Proton Nuclear Magnetic Resonance Studies of 8α-*N*-Imidazolylriboflavin in Its Oxidized and Reduced Forms†

Gary Williamson and Dale E. Edmondson*
Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322
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ABSTRACT: The oxidized and hydroquinone forms of synthetic 8α-*N*-imidazolylriboflavin have been investigated by proton nuclear magnetic resonance spectroscopy at 360 MHz. Proton resonances due to the imidazole ring, isoalloxazine ring, and ribityl side chain have been assigned on the basis of two-dimensional ^1H–^1H correlated spectra (COSY), selective decoupling, and nuclear Overhauser effect difference spectra and by comparison of computer-simulated with experimental spectra. The effect of pH on the imidazolyl resonances shows a pK_a for the unsubstituted imidazole nitrogen of 6.0 ± 0.1 for the oxidized form and a value of 7.0 ± 0.1 for the reduced form, in good agreement with the values obtained from oxidation–reduction potential data in a previous paper [Williamson, G., & Edmondson, D. E. (1985) *Biochemistry 24*, 7790–7797]. Slow exchange of the flavin 8α-methylene and imidazolyl C(2) protons was observed at pH 6.1 but not at pH values below 4.0 for the oxidized form of the flavin. The reduced form, but not the oxidized form, of the flavin exhibits geminal coupling of the 8α-methylene protons and of the C(1′) methylene protons of the ribityl side chain. The magnetic nonequivalence of the protons of these two methylene groups is suggested to result from intermolecular association of the reduced flavin in aqueous solutions at the concentrations required for the spectral experiments.

Results from studies of the pH dependence of the oxidation–reduction potential of 8α-*N*-imidazolylriboflavin carried out in this laboratory (Williamson & Edmondson, 1985a) have shown that the pK_a of the 8α-imidazole substituent is increased by approximately 1 pH unit on reduction of the flavin to the hydroquinone level. This change in pK_a has been attributed to alterations in the inductive effect of the isoalloxazine ring on the imidazole ring upon transformation from the electron-deficient oxidized form to the reduced form. To provide confirmatory evidence for these conclusions as well as to provide additional insights into the relative structures of the oxidized and reduced forms of the 8α-substituted flavin, a high-resolution ^1H NMR study was carried out on both the oxidized and reduced forms as a function of pH. It is known that the chemical shift values of the imidazole protons are sensitive to the state of protonation of the imidazole nitrogens (Markeley, 1975). However, it is not known what effect (if any) the state of ionization of the imidazole ring would have on the chemical shift values of the flavin protons. Such information would be useful to monitor changes in flavin electron density (Sterk & Holzer, 1974) resulting from perturbations of the 8α-imidazole substituent.

Previous high-resolution ^1H NMR spectra of 8α-*N*-histidylriboflavin isomers (Edmondson et al., 1976) have been published as part of the structural elucidation of 8α-*N*^{1}-histidylriboflavin. An extensive ^1H NMR study of oxidized alloxazines and isoalloxazines (Grande et al., 1977a,b) serves as a comparative basis for the results presented here. The NMR data reported here show that (a) the 8α-imidazole pK_a shifts from a value of 6.0 in the oxidized flavin to 7.0 in the hydroquinone form in agreement with the electrochemical data presented in a previous paper (Williamson & Edmondson, 1985a), (b) assignments of the proton resonances both in the imidazole and isoalloxazine rings and in the ribityl side chain are made in both redox forms of the flavin, and (c) the reduced flavin but not the oxidized form appears to exhibit intermolecular "stacking", which complicates efforts to compare the respective conformations of the oxidized and reduced forms in a detailed manner.

Experimental Procedures

Materials

8α-*N*-Imidazolylriboflavin was synthesized and purified as described previously (Williamson & Edmondson, 1985a). N^3-(Carboxymethyl)riboflavin was the generous gift of Dr. D. B. McCormick of our department. Deuterated water (99.8% and 99.96% isotopic enrichment) was purchased from Sigma, sodium 3-(trimethylsilyl)tetradeuteriopropionate (TSP) and 40% NaO^{2}H (>99% ^2H) were purchased from Wilmad, and [$^2H_4$]acetic acid was obtained from Aldrich.

Over the pH range (1.5–11.5) of the experiments presented here, samples were dissolved in ^2H_2O containing the following buffer salts (0.1 M) in the appropriate pH range: deuterated sodium acetate/acetic acid, potassium phosphate, and sodium carbonate. Sodium carbonate was oven-dried before use, and KH_2PO_4 and K_2HPO_4 were deuterated by dissolution in ^2H_2O and lyophilization before use.

Methods

NMR Samples. All flavin solutions used in this work were freshly prepared before spectral analysis and protected from light at all times. Solution pH values were measured directly by using an Orion 8-mm-diameter combination pH electrode and an Orion Model 801 pH meter. The deuterium isotope correction for the activity of ^2H at the electrode was estimated by comparing the readings of known potassium phoshate buffers in H_2O and ^2H_2O. The observed difference was found to be 0.16, which is incorporated as a correction for all pH values reported here.

Due to differences in flavin solubility with pH, in the oxidized form, flavin concentrations of 1–2 mg/mL were used in the pH range of 6–9, and 5 mg/mL concentrations were used at pH values outside of this range. For the flavin hydroquinone studies, 5–8 mg/mL flavin was used below pH 5,

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and concentrations of 1–3 mg/mL were used above pH 5. All flavin samples were placed in 5-mm NMR tubes by using amber tubes (Wilmad 507-PP) for the oxidized flavin and sample tubes with screw-cap enclosures with a Teflon/rubber septum (Wilmad (507-TR) for the flavin hydroquinone. In the later system, flavin solutions were degassed by alternate evacuation and flushing with purified argon for several cycles, left under a positive argon atmosphere, and reduced by the addition of excess sodium dithionite (3–5 mol/mol of flavin) in a ^2H_2O buffer solution.

NMR Spectral Measurements. All ^1H NMR spectral data were obtained by using a Nicolet spectrometer operating at 361.05 MHz in the pulsed Fourier transform mode with quadrature phase detection. For one-dimensional spectra, the following parameters were used: temperature, 22 °C; sweep width, ±2000 Hz; 16 384 data points; 3.0-s recycle time; and between 4 and 128 acquisitions, depending on sample concentration. All chemical shifts were measured relative to an internal standard of 0.05–0.1% TSP. Spectral simulations were performed by using the "NMCSIM" program on the Nicolet spectrometer system.

Two-Dimensional NMR Experiments. ^1H–^1H correlated spectra (COSY) were recorded by using the Jeneer pulse sequence (Benn & Gunther, 1983) $[90°-t_1(+x)-90°x-(x)-t_2]_n$, where t_1 and t_2 are the evolution and observation periods, respectively. Couplings of less than $\sim x/4$ were emphasized by choosing values of x between 0 and 126 ms (Bax et al., 1981). The sweep width employed was 4000 Hz in each dimension with 1024 data points obtained from 4 acquisitions and repeated for 512 equidistant values of t_1. No symmetrization procedures were employed in spectral manipulations. Two-dimensional ^1H–^1H nuclear Overhauser effect spectra (NOESY) were obtained by using the pulse sequence (Bosch et al., 1981; Benn & Gunther, 1983) $[90°x-t_1-90°x-t_m-90°-t_2]_n$. A sweep width of ±2000 Hz in each dimension used 1024 data points from 16 accumulations. A mixing time (t_m) of 400 ms was used in the data acquisition.

Measurement of Nuclear Overhauser Effect (NOE) Enhancements. Semiquantitative NOE enhancements were measured by NOE difference spectra (Saunders & Bell, 1970) from 16 or 32 accumulations recorded with and without selective double irradiation (27–29-dB decoupler power) and averaged by 10 repetitions of the sequence ("Presat" routine on the Nicolet spectrometer). NOE enhancements were measured planimetrically (Kainosho & Kyogoku, 1972; Schirmer et al., 1970).

RESULTS

^1H NMR Spectral Properties of the Ring Protons of Oxidized 8α-N-Imidazolylriboflavin. As an aid in the discussion of the ^1H NMR spectral properties of 8α-*N*-imidazolylriboflavin, the structure and numbering system of this compound are shown in Figure 1. In accord with standard nomenclature, the alkylated imidazole nitrogen is assigned as the 1-position and the unsubstituted imidazole nitrogen as the 3-position.

The low-field ^1H NMR spectrum of the hydrochloride salt of oxidized 8α-*N*-imidazolylriboflavin is shown in Figure 2. Resonances due to the imidazole (Im) protons are designated as A [Im C(2)-H, 8.92 ppm], D [Im C(4)-H, 7.62 ppm], and E [Im C(5)-H, 7.58 ppm]. Each proton resonance is a doublet of doublets due to coupling to the other imidazole protons. Selective irradiation of the Im C(2)-H resonance results in alteration of the Im C(4)-H and Im C(5)-H resonances into an AB type spectrum with J_{4H-5H} = 2.0 Hz. Coupling constants estimated for the Im C(2)-H with the other imidazole protons are J_{2H-4H} = 1.5 Hz and J_{2H-5H} = 1.4 Hz. These

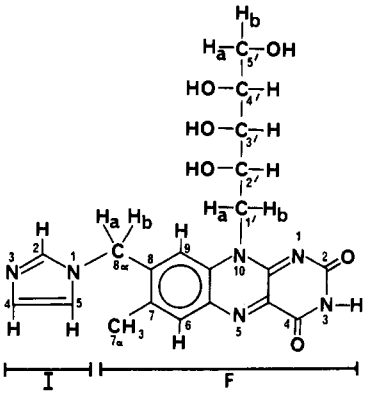

FIGURE 1: The structure of 8α-*N*-imidazolylriboflavin.

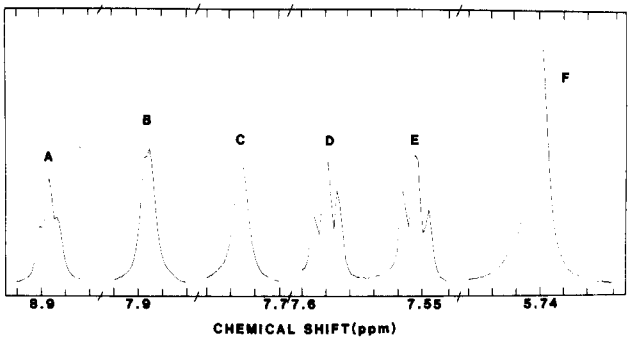

FIGURE 2: Expanded scale of the low-field ^1H NMR spectrum of 8α-*N*-imidazolylriboflavin. The hydrochloride salt of the flavin (20 mg) was dissolved in ^2H_2O (1 mL) (pH 1.4). The peaks are labeled as follows: A, Im C(2)-H; B, Fl C(6)-H; C, Fl C(9)-H; D, Im C(4)-H; E, Im C(5)-H; F, 8α-CH_2. The high (~40 mM) concentration used gives rise to chemical shifts that are slightly different from the corrected values listed in Table I and referred to in the text.

values were verified by the agreement of the simulated spectrum with the experimental spectrum and are comparable to the known coupling constants for free protonated imidazole ($J_{2H-4H} = J_{2H-5H}$ = 1.0 Hz) (Wang & Li, 1966) and to those reported for protonated 1-methylimidazole ($J_{2H-5H} = J_{2H-4H}$ = 1.7–1.8 Hz) (Caesar & Overburger, 1968; Borne et al., 1972). The chemical shift values of the 8α-imidazole protons are very similar to those observed for protonated imidazole (Sachs et al., 1971) (see Table I).

The resonances due to the flavin (Fl) 6- and 9-position aromatic protons are singlets and are denoted in Figure 2 as B (7.98 ppm) and C (7.76 ppm), respectively. These observed chemical shifts are at lower field than the analogous protons in flavin mononucleotide (FMN) by 0.30 and 0.44 ppm, respectively (Kainosho & Kyogoku, 1972), but are comparable to those reported for N^3-methyllumiflavin (Grande et al., 1977a,b). The assignment of the lower field resonance to the Fl C(6)-H is confirmed by two-dimensional *J*-correlated spectra (Figure 4, see below). Over a 30-fold range in flavin concentration (1.1–34 mM), the Fl(6)-H resonance was always at a lower field than the Fl(9)-H resonance. The chemical shift values of both resonances exhibited a small linear dependence on the flavin concentration. The Fl(6)-H resonance moved upfield by 0.028 ppm per 10 mM increase in concentration while the Fl(9)-H resonance moved upfield by 0.01 ppm per the same concentration increment. All other resonances were altered by less than 0.01 ppm per 10 mM increase in concentration.

The flavin 8α-CH_2 resonance (denoted F in Figure 2) is a singlet (5.74 ppm), showing that the two methylene protons are magnetically equivalent as observed previously in ^1H NMR studies of 8α-N^3-histidylriboflavin at 300 MHz (Edmondson

Table I: Comparison of Proton Chemical Shift Values of Imidazoles with Those of 8α-*N*-Imidazolylflavins

	chemical shifts (ppm)		
	C(2)-H	C(4)-H	C(5)-H
imidazole[a]			
protonated	8.88	7.66	7.66
deprotonated	7.90 (7.73)[b]	7.27 (7.14)[b]	7.27 (7.14)[b]
difference	0.98	0.39	0.39
1-methylimidazole[c]			
protonated	8.73	7.60	7.52
deprotonated	7.47	7.08	6.88
difference	1.26	0.52	0.64
8α-*N*-imidazolylriboflavin			
protonated (pH 1.4)	8.92	7.62	7.58
deprotonated (pH 7.5)	7.90	7.17	7.23
difference	1.02	0.45	0.35
8α-*N*-imidazolyl-1,5-dihydroriboflavin			
protonated (pH 1.4)	8.66	7.48	7.42
deprotonated (pH 7.5)	7.68	7.02	7.08
difference	1.00	0.46	0.34
8α-(N^3-methyl-N^1-imidazolium)tetra-*O*-acetylriboflavin (chloride salt)	8.94	7.63	7.58

[a] Sachs et al., 1971. [b] Grimmett, 1970. [c] Barlin & Batterham, 1967.

et al., 1976). On prolonged storage (up to 96 h) of 8α-*N*-imidazolylriboflavin in buffered ^2H_2O (pH 6.1) at room temperature, the 8α-methylene protons and the imidazole C(2)-H undergo extensive ^1H–^2H exchange with the solvent (Figure 3). The slow exchange of the imidazole C(2)-H is well-known (Markeley, 1975); however, the exchange of the 8α-methylene protons of 8α-substituted flavins at ambient temperatures has not been previously observed. Solvent exchange of the 8α-CH_3 protons of FMN has been known for some time (Bullock & Jardetzky, 1965); however, it is observable only at elevated temperatures in the presence of phosphate. It should be noted that the observed exchange of the 8α-methylene protons is dependent on the pH and is observed only at pH values above 4.0.

The 8α-methylene resonance (peak F in Figure 3) shows an upfield shift of 0.017 ppm on substitution of one of the methylene protons with a deuterium. This observed geminal deuterium isotope shift is of a similar magnitude as the geminal deuterium isotope shifts observed in styrene (Baird, 1974). The only other resonance exhibiting a deuterium isotope dependent chemical shift is the Fl C(9)-H (peak C, Figure 3). Examination of this resonance at various levels of deuterium exchange at the 8α-CH_2 position shows downfield shifts of 0.011 and 0.023 ppm while the total integrated intensity does not change. Thus, the downfield shifts of the C(9) resonance appear to be additive with respect to whether one or two deuterium atoms are incorporated into the 8α-methylene group. These isotope-induced shifts were also observed at pH 7.5 as well as at pH 6.1, which demonstrates their origin to be due to the effect of deuterium substitution on the magnetic shielding of the observed protons rather than alterations in imidazole pK_a on deuterium substitution.

The isotope shifts on the flavin C(9) resonance are unusual in that they are in a negative direction rather than the usual positive direction (Batiz-Hernandez & Bernheim, 1967) and are also unusual in that this shift is exhibited over four bonds. Negative isotope shifts have been observed with benzenes (Savitsky et al., 1969) and are thought to arise from solvent effects (Ford et al., 1970). It remains for future work to investigate the reason for this observed negative isotope shift induced by deuteration of the 8α-methylene position on the Fl C(9)-H resonance.

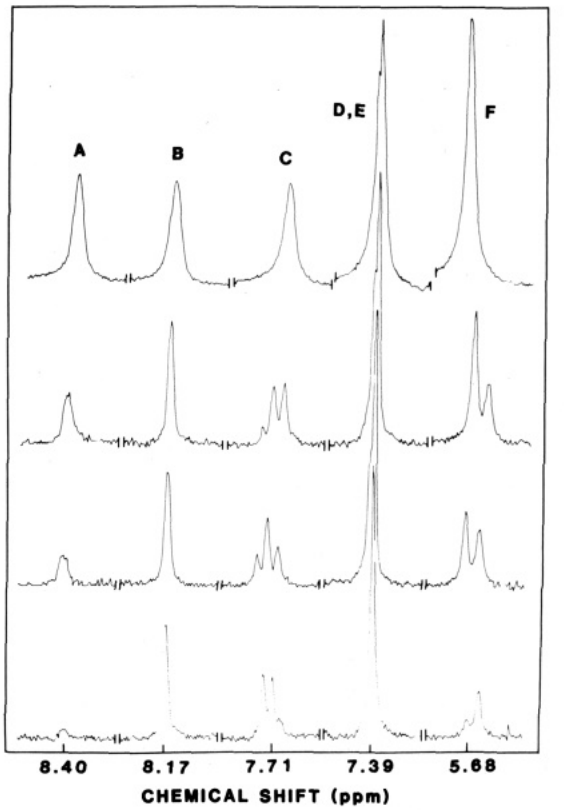

FIGURE 3: Demonstration of slowly exchanging protons of oxidized 8α-*N*-imidazolylriboflavin in ^2H_2O. A saturated solution of flavin in 0.1 M potassium phosphate, pH 6.1, was stored at 20 °C in the dark, and spectra were recorded after 15 min, 28 h, 52 h, and 96 h (spectra from top to bottom, respectively). Peaks are labeled as in Figure 2.

The resonance due to the Fl(7α)-CH_3 protons is observed as a singlet at 2.47 ppm (not shown). This position is slightly downfield from the analogous resonances in FMN (2.23 ppm) (Bullock & Jardetzsky, 1965) and in flavin adenine dinucleotide (FAD) (2.05 ppm) (Kainosko & Kyoguku, 1972) but very close to that observed for N^3-methyllumiflavin (2.43 ppm) (Grande et al., 1977a).

To verify the above resonance assignments as well as to investigate the level of weak, long-range couplings of the various positions in both the 8α-imidazole ring and the flavin, two-dimensional COSY experiments were performed as shown in Figure 4. The diagonal represents the one-dimensional spectrum, and *J*-coupled protons are plotted as off-diagonal cross-peak contours. In this experiment, the acquisition parameters (see Experimental Procedures) were such that coupling constants of ≤2Hz are emphasized. Thus, for example, the Im C(2)-H–Im C(4)-H *J* coupling (1.5 Hz) appears as more contour lines than the ribityl side chain C(1′)-H–C(2′)-H *J* coupling (5.4 Hz) (see below).

On examination of Figure 4, it is apparent that extensive weak couplings are present for the protons of the imidazole and flavin rings, but no observable coupling is apparent between the flavin ribityl side chain and the Fl C(9)-H as observed by Lanterwein et al. (1975) for tetraacetylriboflavin in acetone. The Fl(8α)-CH_2 protons are weakly *J*-coupled to the imidazole C(2)-H and to the upfield imidazole proton. This confirms the assignment of this resonance to the Im C(5)-H. The Fl(8α)-CH_2 protons are also *J*-coupled to the flavin protons at 7α-CH_3 and to both the 6- and 9-position protons. The larger *J* coupling to the upfield aromatic flavin resonance confirms its assignment as due to the Fl C(9)-H. Consistent with this is the stronger coupling of the Fl (7α)-CH_3

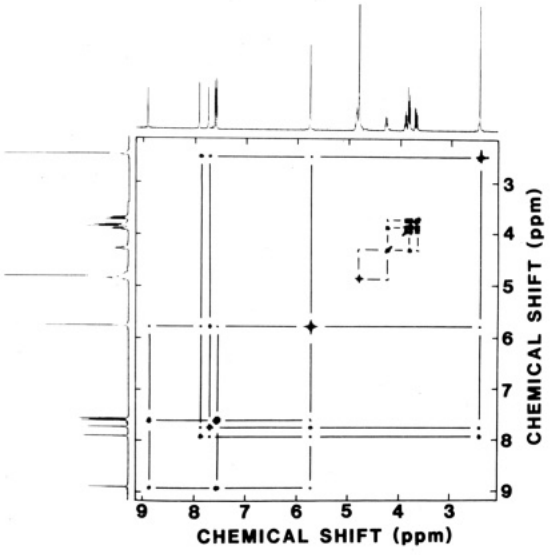

FIGURE 4: Two-dimensional homonuclear COSY contour plot of 8α-*N*-imidazolylriboflavin. The axes show the one-dimensional spectra of the flavin used (20 mg/mL at pH 1.4) for the contour plot. The pulse sequence was modified to emphasize couplings of ~2 Hz (see Experimental Procedures).

to the downfield aromatic flavin proton or Fl C(6)-H. Weak para *J* coupling of Fl C(6)-H and Fl C(9)-H is also apparent as observed for several lumiflavin analogues (Grande et al., 1977a).

The COSY spectral data provide confirmation of the above resonance assignments and provide evidence for weak, long-range couplings that are not easily obtained with selective decoupling approaches. Both one-dimensional and two-dimensional spectral approaches are consistent with a model for 8α-*N*-imidazolylriboflavin in solution in which the isoalloxazine and imidazole rings are rigid planar rings that rotate rapidly with respect to one another on the NMR time scale about the 8α-CH_2 linkage. This results in the magnetic equivalency of the two methylene protons. Previous ^{1}H NMR studies of 8α-N^3-histidylriboflavin (Edmondson et al., 1976) show a similar behavior while 8α-N^1-histidylriboflavin shows a geminal coupling of the two 8α-methylene protons. This geminal coupling is not observed (Edmondson et al., 1976) when the ribityl side chain of the N^1-histidylflavin undergoes an acid-catalyzed cyclization to the 2′,5′-anhydroflavin (Edmondson, 1977). These data suggest that the geminal coupling of the 8α-N^1-histidylriboflavin isomer reflects a sterically restricted rotation of the two ring systems rather than an alternate interpretation in which the diastereotopicity is induced by the proximity of the chiral center of the histidyl side chain.

^{1}H NMR Spectral Properties of the Ring Protons of 8α-N-Imidazolylriboflavin Hydroquinone. On two-electron reduction of 8α-*N*-imidazolylriboflavin by an excess of sodium dithionite at pH 1.4, the ^{1}H NMR spectrum shows all of the imidazole and flavin resonances to be shifted upfield (Figure 5) as compared to the oxidized form (Figure 2). The imidazole protons, denoted A [Im C(2)-H], D [Im C(4)-H], and E [Im C(5)-H] as before, moved upfield by 0.26, 0.14, and 0.16 ppm, respectively (Table I). The chemical shift of the Im C(2)-H is almost identical with that of 1-methylimidazole. The 8α-imidazole substituent therefore reflects the increased electron density of the reduced flavin ring. The relative coupling constants of the imidazole protons are relatively unaffected by flavin reduction: J_{2H-4H} = 1.4 Hz, J_{2H-5H} = 1.5 Hz, and J_{4H-5H} = 2.0 Hz. These data show no major alterations in imidazole ring conformation on flavin reduction.

The flavin C(6)-H and C(9)-H resonances exhibit upfield

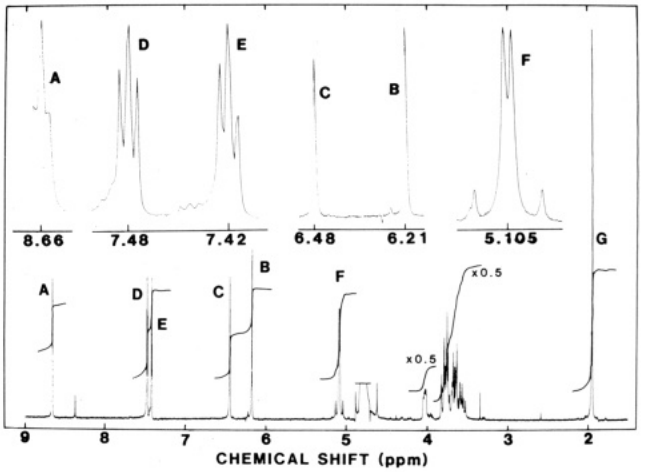

FIGURE 5: Proton NMR spectrum of 8α-*N*-imidazolylriboflavin hydroquinone at pH 2.5. Sodium dithionite (5 mg) in 50 μL of ^2H_2O was added anaerobically to 8 mg of 8α-*N*-imidazolylriboflavin in 0.7 mL of O_2-free ^2H_2O. The spectrum was recorded within 30 min of adding the dithionite. Peaks are labeled as described in the legend to Figure 2.

chemical shifts of 1.77 and 1.28 ppm, respectively, on reduction. Unlike that of the oxidized form, the Fl(9)-H of the reduced form appears at a lower field (6.48 ppm) than the Fl(6)-H (6.21 ppm) (Figure 5). These assignments were determined by selective decoupling and by NOE (see below). Two-dimensional COSY experiments on the reduced flavin were found to be relatively uninformative due to differential relaxation properties of the relevant imidazole and flavin protons. Recent ^{13}C NMR studies on oxidized and reduced flavins (Van Schagen & Muller, 1980; Kawano et al., 1978) showed that the π-electron density at the C(9) position decreased while that at the C(6) position increased on flavin reduction in apolar solvents. The ^{1}H NMR data presented here on 8α-*N*-imidazolylriboflavin in ^2H_2O suggests that the total (σ + π)-electron density increases at both the 6- and 9-positions on flavin reduction. Whether this is a result of altered electron density of reduced 8α-substituted flavins as compared with "normal" flavins or simply an effect of solvent polarity remains to be determined.

The Fl(8α) methylene proton resonance undergoes a 0.64 ppm upfield shift to 5.10 ppm on flavin reduction (Figure 5). In addition, the two protons are magnetically nonequivalent as demonstrated by the observed geminal coupling (J = 15.5 Hz, H_a–H_b = 0.043 ppm). A geminal coupling constant of the value observed here is typical of a tetrahedral SP^3 carbon (Gutowsky et al., 1959). The observation that the 8α-CH_2 protons in the reduced form are nonequivalent while in the oxidized form they are equivalent is of interest in that it suggests, by analogy with previous ^{1}H NMR data on the oxidized forms of 8α-N^1-histidylriboflavin and on 8α-N^1-histidyl-2′,5′-anhydroriboflavin (Edmondson et al., 1976), an alteration in respective ring mobility on flavin reduction. This could result from (a) an altered geometry of the two ring systems resulting in a steric barrier to free rotation or (b) an increase in the degree of flavin aggregation on reduction. As will be demonstrated below, the latter possibility is a more reasonable explanation for this observation than the former. The Fl(7α)-CH_3 resonance (peak G, Figure 5) undergoes an upfield shift of 0.51 ppm (1.96 ppm) on flavin reduction with all of the protons showing magnetic equivalency.

Comparison of NOE Enhancements between Oxidized and Reduced Forms. In principle, the information regarding the molecular geometry of 8α-substituted flavins can be obtained

Table II: Proton–Proton NOE Enhancements Measured for Oxidized and Reduced Forms of 8α-*N*-Imidazolylriboflavin in ^2H_2O

resonance irradiated	enhancement (%)							
	imidazole protons			isoalloxazine ring protons				ribityl side chain protons,
	C(2)-H	C(4)-H	C(5)-H	C(6)-H	C(7α)-CH	C(8α)-CH	C(9)-H	C(1′)-H
oxidized form								
Fl(8α)-CH_2	6	0	5	0	2.5		3	0
Fl(7α)-CH_3	1	0	0	6		2	0	0
Fl(9)-H	1	0	1	0	0	1		2.5
hydroquinone form								
Fl(8α)-CH_2	2	0	0	0	1		2	0
Fl(7α)-CH_3	2	0	0	5		1	0	0
Fl(9)-H	1	0	1	0	0	1		4

from a detailed study of NOE enhancements of the flavin and imidazole protons. NOE measurements are quantitative for a rigid molecule (Young & James, 1984) but are only qualitative for conformationally mobile groups (Saunders & Bell, 1970). We have determined NOE enhancements for the oxidized and reduced forms of 8α-*N*-imidazolylriboflavin to confirm spectral assignment of the individual resonances as well as to provide qualitative insights into any major alterations in conformation that may occur on reduction of the flavin. NOE enhancements were determined by NOE difference spectroscopy (Schirmer et al., 1970) after defining the individual through-space interactions by two-dimensional NOESY spectra (not shown). Peak height enhancements were reproducible to 1% and were estimated from the selective irradiation of the designated resonances (Table II).

As shown in Table II, relatively weak NOE enhancements are observed for the protons of interest (a theoretical maximum of 50% is predicted for ^1H–^1H NOE enhancements). The 8α-CH_2 resonance in the oxidized form shows observable NOE enhancements with the imidazole C(2)-H and C(5)-H and with the flavin 7α-CH_3 and C(9)-H. These data confirm our assignment of the flavin C(9)-H and the imidazole C(5)-H. Weaker NOE enhancements between the 8α-CH_2 and the above protons are observed on reduction of the flavin with no observable NOE interaction with the imidazole C(5)-H (Table II). Inasmuch as there are no major structural alterations of the proximity of the Im C(5)-H to the flavin 8α-CH_2 and the observed geminal coupling of the 8α-CH_2 in the reduced flavin is small in comparison with the chemical shift so that no large changes in the energy levels in the spin system occur (Schirmer et al., 1970), this change in NOE (which is outside the error of the measurement) is most probably due to intermolecular interactions that induce other relaxation pathways and thereby decrease the observed enhancement. We have observed a distinct decrease in solubility of the flavin on reduction as contrasted to the oxidized form, which is consistent with an enhancement of intermolecular interactions. Evidence for stacking of the reduced flavin but not the oxidized flavin is found on analysis of the ribityl side chain resonances (see below).

The NOE data in Table II also provide additional support for the assignments of the flavin C(6)-H and C(9)-H. NOE enhancements are observed between the 7α-CH_3 and the C(6)-H in both the oxidized and reduced forms, whereas NOE enhancements are observed between the C(9)-H and the ribityl C(2′)-H in both redox forms (Table II). No NOE enhancements were observed between any other of the flavin side chain protons and the ring protons.

pH Dependence of ^1H NMR Chemical Shifts for Oxidized and Reduced 8α-N-Imidazolylriboflavin. With the assignment of the ring portion resonance of both the oxidized and reduced forms of the flavin, the pH dependence of their respective chemical shift values could (a) provide information on the relative pK_a values of the imidazole ring of the oxidized and reduced forms and (b) also provide insights into the effect of protonation–deprotonation on the chemical shift values of the flavin resonances.

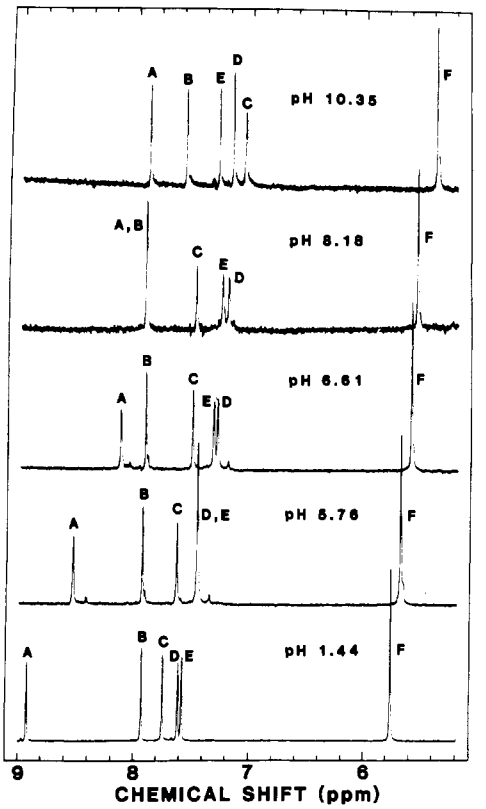

FIGURE 6: Effect of pH on the low-field NMR spectrum of 8α-*N*-imidazolylriboflavin. Spectra were recorded at the pH indicated and assignments made as described in the text. Peaks are labeled as in Figure 2.

In a previous paper (Williamson & Edmondson, 1985a) the pK_a of the 8α-imidazole substituent in the oxidized flavin was determined to be 6.0. The spectra in Figure 6 show that deprotonation of the imidazole ring is accompanied by upfield chemical shifts of 1.02, 0.45, and 0.35 ppm for the Im C(2)-H, the Im C(4)-H, and the Im C(5)-H, respectively. Comparison of these upfield shifts with those of other imidazoles (Table I) shows that the magnitude of the chemical shift changes upon deprotonation are consistent with an amidinium type of imidazole resonance structure as reported previously for 1-methylimidazole (Barlin & Batterham, 1967) (Figure 7). The positive charge is higher on N(3) than on N(1) for the protonated imidazole since the Im C(4)-H resonance undergoes a larger upfield shift on imidazole deprotonation than the Im C(5)-H.

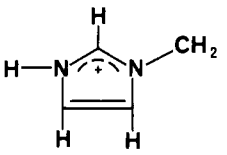

FIGURE 7: Postulated structure of the cationic form of the imidazole moiety of 8α-*N*-imidazolylriboflavin.

The chemical shift values of the flavin aromatic protons have been shown to correlate reasonably well with the total electron densities at the respective positions of substitution (Grande et al., 1977a,b). Therefore, the effect of imidazole ionization on the isoalloxazine resonances should provide some insights into the alterations of flavin electron densities. Deprotonation of the 8α-imidazole substituent resulted in an upfield shift of the Fl(8α)-CH_2 and Fl(9)-H resonances by 0.30 and 0.29 ppm, respectively. No change in chemical shift of the Fl(6)-H and Fl(7α)-CH_3 resonances was observed on imidazole deprotonation (Figure 6). Since the ribityl side chain C(1′) methylene proton resonance (see below) also shifted upfield 0.12 ppm on imidazole deprotonation, it is apparent that alterations in ($\sigma + \pi$)-electron density occur at the "top" of the flavin ring [comprised of the C(8), C(9), C(11), and N(10) positions] on imidazole protonation–deprotonation.

As might be anticipated, flavin N(3) deprotonation in the pH range of 8.2–10.34 exerts a greater effect on the flavin proton than on the imidazole proton chemical shift values. The Fl C(6)-H, 7α-CH_3, 8α-CH_2, and C(9)-H chemical shifts move upfield by 0.56, 0.17, 0.35, and 0.50 ppm, respectively, on oxidized flavin monoanion formation. The Im C(5)-H moves upfield by 0.08 ppm while the chemical shift values of the Im C(2)-H and Im C(4)-H resonances are unaffected.

The pK_a values for the oxidized form of 8α-*N*-imidazolylriboflavin were thus determined from the pH dependence of the respective chemical shift values discussed above as shown in Figure 8. The imidazole pK_a was found to be 6.0 ± 0.1, and the flavin N(3) pK_a was found to be 9.7 ± 0.1. These values are in excellent agreement with those values determined by other methods (Williamson & Edmondson, 1985a).

The pH dependence of the respective chemical shift values of 8α-*N*-imidazolyl-1,5-dihydroriboflavin (Figure 9) shows a single proton ionization due to the 8α-imidazole substituent with a pK_a of 7.0. This value is also in excellent agreement with that found by the pH dependence of the oxidation–reaction potential (Williamson & Edmondson, 1985a). The upfield chemical shifts of the imidazole protons on imidazole deprotonation (Table I) are quite similar to those changes observed in the oxidized flavin. The Fl(8α)-CH_2 and Fl C(9)-H resonances are shifted upfield by 0.21 and 0.15 ppm, respectively, which are ~0.1 ppm less than those observed with the corresponding oxidized form. As found with the oxidized flavin, the Fl C(6)-H and Fl(7α)-CH_3 chemical shifts in the reduced flavin are unaffected by the protonation–deprotonation of the 8α-imidazole substituent. It is of interest to note that N(1) ionization (pK_a = 5.5; Williamson & Edmondson, 1985a) has no influence on the chemical shift values of either the reduced flavin or the imidazole proton resonances.

Assignment and Effect of pH on Ribityl Side Chain Proton Resonances of Oxidized and Reduced Forms of 8α-N-Imidazolylriboflavin. To provide further information on the unusual NMR spectral properties described above for the reduced flavin (reduced NOE enhancement, geminal coupling of the 8α-CH_2 resonances), a detailed study was undertaken on the ribityl side chain NMR spectral properties of the oxidized and reduced forms. ^{1}H NMR spectral data for FMN and FAD (Kainosho & Kyogoku, 1972; Sarma et al., 1968;

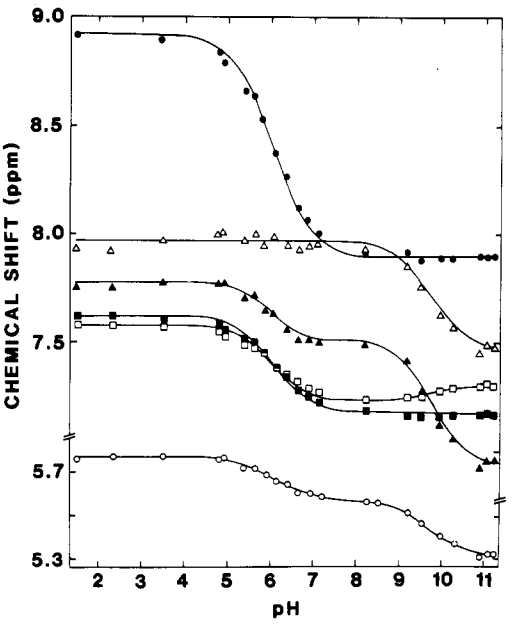

FIGURE 8: Estimation of pK_a values for 8α-*N*-imidazolylriboflavin. The data points are the chemical shifts of Im C(2)-H (●), Fl C(6)-H (△), Fl C(9)-H (▲), Im C(5)-H (■), Im C(4)-H (□), and Fl (8α)-CH_2 (○). The solid lines are theoretical curves for two one-proton ionizations of pK_a = 6.04 and 9.70.

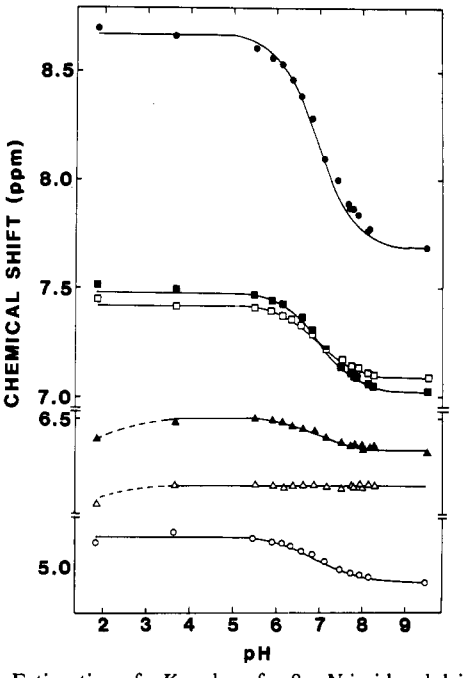

FIGURE 9: Estimation of pK_a values for 8α-*N*-imidazolylriboflavin hydroquinone. The symbols used are as in Figure 8. The solid lines are theoretical curves for a one one-proton ionization of pK_a = 7.0.

Kotowycz et al., 1969) have shown the properties of the side chain C(1′) methylene protons to be sensitive indicators of intermolecular stacking interactions of the flavin in aqueous solution. Indeed, the formation of flavin dimers in both the oxidized and reduced forms in aqueous solutions has been known since the work of Gibson et al. (1962). Recent work in this laboratory (Williamson & Edmondson, 1985b) has shown that the stacking of riboflavin analogues in aqueous solution leads to a geminal coupling of the two protons on the C(1′) side chain position.

The experimental evidence to support this finding is the demonstration that no C(1′) methylene geminal coupling is observed with aqueous acidic solutions of riboflavin in which aggregation is prevented by protonation of the isoalloxazine

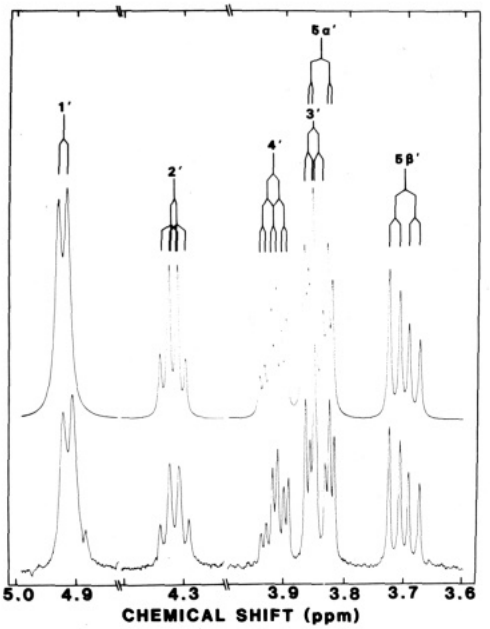

FIGURE 10: NMR spectrum of the ribityl side chain protons of 8α-*N*-imidazolylriboflavin (oxidized form). The side chain resonances of 8α-*N*-imidazolylriboflavin (10 mg/mL) at pH 1.4 (lower spectrum) were analyzed by decoupling, and the information was used to construct the simulation (upper spectrum; 1′ protons, 4-Hz line width; other protons, 2-Hz line width).

ring. The chirality of the C(2′), C(3′), and C(4′) centers of the ribityl side chain does not contribute to magnetic nonequivalence of the C(1′) methylene protons when flavin dimerization is prevented. Geminal coupling of the C(1′) methylene protons is observed, however, in neutral aqueous solutions of N^3-(carboxymethyl)riboflavin, in which, like FMN, the anionic charge is sufficiently remote from the isoalloxazine ring so as to be ineffective in the prevention of flavin dimerization in aqueous solution. This geminal coupling as well as the individual couplings of the H_a and H_b protons of the C(1′) to the C(2′)-H is supportive of a constrained conformation of the ribityl side chain such as that observed in the folded form of FAD and in the stacking of FMN molecules in aqueous solution. Unhindered rotation about the N(10)–C(1′)–C(2′) bonds would result in the magnetic equivalency of the C(1-) protons leading to simple first-order coupling. Such a behavior is, in fact, observed in acidic solutions of riboflavin in which the ring system is protonated, thereby preventing intermolecular stacking (Williamson & Edmondson, 1985b).

Examination of the side chain resonances of oxidized 8α-*N*-imidazolylriboflavin in Figure 10 shows that the 1′-CH_2 protons are magnetically equivalent. The 1′-CH_2 resonance is split into a doublet and integrates to two protons. The C(2′)-H integrates to a single proton and is an apparent quartet due to coupling to the 1′-CH_2 and the C(3′)-H (Figure 9, Table III). On irradiation of the C(1′) protons, the C(2′) resonances collapse into a doublet (J = 4.7 Hz) due to its coupling to the C(3′)-H. Conversely, irradiation of the C-(2′)-H results in a collaspe of the 1′-CH_2 doublet to a singlet, which confirms the assignments listed in Table III. Simulation of the spectral data (Table III, Figure 10) gave good agreement with the experimental spectrum (Figure 9). Confirmation of the assignment of the 5′-CH_2 resonances was achieved by comparing its position and splitting pattern with those of riboflavin and 5′(*RS*)-monodeuterioriboflavin (Williamson & Edmondson, 1985b).

The spectrum in Figure 11 shows the portion of the ^1H NMR spectrum of reduced 8α-*N*-imidazolylriboflavin due to

Table III: Proton–Proton Spin–Spin Coupling Constants for Ribityl Side Chain Protons of Oxidized and Reduced 8α-*N*-Imidazolylriboflavin and Comparison with Those Values Determined for Other Flavin Analogues[a]

	coupling constant (Hz)				
	8α-*N*-imidazolyl-riboflavin		N^3-(carbo-xymethyl)-riboflavin	FMN[b]	FAD[b]
assignment	oxidized	reduced	(oxidized)	(oxidized)	(oxidized)
$1'H_a$–$1'H_b$	0	16	12.5	14.0[c]	14.5[c]
$1'H_a$–2′H	5.4	9.0	9.6	10[c]	10[c]
$1'H_b$–2′H	5.4	1.8	2.6	2.3[c]	2[c]
2′H–3′H	4.7	5.0	4.8	4.5[c]	4.5[c]
3′H–4′H	6.7	7.0	6.5	ND[d]	8.0[c]
4′H–$5'H_a$	2.95	2.5	2.9	ND[d]	ND[d]
4′H–$5'H_b$	6.7	5.7	6.5	ND[d]	ND[d]
$5'H_a$–$5'H_b$	11.9	11.6	12.0	ND[d]	ND[d]

[a] Values were those used in successful simulations of experimental spectra recorded at 22–25 °C. [b] Values taken from Kainosho & Kyogoku (1972). [c] These values were determined at 51 °C. [d] ND, not determined.

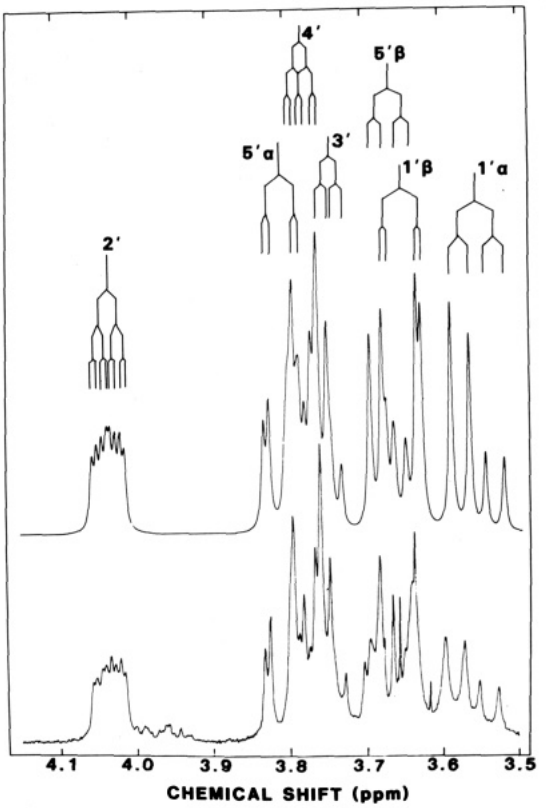

FIGURE 11: NMR spectrum of the ribityl side chain protons of 8α-*N*-imidazolylriboflavin hydroquinone. Oxidized flavin (8 mg/mL) at pH 2.5 was reduced by a 3-fold molar excess of sodium dithionite (lower spectrum). The upper spectrum is the best-fit simulation (2-Hz line width).

the ribityl side chain protons (3.5–4.15 ppm). Assignments were made by selective decoupling experiments and by agreement of the simulated with the experimental spectrum. The fit of the simulated spectrum is not as precise as is the case for the oxidized flavin due to extensive overlap of the resonances and to the variety of resonance line widths observed. With the exception of the C(5′) protons, all of the ribityl resonances are shifted downfield on reduction of the isoalloxazine ring (Table III). The C(1′) protons exhibit the largest downfield shift (~1.2 ppm). The estimated side chain ^1H–^1H coupling constants of the reduced flavin show no major changes when compared with those of the oxidized flavin (Table III), which suggests the side chain conformation to be similar. The

1′-CH_2 protons exhibit a geminal coupling that results in the splitting of the C(2′)-H into a doublet of a doublet of doublets. The observed geminal coupling constant of the 1′-CH_2 protons (16.1 Hz) is of the same order of magnitude as that observed for the 8α-CH_2 protons (see above) and is somewhat larger than those observed for FMN and for FAD (cf. Table III). Thus, by analogy with the oxidized flavin analogues considered above, the 1′-CH_2 geminal coupling is suggestive of intermolecular dihydroflavin stacking, which is not observed for the oxidized flavin. This behavior is then also suggested to give rise to the magnetic nonequivalence of the 8α-CH_2 protons and to the diminished NOE effects observed above for the reduced flavin.

Discussion

The results presented above provide a comprehensive assignment of both ring and side chain resonances in both the oxidized and reduced forms and provide direct confirmation of the conclusions reached from the pH dependence of the oxidation–reduction potential of 8α-*N*-imidazolylriboflavin presented in a previous paper (Williamson & Edmondson, 1985a). That is, the pK_a of the imidazole ring in the oxidized flavin is equal to 6, which is increased by 1 pH unit to a value of 7 on reduction of the flavin by two electrons. These data show that the pK_a values determined by redox potential and other measurements reflect intrinsic pK_a values rather than apparent pK_a values due to linked ionizations. The chemical shift data on the imidazolyl protons (Table I; Figures 8 and 9) show a sensitivity to imidazolyl protonation–deprotonation in both the oxidized and reduced forms but little or no sensitivity to N(1) ionization of the reduced flavin or to N(3) ionization of the oxidized form. In contrast, the chemical shift values of the flavin protons at positions 8α, 9, and C(1′) are sensitive to the ionic form of the 8α-imidazolyl substituent in both the oxidized and reduced forms. These data suggest the distribution of flavin ($\sigma + \pi$)-electron density (at least at the above positions mentioned) to be sensitive to the ionic form of the imidazole ring.

The results on the hydroquinone form of the 8α-*N*-imidazolylriboflavin are, to our knowledge, the first comprehensive ^{1}H NMR data on flavins at this redox level. Previous work has been mainly focused on ^{13}C and ^{15}N NMR studies of flavin analogues in their oxidized and hydroquinone forms (Moonen et al., 1984; Kawano et al., 1978). Care is required in such experiments to prevent even trace semiquinone formation, which would result in extensive line broadening of the resonances. The observed geminal couplings of the methylene protons at the 8α- and 10α-positions appear to be a result of intermolecular ring stacking in contrast to the oxidized form. This behavior occurs when the reduced isoalloxazine is neutral and the 8α-imidazole is positively charged. Due to limited solubility of the reduced flavin at intermediate pH values (around neutrality) where the flavin would be zwitterionic or negative, the complexity of the ribityl side chain spectra and the lower signal to noise ratio of the 8α-methylene group resonance precluded detailed study of the effect of pH on the geminal coupling (and hence ring stacking) of these methylene groups. Further work needs to be done on the physical association of the reduced flavin molecules. It is unlikely they would complex according to the models put forth by Sarma et al. (1968) or by Kainosho & Kyogoku (1972) as this would put two positively charged imidazole rings in an energetically unfavorable close proximity. Whatever the geometry of the interaction, it appears to restrict the rapid rotation of the imidazole ring with respect to the flavin ring and, similarly, also restricts the mobility of the ribityl C(1′) methylene group with respect to the isoalloxazine ring. No major changes in imidazole ring proton coupling constants or in chemical shift values are observed, and thus we can conclude that the pK_a value for the imidazole ring is not influenced to any significant degree.

The chemical shift data presented here in comparison with "normal" flavins suggest that useful information could be obtained on the distribution of π-electron densities in the flavin ring system of this model flavin by employing ^{13}C and ^{15}N NMR spectral approaches such as has been done with "normal" flavins. In addition, such studies may also provide information on the effect of 8α-imidazole substitution and/or ionization on the conformation of the reduced flavin in solution as has recently been published with the unsubstituted flavins (Moonen et al., 1984). These experiments would provide a useful basis for future investigations of flavoenzyme systems containing 8α-substituted histidylflavins.

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Cooperative Binding of Manganese(II) to Chloroplast Coupling Factor 1 Detected by NMR Proton Relaxation Enhancement†

Alice E. Haddy,‡ Wayne D. Frasch,*,§ and Robert R. Sharp‡
Department of Chemistry and Department of Cellular and Molecular Biology, The University of Michigan, Ann Arbor, Michigan 48109
Received April 15, 1985

ABSTRACT: The binding of divalent manganese to soluble latent spinach chloroplast coupling factor 1 (CF_1) was examined by measurements of the water proton spin–lattice relaxation enhancement (PRE), revealing positive cooperativity between high-affinity sites. A method that used only a single enhancement parameter, ϵ_b, for the quantitation of cooperative PRE data was derived. Application to the high-affinity sites yielded a value of 9.01 ± 0.11 for ϵ_b. Two high-affinity sites participated in cooperative binding, although the possibility that a third site was present was not eliminated. The apparent binding constant to site ratio, K/n, was found to be 3.4–3.7 μM, giving a value for K of approximately 7 μM.

The soluble chloroplast coupling factor 1, CF_1,[1] which is the photosynthetic adenosinetriphosphatase, is composed of five distinguishable peptides with the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ (Merchant et al., 1983; Moroney et al., 1983). Evidence that the active site is on the β subunit (Carlier et al., 1979; Bruist & Hammes, 1981) suggests that there are three active sites. In both CF_1 and its mitochondrial counterpart, MF_1, the sites appear to be strongly coupled during the catalytic cycle (Boyer, 1979). This conclusion is supported by the observation that rapid turnover of the enzyme requires the binding of substrate to two of the three active sites (Grubmeyer & Penefsky, 1981; Cross et al., 1982). X-ray diffraction analysis does not support a simple 3-fold symmetry of subunits, suggesting structural inequivalence of the β subunits (Amzel et al., 1982).

Any of several divalent metal ions, including Mg(II), Mn(II), and Ca(II), is required for enzymatic activity (Nelson et al., 1972; Hochman et al., 1976). The metal cofactor coordinates to the phosphates of the nucleotide substrate at the active site with a specific stereochemistry to enable catalysis (Frasch & Selman, 1982). The most effective cofactor depends on the method of activation of the CF_1 ATPase. The thiol-activated ATPase has the highest rate of activity in the presence of Ca(II) (Farron & Racker, 1970); Mn(II) and Mg(II) are more effective at submillimolar concentrations but become inhibitory at higher concentrations (Hochman et al., 1976). Recently, it has been found (Pick & Bassilian, 1981) that octyl glucoside activates a Mg(II)- or Mn(II)-ATPase of soluble CF_1 that is not inhibited by high concentrations of these metals and, thus, more closely resembles the membrane-bound ATP synthase.

A recent study of the binding of Mn(II) to latent lettuce CF_1 was carried out with ESR to measure free Mn(II) (Hiller & Carmeli, 1985). In this study, CF_1 exhibited three cooperating Mn(II) binding sites with an apparent dissociation constant of 15 μM and a Hill coefficient of 2.9. Three low-affinity Mn(II) binding sites with a dissociation constant of 47 μM were also found, as well as several lower affinity nonspecific sites. However, the degree of cooperativity and the binding constants were found to depend on the previous history of the enzyme.

We have studied the binding of Mn(II) to latent CF_1 by using proton relaxation enhancement (PRE) of the solvent (Mildvan & Cohn, 1970). This technique provides a direct, sensitive probe of enzyme-bound Mn(II) and is particularly useful for characterizing metal binding at low mole ratios of metal to enzyme, where sigmoidicity due to cooperative interactions is most pronounced. The PRE data show clear evidence of positive cooperativity of Mn(II) binding to CF_1.

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‡ Department of Chemistry.
§ Department of Cellular and Molecular Biology.

[1] Abbreviations: PRE, proton relaxation enhancement; CF_1, chloroplast coupling factor 1; MF_1, mitochondrial coupling factor 1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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