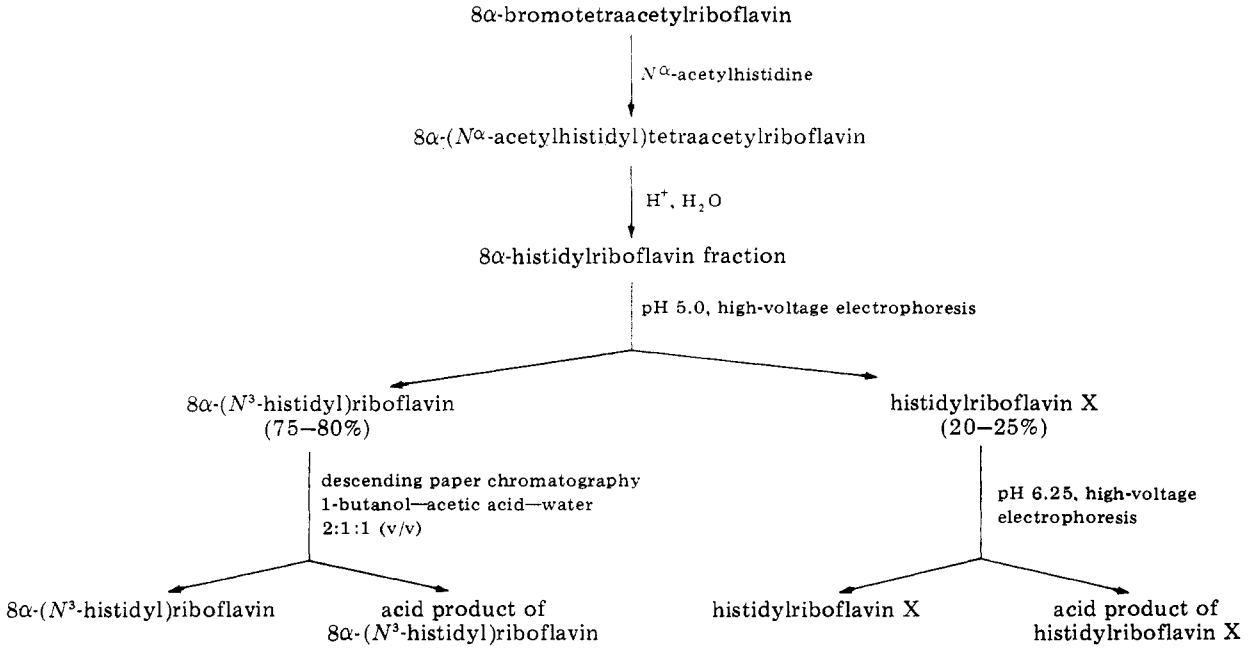
Scheme I: Isolation of Synthetic Histidylriboflavin X.



isomer, has not been fully elucidated, although acid modification has been shown to alter the ribityl side chain, rather than the histidylflavin linkage.

Experimental Section

Flavin Analogues. 8α-Bromotetraacetylriboflavin was synthesized and condensed with N^{α}-acetylhistidine, as described by Walker et al. (1972). The protecting groups were removed by hydrolysis in 6 N HCl at 100 °C following the course of the reaction by TLC[1] (system A, see below). As seen in Scheme I, the isolation of 8α-(N^3-histidyl)riboflavin and 8α-(N^1-histidyl)riboflavin from the reaction mixture was accomplished by preparative high-voltage electrophoresis at pH 5.0, 40 V/cm, 2 h. Separation of the acid-modified histidylflavins from the unmodified N^3 and N^1 histidylflavins involved descending paper chromatography in 1-butanol–acetic acid–water (2:1:1, v/v) and electrophoresis at pH 6.25, 40 V/cm, 2.5 h, respectively (Scheme I).

Because of the tendency of 8α-(N^1-histidyl)riboflavin to break down to 8-formylriboflavin in neutral, aqueous solution, all solutions of this compound were kept frozen in 5% acetic acid when not in use. Purity was routinely monitored by TLC (system B, see below) or by high-voltage electrophoresis at pH 5.0.

Lumiflavin was a gift from Dr. S. Ghisla, University of Konstanz, Germany. 8-Formylriboflavin was synthesized according to the procedure of McCormick (1970). 8α- and 7α-methylriboflavins were gifts from Dr. J. Lambooy, University of Maryland.

Chemical Degradation Studies. For studies involving cleavage of the flavin–histidine bond, the following procedure was followed. The histidylflavin (1 mM) was acetylated by incubation with 100 mM acetic anhydride in 0.2 M *N*-ethylmorpholinium acetate buffer, pH 8.0, at room temperature for 1 h. The solvents were removed in vacuo. Methylation of the imidazole ring was achieved by incubation of the acetylated histidylflavin with 0.1 ml of methyl iodide in 0.3 ml of dimethylformamide. The reaction was complete when the fluorescence at pH 7.0 and 3.3 became equal (about 60 h). Excess solvent and methyl iodide were then removed in vacuo. In some experiments the methylhistidylflavin was purified after deacetylation, prior to cleavage, by high-voltage electrophoresis in pyridine acetate buffer, pH 6.25 (30 V/cm, 2 h). The cationic flavin band was eluted with water and lyophilized.

Reductive cleavage of the methylhistidine–flavin bond was performed at 100 °C in 9:1 (v/v) glacial acetic acid–trifluoroacetic acid by adding ~1-mg portions of Zn dust three times over a 45 min period. After a twofold dilution with water, the acetylated flavin was isolated by $CHCl_3$ extraction. The acetylated flavin could be readily deacylated by incubation at 38 °C for 1 h in 6 N HCl. This procedure was used for identification of the flavin moiety. The methylhistidine–flavin bond could also be cleaved by hydrolysis in 6 N HCl at 150 °C for 24 h or longer, resulting in a good yield of the methylhistidine but complete destruction of the flavin.

Methylhistidines were identified by their ninhydrin color after high-voltage electrophoresis at pH 6.25, 50 V/cm, 60 min, while flavins were identified by TLC. Conversion of the flavins liberated by Zn to their lumiflavin form was achieved by alkaline irradiation (Koziol, 1971). The lumiflavin formed was identified by TLC.

Methods. EPR spectra were recorded on flavin samples dissolved in 88% formic acid under argon in glass capillaries after reduction with $TiCl_3$. Spectra were recorded with a Varian E-4 instrument at room temperature (microwave frequency, 9.07 GHz; modulation frequency, 100 kHz; microwave power, 3 mW; and modulation amplitude, 0.5 G). Absorption spectra were recorded with a Cary 14 spectrophotometer and corrected fluorescence spectra with a Hitachi MPF-3 spectrofluorometer. ^1H NMR spectra were measured at Varian Instruments, Palo Alto, Calif. on a 300-MHz Fourier transform spectrometer in 100% D_2O, using sodium 4,4-dimethyl-4-silapentanesulfonate as an internal standard. ENDOR spectra were also recorded at Varian Instruments on the flavin cationic semiquinone form (produced by $TiCl_3$ reduction) in 6 N HCl under the following conditions: temperature, −160

[1] Abbreviations used are: ENDOR, electron–nuclear double resonance; EPR, electron paramagnetic resonance; ^1H NMR, proton magnetic resonance; TLC, thin-layer chromatography; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

°C; microwave frequency, 9.21 GHz; microwave power, 3 mW; modulation frequency, 34.7 Hz; modulation amplitude, 40 G.

Oxidation–reduction potentials and sulfite affinities were measured as previously described (Edmondson and Singer, 1973). TLC system A was: silica gel plate, 1-butanol–acetic acid–H_2O (2:1:1, v/v); system B was: cellulose plate, 1-butanol–acetic acid–H_2O (4:1:5, v/v) (upper phase). Other solvent systems used are described in the legends of the figures.

Results

Properties of Different Forms of Histidylriboflavin. Until relatively recently two forms of histidylriboflavin were thought to exist: 8α-(N^3-histidyl)riboflavin, orginally isolated from succinate dehydrogenase and subsequently synthesized (Walker et al., 1972), and its acid modification product, which has been thought to be 8α-(N^1-histidyl)riboflavin (Walker et al., 1972). While the structure of the former has been unambiguously established, that of the acid modification product has not. Evidence presented later in this paper shows that this compound is, in fact, an N^3 derivative, in which the ribityl portion is modified, rather than the site of attachment of the flavin to the imidazole ring.

Subsequent studies on the covalently bound FAD component of thiamine dehydrogenase and of β-cyclopiazonate oxidocyclase (Kenney et al., 1974a,b) brought to light the existence of another type of histidyl riboflavin. Linkage of the flavin to the imidazole ring of histidine in flavin peptides isolated from these two enzymes was first indicated by the characteristic quenching of the fluorescence at pH values above 3.4 and later proven by the demonstration that the aminoacyl flavin, derived from these flavin peptides, liberated 1 mol of histidine on drastic acid hydrolysis.

There were significant differences in properties, however, between the flavin component of thiamine dehydrogenase or of β-cyclopiazonate oxidocyclase and of 8α-(N^3-histidyl)riboflavin, obtained synthetically or isolated from natural sources. The first difference noted was that the pK_a of the imidazole, as measured by the variation in the quantum yield of fluorescence with pH, is some 0.5 pH unit higher in the histidylriboflavin (or histidyl FMN) isolated from thiamine dehydrogenase and β-cyclopiazonate oxidocyclase than in authentic 8α-(N^3-histidyl)riboflavin (Kenney et al., 1974a,b). While this difference is small, it suffices for complete separation of the two compounds by electrophoresis at pH 5.0 (Scheme I). The compound also differed in its pK_a from the acid-modified form of 8α-(N^3-histidyl)riboflavin. This new form of histidylriboflavin will be referred to as histidylriboflavin X in the present paper and its structure will be shown to be 8α-(N^1-histidyl)riboflavin.

In our preliminary reports on histidylriboflavin X the pK_a of the imidazole nitrogen was reported to be 5.1 ± 0.1 in the aminoacylflavin derived by acid hydrolysis (6 N HCl, 95 °C) of the flavin peptide from thiamine dehydrogenase and 5.0 ± 0.1 in the compound similarly obtained from β-cyclopiazonate oxidocyclase (Kenney et al., 1974a,b). It was not recognized at the time that histidylriboflavin X, like the N^3 isomer of histidylriboflavin, can undergo acid modification and, therefore, that preparations derived by acid hydrolysis of flavin peptides contain varying amounts of acid-modified histidylriboflavin X. Following separation of this acid modification product by high-voltage electrophoresis at pH 6.25 (Scheme I), the true pK_a of histidylriboflavin X is found to be 5.2, while that of its acid modification product 5.0 (Figure 1A). A pK_a of 5.0–5.1 was also found in preparations obtained by acid hydrolysis from thiamine dehydrogenase, which contained 80–90% acid-modified histidylriboflavin X, while a separate sample of the compound, hydrolyzed under conditions leading to little or no acid modification, gave a pK_a of 5.25 (Figure 1A). As expected for a one proton ionization, plots of $[FH^+]/[F]$ vs. $[H^+]$ are linear (Figure 1B).

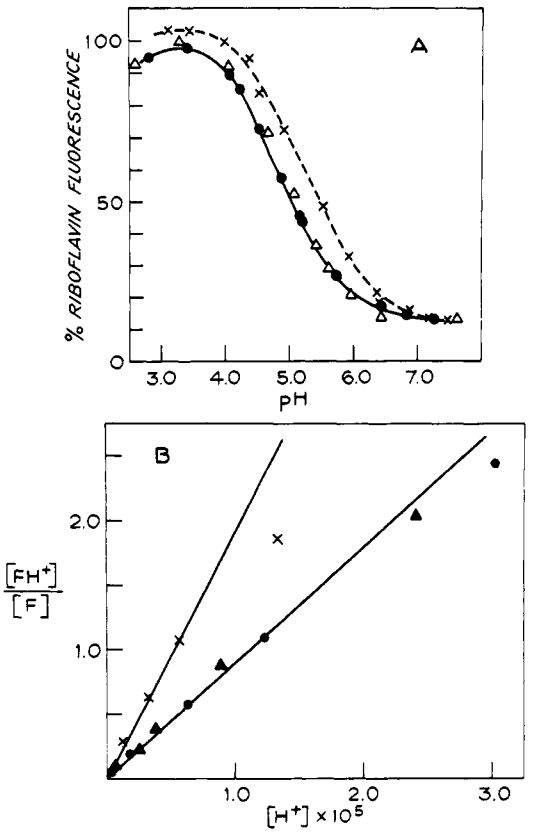


FIGURE 1: (A) pH–fluorescence profiles of histidylriboflavin X and of its acid modification product. Symbols: (●) acid-modified histidylriboflavin X; (△) histidylflavin obtained from thiamine dehydrogenase after acid hydrolysis (6 N HCl, 95 °C, 16 h) of the flavin peptide; (x) unmodified histidylriboflavin X obtained synthetically or from careful acid hydrolysis of the flavin peptide from thiamine dehydrogenase. The fluorescence values were measured at the 445-nm corrected excitation peak, with the emission wavelength at 525 nm, in 1 mM sodium citrate–sodium phosphate. The pH was adjusted by the addition of 6 N HCl or 6 N NaOH. (B) Graphical determination of the pK_a values from the data of A. The symbols are the same as in Figure 1A, with the exception that the histidylflavin from thiamine dehydrogenase is denoted by (▲).

The difference of 0.2 pH unit in the pK_a values of histodylriboflavin X and its acid modification product equals that reported by Walker et al. (1972) to distinguish 8α-(N^3-histidyl)riboflavin from its acid-modified form (pK_a = 4.7 vs. 4.5). Another similarity to 8α-(N^3-histidyl)riboflavin (Walker et al., 1972) is that deprotonation of histidylriboflavin X brings about a shift in the near-ultraviolet absorption band of the flavin from 344 to 355 nm, with an isosbestic point at 350 nm. Calculations of the pK_a value of the imidazole in histidylriboflavin X from absorption difference spectra gave a value identical with that calculated from the pH dependence of fluorescence quenching, showing that deprotonation of the flavin in the ground state is the cause of the lower fluorescence yield. This is in accord with the interpretation of similar data reported for the N^3 isomer (Salach et al., 1972).

In addition to the pK_a differences discussed above, histidylriboflavin X differs from 8α-(N^3-histidyl)riboflavin in reactivity with $NaBH_4$. While the latter is unaffected by treatment with $NaBH_4$, the former is reduced to a form whose

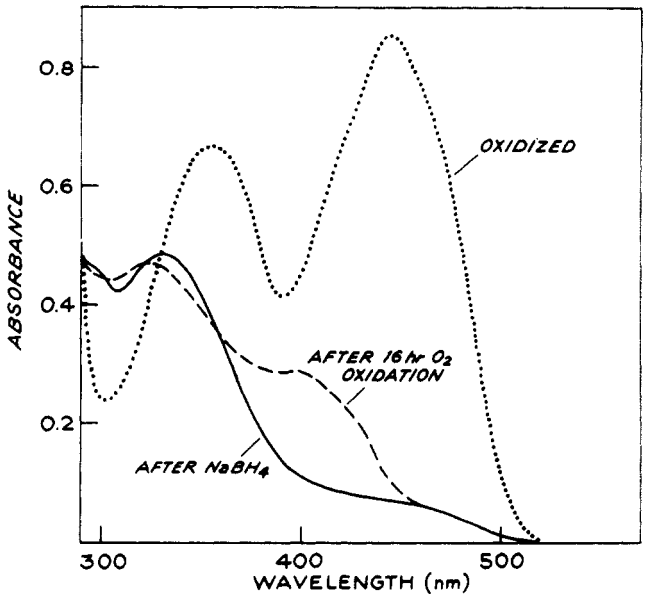


FIGURE 2: Effect of $NaBH_4$ on the absorption spectrum of histidylriboflavin X. The flavin was dissolved in air-saturated, 25 mM sodium phosphate, pH 7.0, at room temperature. After recording the spectrum of the reduced form, the sample was aerated, and allowed to remain in air-saturated buffer for 16 h in the dark before recording the spectrum given by the dashed curve.

spectral properties are shown in Figure 2. This reduced form does not react rapidly with oxygen, since no fluorescence was observed at either pH 3.3 or 7.0 when the reduced compound was diluted with 30 volumes of air-saturated buffers. Upon exposure to air overnight, the intensity of the absorbance at 400 nm increased, suggesting a partial transformation to the 3,4-dihydro form (Figure 2). The 3,4-dihydro forms of lumiflavin and of riboflavin have been shown to have absorption maxima at 400 nm (Müller et al., 1969). As will be documented elsewhere, the flavin peptides from thiamine dehydrogenase and β-cyclopiazonate oxidocyclase are also reduced rapidly by $NaBH_4$, while that from succinate dehydrogenase is not.

The third major difference between histidylriboflavin X and (N^3-histidyl)riboflavin is stability. While the latter compound may be stored over prolonged periods at room temperature, with no evidence of breakdown (Table I), the former undergoes extensive decomposition on storage in neutral, aqueous solution at room temperature. This decomposition may be followed by high-voltage electrophoresis at pH 5.0, since, as will be documented later, the decomposition product of the flavin moiety is 8-formylriboflavin, which does not migrate at this pH. The compound is somewhat more stable at -10 °C in neutral solution, but significant decomposition has also been observed at this temperature over a period of a few weeks.

These and some additional differences between histidylriboflavin X and 8α-(N^3-histidyl)riboflavin are summarized in Table I.

^1H NMR Studies. As mentioned above, early in the course of this study it was ascertained that histidylriboflavin X differs in the pK_a of fluorescence quenching from both 8α-(N^3-histidyl)riboflavin and from its acid modification product that was thought to be 8α-(N^1-histidyl)riboflavin at the time. One possibility that arose (Kenney et al., 1974a) was that the flavin might be attached to C-2 or C-4 of the imidazole ring.

This possibility was ruled out when Dr. A. Schonbrunn, Brandeis University, kindly examined a sample of the synthetic compound by ^1H NMR and found that both the C-2 and C-4 protons were present in the compound. The same ^1H NMR data also seemed to indicate, however, the presence of a single proton, rather than the expected two, on the 8α carbon of the flavin. This observation appeared to suggest that histidylriboflavin X is an adduct of histidine and 8-formylriboflavin, analogous to the covalently bound flavin thiohemiacetal of *Chromatium* cytochrome c_{552} (Walker et al., 1974).

Some evidence for this structure came from the observation that histidylriboflavin X yields 8-formylriboflavin on storage at room temperature (Table I) or, much more rapidly, on refluxing aqueous solutions in the dark for 8 h. 8-Formylriboflavin was identified in these experiments by TLC in system B, by its characteristic cationic hydroquinone absorption spectrum (Figure 3), and by the EPR spectrum of its cationic semiquinone (Figure 4, curves C, D).

Although these observations were compatible with the contemplated histidylhydroxyriboflavin structure (Singer and Kenney, 1974; Singer and Edmondson, 1974) optimally resolved ^1H NMR spectra were needed to confirm the preliminary data which had suggested the presence of one proton on

TABLE I: Comparison of the Properties of 8α-(N^1-Histidyl)riboflavin with Those of 8α-(N^3-Histidyl)riboflavin.

Property	8α-(N^1-Histidyl)-riboflavin[a]	8α-(N^3-Histidyl)riboflavin
pK_a of imidazole	5.2	4.7[b]
pK_a of imidazole acid-modified product	5.0	4.5[b]
Oxidation-reduction potential ($E_{m,7}$)	−165 mV	−160 mV[c]
K_d for sulfite adduct formation	0.087 M	0.056 M[c]
Second-order rate constant for sulfite adduct formation	1.64 M^{-1} min^{-1}	1.94 M^{-1} min^{-1} [c]
Reduction by BH_4^-	Yes	No
Electrophoretic mobility at pH 5.0 (FMN = +1)	−1.1	−0.88
Stability to storage in H_2O (30 days at room temperature in the dark)	80% decomposed to 8-formylriboflavin	97% recovery of original material

[a] This compound is referred to as histidylriboflavin X in the text. [b] Data of Walker et al. (1972). [c] Data of Edmondson and Singer (1973).

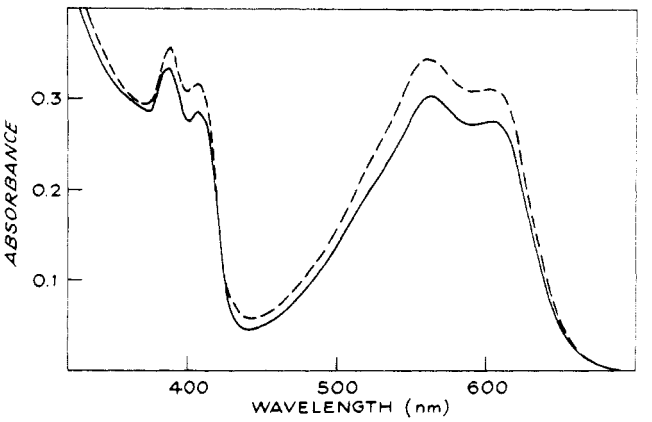


FIGURE 3: Comparison of the absorption spectra of the cationic hydroquinone form of the flavin arising from breakdown of histidylriboflavin X and of 8-formylriboflavin. Histidylriboflavin X was refluxed for 8 h in water and the flavine product separated by high-voltage electrophoresis at pH 5.0. 8-Formylriboflavin (- - -) and the product of breakdown of histidylriboflavin X (—) were dissolved in 6 N HCl and reduced with 1 mM $TiCl_3$.

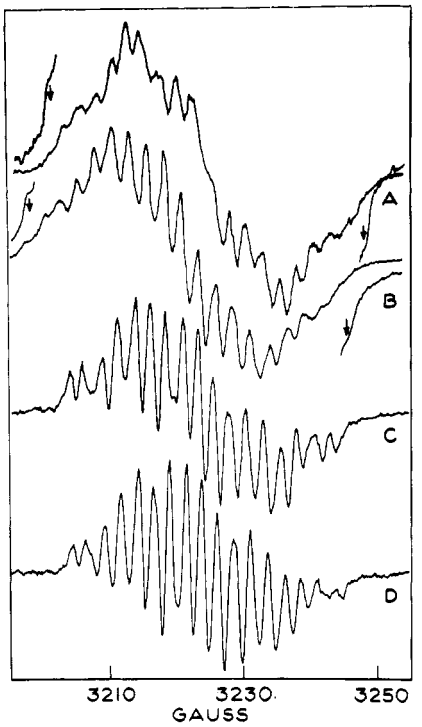


FIGURE 4: EPR spectra of the cationic semiquinone forms of (A) 8α-(N^3-histidyl)riboflavin, (B) histidylriboflavin X, (C) 8-formylriboflavin, and (D) the degradation product from histidylriboflavin X. All flavin concentrations were 5 mM in 88% formic acid.

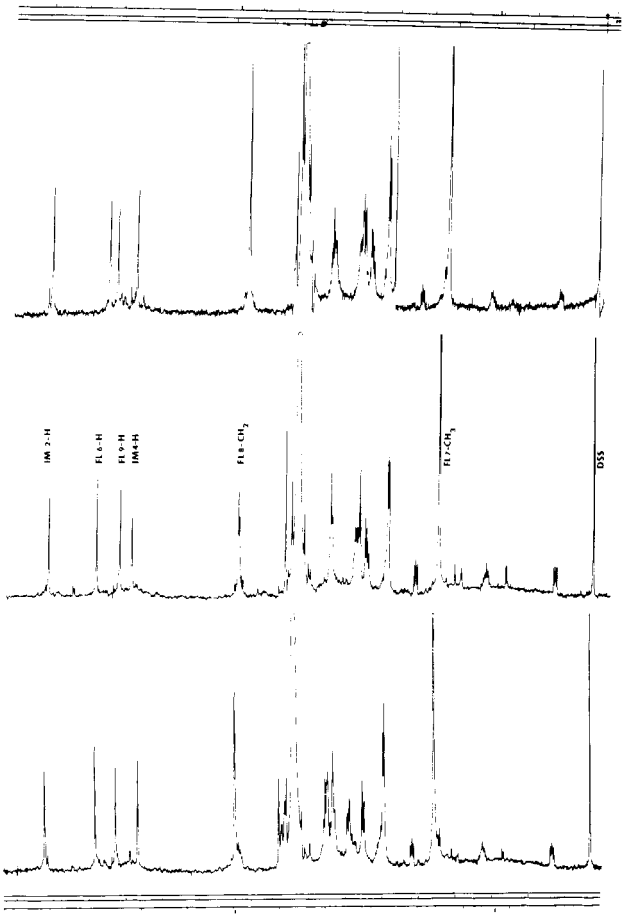


FIGURE 5: ^{1}H NMR spectra at 300 MHz, Fourier transform, of histidyl flavins. The flavins were dissolved in D_2O at 13 mM concentration. Top spectrum, 8α-(N^3-histidyl)riboflavin; middle spectrum, histidylriboflavin X; bottom spectrum, acid-modified histidylriboflavin X. The chemical shifts for the protons of the various groups in ppm are as follows: 8α-(N^3-histidyl)riboflavin, Fl(7)-CH_3, 2.48; Fl(8)-CH_2-, 5.75; Im(4)-H, 7.56; Fl(9)-H, 7.87; Fl(6)-H, 8.0; and Im(2)-H, 8.94; histidylriboflavin X, Fl(7)-CH_3, 2.58; Fl(8)-CH_2-, 5.80; Im(4)-H, 7.53; Fl(9)-H, 7.39; Fl(6)-H, 8.10; Im(2)-H, 8.88; acid-modified histidylriboflavin X, Fl(7)-CH_3, 2.58; Fl(8)-CH_2-, 5.80; Im(4)-H, 7.39; Fl(9)-H, 7.76; Fl(6)-H, 8.10; Im(2)-H, 8.93. The proton resonance peaks from the histidyl and flavin side chains are located in the area between the HDO peak and the Fl(7)-CH_3 peak.

the 8α carbon. Figure 5 compares the 300 MHz spectra of 8α-(N^3-histidyl)riboflavin (upper trace), histidylriboflavin X (middle trace), and its acid modification product (bottom trace).

Particularly striking is the splitting of the 8-methylene protons in histidylriboflavin X, which is not seen in the other two compounds. This geminal coupling is small (6 Hz at 300 MHz) and was, therefore, not observed with an 80 MHz instrument. The interpretation of this coupling is given in the Discussion.

Table II presents the results of mechanical integration of the areas under the peaks in the three spectra of Figure 5, using the value for 3.0 for the number of protons in the 7-CH_3 peak as a reference. The data proved unambiguously the presence of two protons in the 8α position of histidylriboflavin X, eliminating further consideration that its structure is 8α-hydroxy-8α-histidylriboflavin.

Proof of Site of Attachment of Histidine. Having demonstrated the nonidentity of histidylriboflavin X with either 8α-(N^3-histidyl)riboflavin or the compound thought to be its N^1 isomer, and having eliminated a higher oxidation state of the 8α carbon or attachment of the flavin to a carbon, rather than a nitrogen, of the imidazole as the basis of the difference, it became essential to examine the evidence that the histidine is indeed linked to the 8α carbon of the flavin, the site of attachment of all other covalently bound flavins to their proteins (Singer and Edmondson, 1974). Up to this time the evidence for 8α attachment in histidylriboflavin X rested on the hypsochromic shift of the second band of the fluorescence excitation and of the absorption spectra of the compound (Kenney et al., 1974a,b; Singer and Edmondson, 1974).

The possibility was considered that substitution might be on the 7α-CH_3 group. This could arise, in the case of the synthetic compound, if the 8α-bromotetraacetylriboflavin sample contained 20–25% of 7α-bromotetraacetylriboflavin. On condensation with histidine one would obtain an adduct analogous to 8α-(N^3-histidyl)riboflavin but substituted in the 7α position. Some of the data available seemed to argue against this possibility, but not in a conclusive way. Thus, the EPR spectra of the cation radicals of 8α-(N^3-histidyl)riboflavin and of histidylriboflavin X are identical (Figure 4) and differ from that of riboflavin, suggesting that appreciable spin density occurs in the position of the flavin which is substituted. It has been emphasized (Müller et al., 1971; Salach et al., 1972) that the spin density of the C-7 is very low. However, the possibility could not be ruled out that the presence of a bulky histidyl residue on the 7α carbon altered this spin density. Evidence has also been presented that histidylriboflavin X breaks down to 8-formylriboflavin. Since the TLC properties and the reduced spectrum of 7-formylriboflavin are not known, the possibility remains that the techniques used do not distinguish between 7- and 8-formylriboflavins. Further in the ^{1}H NMR spectrum of histidylriboflavin X (Figure 5) the peak at 2.58 ppm, nearest to DSS peak, is denoted as 7-CH_3 and is assumed in Table II to represent three protons. This assignment, however, is based on analogy with the ^{1}H NMR spectrum of 8α-(N^3-histidyl)-

TABLE II: Integration of Flavin and Imidazole Protons of Synthetic Histidyl Flavins.

Flavin	Fl(7)-CH_3	Fl(8)-CH_2	Fl(6)-H	Fl(9)-H	Im(4)-H	Im(2)-H
8α-(N^3-Histidyl)riboflavin	3.0	2.0	0.8	0.6	0.6	0.9
8α-(N^1-Histidyl)riboflavin[a]	3.0	1.8	0.8	0.9	0.7	0.9
Acid modification product of 8α-(N^1-Histidyl)riboflavin[a]	3.0	2.1	0.7	0.6	0.7	0.8

[a] In the text, 8α-(N^1-histidyl)riboflavin is referred to as histidylriboflavin X.

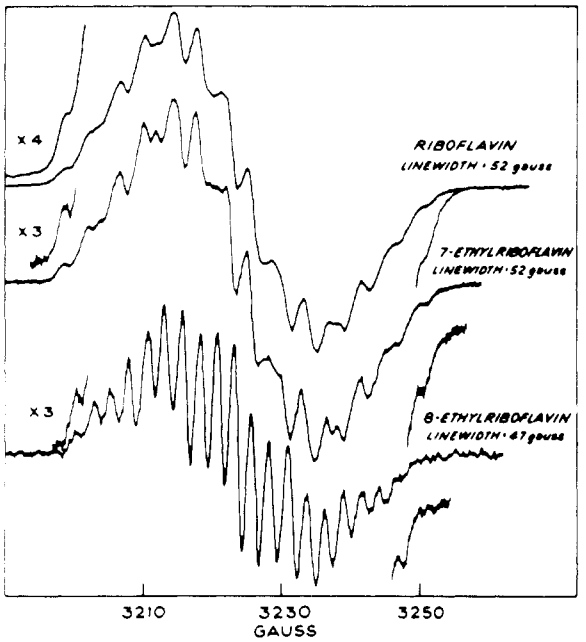


FIGURE 6: EPR spectra of the cationic semiquinone forms of riboflavin, 7α-methylriboflavin, and 8α-methylriboflavin. All flavin concentrations were 5 mM in 88% formic acid.

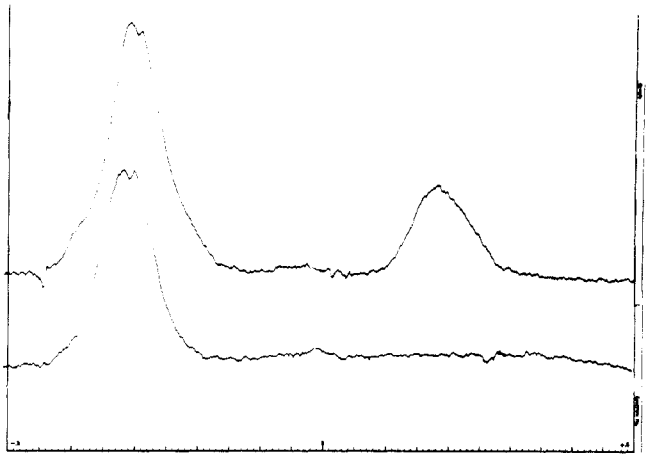


FIGURE 7: ENDOR spectra of the cationic semiquinone forms (2 mM) of riboflavin (top spectrum) and histidylriboflavin X (bottom spectrum) in 6 N HCl. The intense signal in each spectrum is the matrix ENDOR, which is centered at 13.85 MHz. The total scan range for the spectra is 10 MHz.

riboflavin (Walker et al., 1972 and Figure 5). In flavins in which the methyl groups are unsubstituted the resonances of the 7- and 8-CH_3 groups are very close (Bullock and Jardetzky, 1965; Kainosho and Kyogoku, 1972) and it is the substitution by the histidyl residue that causes the considerable downfield shift of one of the two CH_3 peaks. Thus, if the 7-CH_3 group were substituted, the resonance at 5.80 ppm in the middle spectrum of Figure 5 would belong to the 7-CH_2 group, and that at 2.58 ppm to the unsubstituted 8-CH_3 group. This would in no way invalidate the data in Table II, but might, in the light of the data of Bullock and Jardetzky (1965), explain the *slight* downfield shift of the CH_3 resonance nearest the DSS peak in histidylriboflavin X, as compared with 8α-(N^3-histidyl)riboflavin (Figure 5).

In order to remove these uncertainties, two approaches were used. First, the EPR spectra of the cation radicals of 7α- and of 8α-methylriboflavins were compared with that of riboflavin (Figure 6). It is seen that the replacement of the methyl group in the 7 position with an ethyl does not alter the hyperfine spectrum, i.e., the spectral width remains at 52 G, as in riboflavin, but the same replacement in the 8 position collapses the spectrum to 47 G. This confirms the fact that the 8 position is the one of high spin density. Since a formyl group would not be expected to hinder rotation any more than the insertion of a methylene group does, if histidylriboflavin X were 7α-substituted, the formylriboflavin derived from it would be expected to show as great a difference from 8-formylriboflavin in the EPR spectra as do 7α- and 8α-methylriboflavins. As shown in Figure 4, curves C and D, this was not the case, since the two formylflavins gave identical EPR spectra.

Additional evidence for 8α substitution came from comparison of the ENDOR spectra of the radical cation forms of riboflavin and histidylriboflavin X (Figure 7). The signal in the riboflavin spectrum at 4.75 MHz from the free proton frequency, which is due to the 8-CH_3 group (Walker et al., 1969; Salach et al., 1972), is missing in the spectrum of histidylriboflavin X, thus showing that the 8-CH_3 group is indeed substituted.

Proof of the Structure of Histidylriboflavin X by Methylation and Cleavage. Since the evidence presented seemed to eliminate all reasonable structures for histidylriboflavin X, it became essential to scrutinize all the assumptions made in the course of this study. The only assumption remaining concerned the identity of the sample of 8α-(N^1-histidyl)riboflavin used for comparison with the unknown compound. This compound was known to be an acid modification product of authentic 8α-(N^3-histidyl)riboflavin and its structure was not conclusively established in our earlier studies (Walker et al., 1972) but was thought to represent an acid-catalyzed migration of the flavin from N^3 to N^1 of the imidazole ring. It was recognized, however, in the paper of Walker et al. (1972), that both the shift from a less hindered structure to a more hindered one and the unidirectional nature of the presumed acid-catalyzed migration from N^3 to N^1 are contrary to expectation and require further study.

An unambiguous procedure for proving that acid-modified 8α-(N^3-histidyl)riboflavin is indeed the N^1 isomer would be to methylate the free imidazole nitrogen, cleave the resulting compound by reduction with Zn, and identify riboflavin and 3-methylhistidine as the products. This procedure, used earlier by Walker et al. (1972) for identifying 8α-(N^3-histidyl)ribo-

TABLE III: Characterization of the Flavin Moiety after Removal of Histidine.

Flavin Analogue	Flavin Product after Zn Cleavage: Identity	R_F Values in TLC[a]: System B	System C	Product after Alkaline[b] Irradiation of Zn Cleavage Product
Riboflavin		0.52	0.14	Lumiflavin
8α-(N^3-Histidyl)riboflavin	Riboflavin	0.52	0.14	Lumiflavin
Acid modification product of 8α-(N^3-histidyl)-riboflavin	Flavin X	0.60	0.44	Lumiflavin
8α-(N^1-Histidyl)riboflavin	Riboflavin	0.52	0.14	Lumiflavin
Acid modification product of 8α-(N^1-histidyl)-riboflavin	Flavin X	0.60	0.44	Lumiflavin

[a] The TLC system for B was cellulose plate, 1-butanol–acetic acid–water (4:1:5, v/v) (upper phase) and for C, silica gel, chloroform–methanol–acetic acid (18:1:1, v/v). [b] Lumiflavin was identified by TLC on silica gel, chloroform–methanol (9:1, v/v) and in water-saturated isoamyl alcohol as solvent systems.

flavin, gave satisfactory yields of the flavin moiety (typically 60–80%, occasionally 90%)[2] from both the N^1 and N^3 isomers of histidylriboflavin and from their acid modification products, but the recovery of methylhistidine from these compounds was uniformly low. In contrast, acid hydrolysis (6 N HCl at 125–150 °C for ~24 h) gave satisfactory yields of methylhistidine (~60%) but resulted in complete destruction of the flavin.[3]

Methylation of acid-modified 8α-(N^3-histidyl)riboflavin, followed by acid hydrolysis, resulted in the liberation of 1-methylhistidine, instead of the 3-methylhistidine expected if the structure had been 8α-(N^1-histidyl)riboflavin (Figure 8). Reductive cleavage of the methylated compound, on the other hand, liberated a compound that could be distinguished from riboflavin by greater mobility in TLC (Table III), suggesting a more apolar nature.[4] The compound, referred to as flavin X in Table III, was indistinguishable from riboflavin in absorption, fluorescence emission, and EPR spectra. Collectively, these experiments show that acid treatment of 8α-(N^3-histidyl)riboflavin does not result in conversion to the N^1 isomer, as had been supposed, but in modification of the flavin moiety, leaving the site of attachment at N^3 of the imidazole ring. Alkaline irradiation of this flavin X yielded lumiflavin (Table III), which was identified by cochromatography with synthetic lumiflavin in several solvent systems and by EPR spectrum of its cation radical, which was superimposable on the spectrum of lumiflavin semiquinone cation. Clearly, then, acid modification affects the ribityl moiety of riboflavin.

Figure 8 also shows that methylation and acid hydrolysis of histidylriboflavin X yields 3-methylhistidine, showing that the flavin is attached to N^1 of the imidazole ring in the parent compound. Together with the data presented earlier in this paper, this identifies the compound as 8α-(N^1-histidyl)riboflavin. In accord with this assignment, methylation of this compound, followed by Zn reduction, yields authentic riboflavin (Table III). It may be further seen that the acid modification product of 8α-(N^1-histidyl)riboflavin liberates on methylation and reductive cleavage flavin X, the same product as was obtained from acid-modified 8α-(N^3-histidyl)riboflavin.

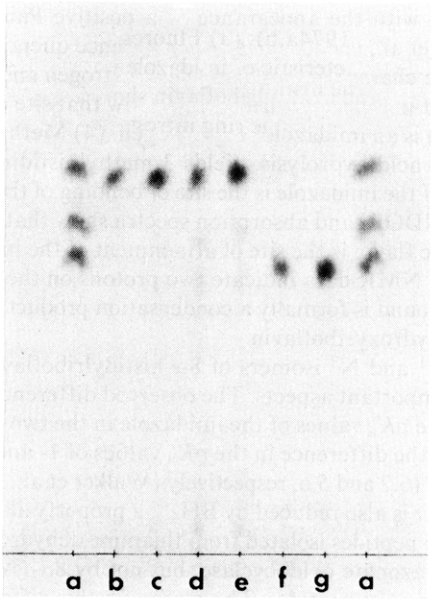


FIGURE 8: Electrophoretic identification at pH 6.25 of the methylhistidines liberated from methylated histidylflavins by acid hydrolysis. The spots, visualized with ninhydrin spray, were: (a) standards (20 nmol each, in order of increasing migration from the origin to the negative pole): 3-methylhistidine, histidine, and 1-methylhistidine; (b) hydrolysis product from 25 nmol of methylated 8α-(N^3-histidyl)riboflavin; (c) sample (b) plus 20 nmol of 1-methylhistidine; (d) hydrolysis product from 25 nmol of methylated, acid-modified 8α-(N^3-histidyl)riboflavin; (e) sample (d) plus 20 nmol of 1-methylhistidine; (f) hydrolysis product from 37 nmol of methylated histidylriboflavin X; (g) sample (f) plus 20 nmol of 3-methylhistidine.

Discussion

Prior studies (Kenney et al., 1974a,b; Singer and Edmondson, 1974) have shown that thiamine dehydrogenase and β-cyclopiazonate oxidocyclase contain a new type of covalently bound flavin, in which the FAD is linked to a histidine residue and which yields on dephosphorylation a histidylriboflavin with different properties from those of 8α-(N^3-histidyl)riboflavin,

[2] The factor limiting overall yield is the rate of methylation of the imidazole by CH_3I. This is significantly slower with 8α-(N^3-histidyl)riboflavin than with the N^1 isomer. Zinc cleavage itself is essentially quantitative, as far as yield of the flavin is concerned.

[3] Hydrolysis in 6 N HCl for 24 h at 125 °C results in complete cleavage of the histidine–flavin bond of both the N^1 and N^3 isomers of histidylriboflavin and also of methylated (N^1-histidyl)riboflavin but 150 °C was required to hydrolyze methylated N^3-histidylriboflavin.

[4] The increased mobility in TLC cannot be ascribed to the incorporation of methyl groups from the CH_3I into the flavin, since methylation with $^{14}CH_3I$, followed by reductive cleavage, showed no radioactivity in the flavin.

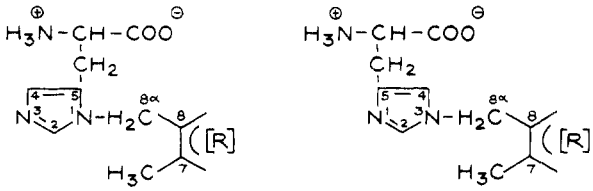


FIGURE 9: Structures of: 8α-(N^1-histidyl)riboflavin (left side) and 8α-(N^3-histidyl)riboflavin (right side). [R] denotes the rest of the flavin moiety.

the flavin component of succinate dehydrogenase (Walker et al., 1972). This new type of histidylriboflavin is formed in 20–25% yield as a by-product of the condensation of histidine with 8α-bromotetraacetylriboflavin. The structure of the compound is shown to be 8α-(N^1-histidyl)riboflavin in the present paper (Figure 9, left side).

The evidence for this structure rests on the following observations. (1) Methylation followed by reductive cleavage yields riboflavin. (2) Drastic acid hydrolysis yields 1 mol of histidine, with the appearance of a positive Pauly reaction (Kenney et al., 1974a,b). (3) Fluorescence quenching with a pK_a value characteristic of imidazole nitrogen and a negative Pauly test in the histidylriboflavin show that site of flavin attachment is an imidazole ring nitrogen. (4) Methylation, followed by acid hydrolysis, yields 3-methylhistidine, showing that N^1 of the imidazole is the site of bonding of the flavin. (5) EPR, ENDOR, and absorption spectra show that the 8α carbon of the flavin is the site of attachment of the histidine. (6) Since ^{1}H NMR data indicate two protons on the 8α carbon, the compound is formally a condensation product of histidine and 8α-hydroxyriboflavin.

The N^1 and N^3 isomers of 8α-histidylriboflavin differ in several important aspects. The observed difference of 0.5 pH unit in the pK_a values of the imidazole in the two compounds parallels the difference in the pK_a values of 1- and 3-methylhistidines (6.2 and 5.6, respectively (Walker et al., 1972)). The N^1 isomer is also reduced by BH_4^-, a property also shown by the flavin peptides isolated from thiamine dehydrogenase and β-cyclopiazonate oxidocyclase, but not by 8α-(N^3-histidyl)-riboflavin or its peptides. The reason for this difference is not apparent. The third major difference between the two isomeric histidylriboflavins is the much greater lability of the N^1 and its tendency to yield 8-formylriboflavin. The compound is more stable to storage in weakly acidic than in neutral aqueous solutions. This may suggest that the acidity of the 8α-methylene protons may be a determining factor in the stability of histidylriboflavins. Ghisla and Hemmerich (unpublished results, cited in Walker et al., 1972) have found that 8α-histidylriboflavin derivatives are extremely labile to base, since the carbanion formed on removal of an 8α-methylene proton undergoes decay by oxidation or disproportionation.

The fourth major difference is in the geminal coupling of the 8α-methylene protons in the ^{1}H NMR spectrum of the N^1 isomer which is not shown either by its acid modification product or by 8α-(N^3-histidyl)riboflavin (Figure 5). This coupling is probably a result of altered rotation of the histidine ring relative to the flavin ring system about the CH_2 axis. This altered rotation could result from steric interaction(s) of the histidine side chain with the ribityl side chain of the flavin moiety, thus creating an asymmetry, which does not exist for the N^3 isomer because of steric reasons. The fact that coupling of the 8α protons is not seen in the acid-modified compound may be the consequence of modification of the ribityl side chain.

Inspection of the chemical shifts of the various protons for both the N^1 and N^3 isomers shows that the largest differences are in the flavin aromatic protons (Figure 5). The 9(H) and 6(H) protons of the flavin in the N^1 isomer are shifted 0.15 ppm upfield and −0.1 ppm downfield relative to these protons in the N^3 isomer. These shifts are consistent with the greater proximity of the histidine side chain to the benzenoid portion of the flavin ring in the N^1 isomer, as compared with the N^3 isomer.

The present investigation also focuses attention on the chemical nature of the acid modification products of the two isomeric histidylriboflavins. Prior to this work only one acid-modified 8α-histidylriboflavin was known to exist (Walker et al., 1972). Its structure had not been investigated but was assumed to be 8α-(N^1-histidyl)riboflavin. It is now clear that there are two isomeric acid modification products, one arising from N^1, the other from the N^3 isomer of histidylriboflavin in strong acid, and that acid modification affects the ribityl side chain, not the site of attachment of the flavin to the histidine. The two acid-modified histidylflavins differ from each other and from the parent histidylriboflavins in their pK_a values of the imidazole nitrogen and may be readily separated on this basis.

The fact that acid modification affects the ribityl side chain was already indicated by major differences in ^{1}H NMR spectra of 8α-(N^3-histidyl)riboflavin and its acid modification product in the region corresponding to the ribityl proton resonances (Walker et al., 1972). A similar difference is seen in Figure 5 of this paper on comparing the ^{1}H NMR spectra of 8α-(N^1-histidyl)riboflavin and its acid-modified form. An even earlier indication that the ribityl chain is affected by exposure to strong acids is an early paper of Baddiley et al. (1957) who reported that FMN and riboflavin are converted in 1 N HCl at 100 °C to a modified form that they assumed to be a 2′,5′-anhydro form of riboflavin, by analogy with the acid-catalyzed dehydration of ribitol and of ribitol 1-phosphate to 1,4-anhydroribitol

Studies in progress on the structure of the acid modification product of 8α-(N^3-histidyl)riboflavin have ascertained, beyond doubt, that the ribityl moiety is altered in this compound, since its periodate titer is lower for the acid-modified 8α-(N^3-histidyl)riboflavin than for the unmodified parent compound. There is reason to believe, however, that the structure proposed by Baddiley et al. (1957) would apply to acid-modified histidylriboflavins, since the flavin moieties of acid-modified riboflavin and acid-modified 8α-(N^3-histidyl)riboflavin (flavin X) appear to be identical.[5]

The last point to be discussed concerns the biological occurrence of the two isomeric histidylriboflavins. Three enzymes have been shown to contain 8α-(N^3-histidyl)FAD at the active site: succinate dehydrogenase (Salach et al., 1972; Walker et al., 1972), D-6-hydroxynicotine oxidase (Möhler et al., 1972), and sarcosine dehydrogenase (Pinto and Frisell, 1975). Similarly, three enzymes are known to contain 8α-(N^1-histidyl)-FAD: thiamine dehydrogenase, β-cyclopiazonate oxidocyclase, and, as judged by a recent paper (Nakagawa et al., 1975), L-gulono-γ-lactone oxidase, a microsomal enzyme from rat liver. The occurrence of as many enzymes containing the sterically hindered N^1 isomer as the sterically favored N^3 isomer might suggest the participation of an enzyme or enzymes in the attachment of the flavin to histidyl residues of the respective apoenzymes during their biosynthesis.

[5] Edmondson, D. E., unpublished data.

Acknowledgments

The authors acknowledge the excellent technical assistance of Ms. D. Stanke and Mr. R. Seng and are grateful to Dr. A. Schonbrunn, Brandeis University, for her help in the preliminary ^{1}H NMR experiments. Special thanks are extended to Dr. J. Shoolery and to Dr. J. Hyde, Varian Instruments, for their help in running the 300 MHz ^{1}H NMR and the ENDOR spectra.

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Solution Conformation of a Retro-D Analogue of Tocinamide†

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ABSTRACT: The solution conformation of a retro-D analogue of tocinamide

H-D-Cys-D-Asn-D-Gln-D-aIle-D-Tyr-NHCH$_2$CH$_2$S

was examined using proton magnetic resonance and circular dichroism spectroscopy. The observations support major contributions to the conformational distribution from structures with a type I β turn in the sequence D-Asp-D-Gln-D-aIle-D-Tyr. This is topologically similar to the β turn proposed for oxytocin, L-Tyr-L-Ile-L-Gln-L-Asn, but with the polarity of the CONH groups reversed along the chain; the peptide is, however, hormonally inert. In conjunction with nuclear magnetic resonance data, the circular dichroism spectra are interpreted to indicate that the region of the peptide ring near the disulfide occurs in at least two different conformations. One of the side-chain carboxamides, probably that of asparagine, appears to be intramolecularly associated rather than freely exposed to solvent.

Incorporation of D-amino acid residues has been one tool in the study of structure–activity relationships of biologically active peptides. Enantiomers of several peptide hormones and active fragments have been found to be essentially inactive (Rudinger, 1971), but examination of peptides made of enantiomeric amino acids coupled in reverse sequence has been more hopeful. Shemyakin and his collaborators (1969) suggested that there might be prepared enzymatically resistant retro-D analogues of peptide hormones (or active fragments) in those cases where the parent molecules are devoid of N- and C-terminal charges and of proline residues. Supporting examples include retro-D-[Gly^{5,10}]gramicidin S (Shemyakin et al., 1967), which has antibiotic activity equal to that of the parent L-antibiotic. Similarly, the synthetic retro-D analogue of [Tyr^6]antamanide sulfate ester was found substantially to retain the activity of the L-compound in antagonizing the lethal effect on mice of the toxin phalloidin (Wieland et al., 1972). However, Vogler et al. (1966) found retro-D-bradykinin to be inactive as an agonist or antagonist of bradykinin. Because bradykinin bears charged termini and contains proline residues, its retro-D analogue would not be expected to be sufficiently

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