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Reinvestigation of the Structure of Oxidized and Reduced Flavin: Carbon-13 and Nitrogen-15 Nuclear Magnetic Resonance Study†

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ABSTRACT: Several chemically substituted flavins are investigated in the oxidized and the reduced state by ^{13}C and ^{15}N NMR techniques. The dependence on the polarity of the solvent and on the concentration is studied. In combination with already published results, a semiempirical theory is developed to interpret the chemical shifts in terms of the solution structure of flavins. Where possible, the results are compared with crystallographic and light absorption data. In contrast to common ideas, the solution structure of the oxidized state is not fully coplanar, but the N(10) atom is situated out of plane to a certain degree. Polarizing the flavin by hydrogen bonds in a high dielectric medium moves the N(10) atom into the molecular plane, and the flavin molecule becomes coplanar. In the coplanar molecule, π electrons are delocalized from the N(10) atom mainly to O(2α) and O(4α). The NMR results show that the solution structure of reduced flavin is mainly governed by sterical hindrance and hydrogen bonds. The findings are in contrast to commonly accepted ideas that reduced flavin is strongly bent. In an apolar solvent, the reduced neutral isoalloxazine is only slightly bent. The formation of hydrogen bonds in a protic solvent of a high dielectric constant decreases the bend. The N(10) atom is now almost fully sp^2 hybridized, and the N(5) atom has an endocyclic angle of 115–117°, indicating its predominant sp^2 character. The results have several important implications for flavin catalysis. Among these, it is shown that the altered redox potential of the semiquinone–fully reduced redox couple of flavodoxin is probably not caused by the planarity of the reduced protein-bound $FMNH^-$.

The flavins are especially remarkable among the various known natural redox coenzymes because of two outstanding features: (i) they can function as one-electron as well as two-electron redox carriers; (ii) they act with considerable efficiency in a wide variety of enzymatic reactions. As a consequence, the flavins are known as very versatile redox coenzymes. This versatility suggests that nature has many possibilities to "tune" the function of the flavin. This has led to the proposal that specific interactions between flavin and apoflavoprotein play a particular role in determining the pathway of flavin catalysis (Müller, 1972; Müller et al., 1970; Hemmerich & Massey, 1982). Other factors such as mobility of the flavin (Moonen & Müller, 1983), microenvironment of the flavin binding site, and the planarity of the reduced

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flavin molecule (Tauscher et al., 1973; Simondsen & Tollin, 1980) may also be important.

Nuclear magnetic resonance is a powerful tool to test these hypotheses as, in principle, the modulation of the structure and electron density can be monitored with ^{13}C and ^{15}N NMR techniques (Van Schagen & Müller, 1981; Franken et al., 1984). Moreover, dynamic information can also be obtained by the NMR method on various time scales (Moonen & Müller, 1983, 1982; Moonen et al., 1982). A detailed ^{15}N (Franken et al., 1984) and ^{13}C (Van Schagen & Müller, 1981) NMR study of *Megasphaera elsdenii* flavodoxin actually showed that some specific interactions could be unmambiguously assigned. No satisfying explanation could be given, however, for some remarkable chemical shifts in ^{13}C and ^{15}N spectra. Among these, we mention the chemical shift of the N(10) atom of protein-bound FMN in both the oxidized and the reduced state. The chemical shifts due to C(10a) and C(4a) could not be explained satisfactorily either. The difficulties in the interpretation of the chemical shifts exist not only for protein-bound flavins but also for free flavins. Although extensive ^{13}C and ^{15}N NMR studies have been carried out on free flavins (Grande et al., 1977a; Van Schagen & Müller, 1980; Yagi et al., 1976; Kawano et al., 1978), some chemical shift changes still remain unexplained. Among these are the chemical shifts due to N(10), C(10a), and C(4a), as well as C(7), C(5a), and C(9a), both in the oxidized and reduced flavin. The difficulties in the interpretation probably originate from the various possibilities to modify the structure and the electron density of flavins by the environment, which might be related to the biological versatility of flavins. We report here a reinvestigation of oxidized and reduced flavins by NMR techniques, using various chemically modified flavins. The aim of this detailed study is to detect the various possibilities of modulating the physical and chemical properties of flavins and to provide a basis for the interpretation of ^{13}C and ^{15}N NMR results of flavoproteins.

Materials and Methods

^{13}C- and ^{15}N-substituted flavins were prepared as described previously (Van Schagen & Müller, 1981; Moonen & Müller, 1983; Müller et al., 1983). Tetraacetylriboflavin (TARF) was prepared from riboflavin by acetylation in a mixture of $CH_3COOH/(CH_3CO)_2O$ in the presence of a small amount of $HClO_4$ (Müller, 1971). Phosphorylation of riboflavin derivatives was done according to the procedure of Scola-Nagelschneider & Hemmerich (1976). The synthesis of all other compounds has been described previously (Grande et al., 1977b; Van Schagen & Müller, 1980).

Wilmad 10-mm precision tubes were used. The samples contained about 3 mM flavin, if ^{13}C- and ^{15}N-enriched compounds were used, unless otherwise stated. Natural abundance ^{13}C and ^{15}N NMR measurements were performed with samples containing 20–200 mM flavin. The sample volume was 1.6 mL. Aqueous samples contained 10% ^2H_2O for ^{13}C NMR and 100% ^2H_2O for ^{15}N NMR (to exchange NH protons for deuterons). pH Measurements were done before and after the NMR measurements and are reported without correction for isotope effects. Aqueous samples contained 100 mM potassium phosphate, pH 8.0. Reduction was conducted by the addition of the desired amount of a dithionite solution to the anaerobic solutions. Anaerobiosis was achieved by carefully flushing the solutions in the NMR tube with argon for about 20 min. The NMR tube was sealed with a serum cap. Reduction of oxidized flavin solutions in C^2HCl_3 to the 1,5-dihydro state was effected directly in the NMR tube by vigorous shaking of a two-phase solution consisting of the flavin solution in C^2HCl_3 and an aqueous solution of 0.5 M potassium phosphate (pH 8.0, saturated with KCl) containing a 10-fold excess of sodium dithionite with respect to the flavin.

Measurements were performed on a Bruker CXP 300 NMR spectrometer operating at 30.4 MHz for ^{15}N and 75.6 MHz for ^{13}C NMR measurements. Broad-band decoupling of 2 W was applied for ^{13}C NMR. No decoupling was applied for ^{15}N NMR. The temperature of all samples was kept constant at 26 ± 2 °C. All spectra were recorded with 30° pulses and a repetition time of 0.8–1.3 s. Dioxan was used as an internal reference in aqueous solution and Me_4Si in C^2HCl_3 solutions for ^{13}C NMR ($\delta_{dioxan} - \delta_{Me_4Si} = 67.8$ ppm). Chemical shifts are expressed relative to Me_4Si. Neat $CH_3{}^{15}NO_2$ was used as an external reference in all solutions for ^{15}N NMR with a coaxial cylindrical capillary (Witanowski et al., 1981). Chemical shifts are reported as true shieldings (i.e., corrected for bulk volume susceptibilities) relative to liquid NH_3 ($\delta_{CH_3NO_2} - \delta_{NH_3} = 381.9$) (Witanowski et al., 1981; Levy & Lichter, 1979).

Results and Discussion

^{13}C NMR studies of substituted benzene derivatives have provided empirical justification for the idea that changes in π-electron densities are primarily responsible for the observed shielding variations (Lauterbur, 1962). Karplus & Pople (1963) developed a theory that supports this notion. Lauterbur (1965) showed that simple aromatic heterocycles also follow this rule. Pugmire & Grant (1971) investigated more complex aromatic heterocycles and concluded that the parameters derived from simple heterocycles can be satisfactorily applied. Up till now, several studies confirmed that the semiempirical relationship between π-electron density and ^{13}C chemical shift in aromatic systems is valid [Levy & Lichter (1979) and references cited therein].

The same rules can be applied for ^{15}N chemical shifts, although with some caution with regard to the marked differences between pyridine- and pyrrole-type nitrogens [Witanowski et al. (1981) and references cited therein]. The explanation of the ^{15}N chemical shift of a deprotonated nitrogen atom in heteroaromatic systems is theoretically more difficult to explain. The investigated systems always follow the same rules however, in that the deprotonated atom shifts considerably downfield, although the electron density is increased (Pugmire & Grant, 1968, 1971; Quirt et al., 1974). These studies showed in addition that the atoms in α-position to the deprotonation site show also a downfield shift; for all other atoms, the π electron increase or decrease governs the chemical shift.

Van Schagen & Müller (1980) showed that, assuming a "butterfly" conformation of reduced flavin along the N(5)–N(10) axis, a good agreement exists between the π-electron density and the ^{13}C chemical shift of a particular atom. In the oxidized state, assuming a flat aromatic system, the agreement is less satisfying, and this fact could not be explained at that time.

In the interpretation of our results we consider, taking into account the above-mentioned semiempirical rules, the following parameters [for a discussion see Levy & Lichter (1979) and Witanowski et al. (1981)]: (i) the degree of hybridization, (ii) the π-electron density, (iii) the different behavior of pyridine-and pyrrole-type nitrogen atoms, and (iv) the influence of a negative charge on the deprotonation site and on the neighboring atoms (only relevant for the interpretation of anionic reduced flavin).

Oxidized Flavin. The oxidized isoalloxazine ring of flavin has commonly been assumed to be a flat aromatic ring system.

Table I: Relevant ^{13}C Chemical Shifts of Various Oxidized Flavins

compd	solvent	^{13}C chemical shift (ppm)[a]											
		C(2)	C(4)	C(4a)	C(5a)	C(6)	C(7)	C(8)	C(9)	C(9a)	C(10a)	C(10α)	C(8α)
[2,4,4a,10a-$^{13}C_4$]FMN[b]	$H_2O/^2H_2O$[c]	159.8	163.7	136.2							152.1		
FMN[d]	$H_2O/^2H_2O$[c]	159.0	162.1	134.9	135.4	132.8	140.6	151.9	118.2	131.5	151.0	48.8	22.0
MeIMN	$H_2O/^2H_2O$[c]	159.2	162.6	136.9	136.8	131.7	140.5	140.2	118.5	132.8	151.3	49.2	
TARF	C^2HCl_3	155.2	159.8	135.6	134.6	132.8	136.6	147.5	115.5	131.2	149.1	44.5	21.4
TARF	C^2HCl_3/MeOH[e]	155.9	161.4	136.0	134.9	133.1	136.8	147.8	115.6	131.9	149.2	45.3	21.4
MeLfl	C^2HCl_3/MeOH[e]	156.7	160.6	135.5	135.0	132.6	137.5	148.8	115.7	132.0	149.2	32.2	21.6
MeLfl	DMF[f]	155.9	160.5	137.1	135.0	132.8	136.7	147.7	117.0	132.0	150.2	32.1	20.8
Me_2IA	C^2HCl_3/MeOH[e]	156.5	160.3	136.7	136.2	132.6	137.9	138.2	115.4	131.7	149.3	32.3	

[a] Relative to Me_4Si. [b] Chemical shifts extrapolated to infinite dilution. [c] Mixture contained 10% ^2H_2O (by volume). [d] Concentration is 40 mM. [e] Mixture contained 10% MeOH (by volume). [f] Dimethylformamide.

Table II: ^{15}N Chemical Shifts of Oxidized Flavins As Dependent on Solvent and Concentration

compd	concn (mM)	solvent	^{15}N chemical shift (ppm)[a]			
			N(1)	N(3)	N(5)	N(10)
[1,3,5-$^{15}N_3$]FMN	4	^2H_2O[b]	190.8	160.5	334.7	
FMN[c]	120	^2H_2O[b]	192.9	161.1	334.4	162.5
[1,3,5,10-$^{15}N_4$]MeIMN	0.6	^2H_2O[b]	190.5	160.4	335.5	164.6
[1,3,5,10-$^{15}N_4$]MeIMN	6	^2H_2O[b]	191.1	160.4	335.6	164.3
TARF[c]	350	C^2HCl_3	199.9	159.8	344.3	150.2
TARF[c]	350	C^2HCl_3/MeOH[d]	198.8	159.8	343.4	151.4
[1,3,5,10-$^{15}N_4$]Me_2TARI[e]	7	C^2HCl_3	201.1	160.7	346.7	150.4

[a] Relative to NH_3 (cf. Materials and Methods). [b] p^{2}H is 7.5. [c] Natural isotope abundance ^{15}N NMR spectra. [d] Mixture contained 10% MeOH (by volume). [e] From Franken et al. (1984).

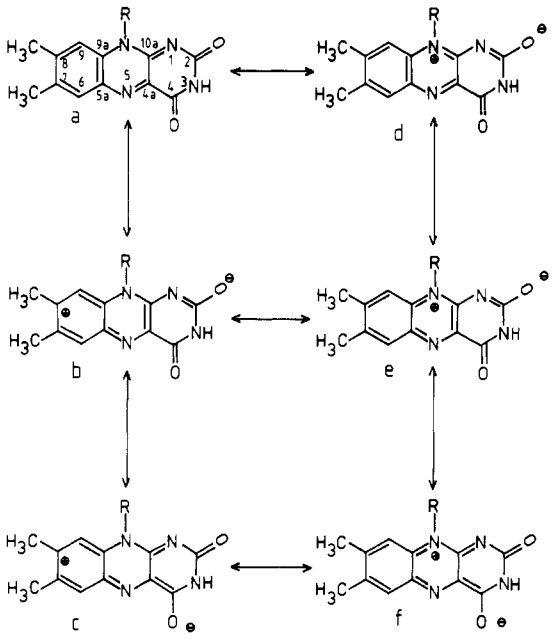


FIGURE 1: Possible mesomeric structures of oxidized flavin as deduced from NMR results.

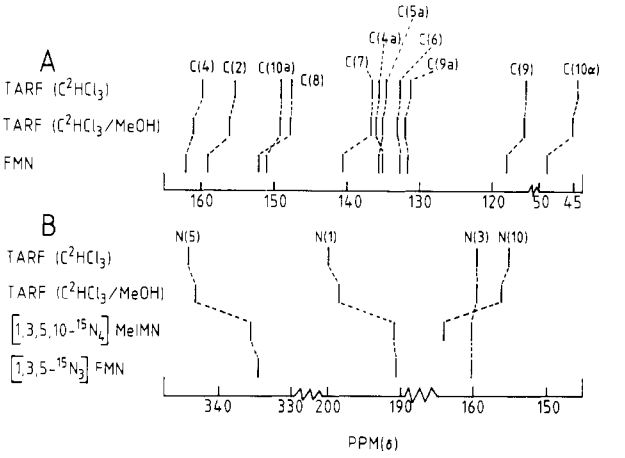


FIGURE 2: Correlation diagram of ^{13}C and ^{15}N NMR chemical shifts of oxidized flavins. The ^{15}N chemical shifts of TARF are from natural isotope abundance ^{15}N NMR spectra.

It is generally accepted that the carbonyl function at C(2) plays a major role when the molecule is polarized in polar solvents (structure b, Figure 1). In keeping with this idea, the partial positive charge is smeared out, *via* mesomeric structures, over the C(8), C(10a), N(5), C(6), and C(9a) atoms. In this generally accepted picture, it is important to note that the N(10), N(3), C(4), C(9a), and C(7) atoms are not involved in the mesomeric structure.

Polarization of the C(2) carbonyl function in aqueous solutions gives rise to a π-electron decrease (hence a downfield shift) at C(2) and subsequently via mesomeric structures at C(10a), N(5), C(6), C(8), and C(9a). The downfield shift of C(7) and C(9) shown in Figure 2A and Table I cannot be explained solely by structure b (Figure 1). Evidently, structure b gives a too simplified picture of the polarized isoalloxazine ring. To describe the polarized structure of oxidized flavin more accurately, it is necessary also to include the ^{15}N chemical shifts.

In heteroaromatic systems the nitrogen atoms can roughly be divided in pyrrole- or α-type and pyridine- or β-type nitrogen atoms (Witanowski et al., 1972). The chemical shifts of the two classes of nitrogen atoms depend differently on the polarity of the solvent. The origin of these effects lies in the orthogonality of the electron lone pair with respect to the aromatic system in pyridine-type nitrogens [N(1) and N(5) in flavin] and in the incorporation of the electron lone pair into the system in pyrrole-type nitrogens [N(3) and N(10) in flavin]. The chemical shifts of pyridine-type nitrogens show a strong upfield shift on going from apolar to aqueous solution whereas those of the pyrrole-type nitrogens exhibit a smaller and opposite effect. As shown in Figure 2B and Table II, the chemical shifts of the N(1), N(5), and N(3) atoms in flavin behave as expected on the basis of the above-given classification. The large upfield shift of the resonances due to N(1) and N(5) observed in going from apolar solvents to aqueous solution indicates hydrogen-bond formation between these atoms and water molecules, as already previously shown (Franken et al., 1984). The chemical shift of the N(3) atom

shows the expected solvent-dependent shift for a pyrrole-type nitrogen. However, the chemical shift of the N(10) atom is unexpected, and a drastic downfield shift, amounting to 15 ppm, is observed. This observation strongly indicates that the N(10) atom is not as isolated as commonly assumed, but is strongly affected by polarization of the molecule.

As the N(10) atom in flavin is a substituted pyrrole-type nitrogen, not able to form hydrogen bonds with the solvent, the large downfield shift must be ascribed to a hybridization effect; i.e., in aqueous solutions N(10) possesses more sp^2 character than in apolar solutions. This implies that N(10) must be out of plane to some degree in apolar solvents and more in plane in polar solvents. This configurational change of the N(10) atom is independent of the substituent at N(10) (Table I) and is therefore an intrinsic property of the isoalloxazine ring. It should be noted that the chemical shift due to the C(10α) atom parallels that of the N(10) atom (Figure 2). The flat structure in polar solutions can be rationalized by the mesomeric structures d–f (Figure 1). The creation of a partial positive charge on the N(10) atom can be expected to lead either to an upfield shift of the resonances due to N(1), N(3), C(4a), C(5a), C(7), and C(9) (increase of π-electron density) or to a downfield shift of these resonances, owing to delocalization of the partial positive charge. Although these effects might influence the resonance position of N(1) and N(3) to some degree, the observed upfield shifts are caused predominantly by hydrogen-bond formation (cf. above). These two nitrogen atoms are therefore not further considered in the following. With respect to the carbon atoms, all of them are shifted downfield, indicating that the π-electron density at these sites is not increased when the flavin is polarized and that the observed downfield shifts are caused by delocalization of the partial positive charge of the N(10) atom. It should be noted that the small sensitivity of the C(4a) resonance to solvent polarity indicates that the two opposing effects are almost cancelling each other at C(4a). It must therefore be concluded from the ^{13}C NMR spectra that O(2α) and O(4α) are the main π-electron acceptors upon polarization of flavin. The resulting mesomeric structures are stabilized by hydrogen bonds to the two oxygen atoms. Mesomeric structures d–f (Figure 1) are thus in agreement with the NMR results.

It could be argued that O(4α) receives π-electron density from N(3). The chemical shift of N(3) is only slightly influenced by the polarity of the solvent (Figure 2, Table II), strongly indicating that O(4α) receives π-electron denstiy mainly from the N(10) atom when the flavin is polarized. This interpretation is in perfect agreement with coherent anti-Stokes Raman spectra of flavins (Müller et al., 1983). These results have shown that the frequencies of the Raman modes are more influenced by protic solvents than by heavy-atom substitution of, e.g., the N(3)H group. This effect has been ascribed to a change of bond hybridization throughout the entire system when the flavin is polarized and hydrogen bonds are formed. The NMR results give now detailed insights into this change of hybridization and should facilitate a more detailed interpretation of the Raman spectra of flavins.

In the following, the effects on the ^{13}C chemical shifts observed on increasing the polarity of the solvent will be briefly discussed. Adding methanol to TARF in chloroform leads to a relatively strong polarization of C(2) and C(4). The indriectly polarizable atoms, e.g., C(7), C(8), C(9), and C(10a), exhibit only minor shifts, if any. In aqueous solutions, on the other hand, large shifts are also evident for the indirectly polarizable atoms, and C(2) and C(4) show an additional shift. This indicates that C(2) and C(4) already polarize when some

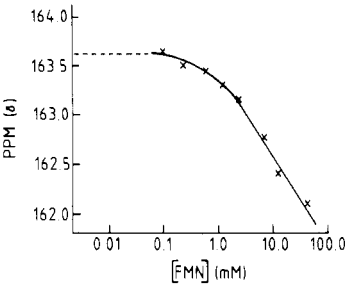


FIGURE 3: Concentration dependence of the ^{13}C chemical shift due to C(4) of [4-^{13}C]FMN in aqueous solution.

methanol is present, i.e., when the solvent is able to form hydrogen bonds. On the other hand, a protic solvent of a high dielectric constant is needed to polarize the indirectly polarizable atoms.

It has already been shown (Sarma et al., 1968; Kotowycz et al., 1969; Kainosho & Kyogako, 1972; Moonen & Müller, 1982; Franken et al., 1984) that FMN in aqueous solution forms aggregates. In Figure 3, the ^{13}C chemical shift of the C(4) atom of FMN is shown in dependence of the concentration. A rather strong concentration dependence of the ^{13}C chemical shift is observed. Similar effects are also observed for C(4a), C(2), and C(10a). The chemical shift values extrapolated to infinite dilution are presented in Table I. The observation that stacking is strongly inhibited at concentrations below 0.1 mM is in excellent agreement with light absorption studies (Müller et al., 1973). The ^{13}C NMR results also indicate that stacking of FMN inhibits its full polarization as evident from the extrapolated ^{13}C chemical shifts (Table I). We suggest that stacking leads to a microenvironment with a lower effective dielectric constant than that of the pure solvent, preventing full polarization of the molecule. It should also be noted that the C(4) resonance is most influenced by stacking. Under this condition, the hydrogen bond to O(4α) is weaker than in dilute solution (Table I). The concentration dependence of the C(4) and C(4a) resonances strongly suggests that C(4a) becomes an important π-electron acceptor in the absence of hydrogen bonding to O(4α) and at the same time renders O(4α) to a less favorable π-electron acceptor. This interpretation is in agreement with the fact that in monomeric FMN C(4a) shifts downfield, indicating transfer of electron density to O(4α).

Similarly, a comparison of the ^{15}N chemical shifts as dependent on the concentration shows that in $CHCl_3$ (Table II) the chemical shifts due to N(1), N(3), and N(5) in TARF (natural isotope abundance spectra) are shifted somewhat upfield as compared to those of Me_2TARI at lower concentration. The latter molecule carries a methyl group at N(3) preventing association by intermolecular hydrogen-bond formation. In the former molecule, such interactions are possible and are reflected in a slight upfield shift of the resonances due to N(1), N(3), and N(5). This interpretation is in full accordance with the results of TARF in $CHCl_3$/MeOH (Table II). More important, however, is the observation that the chemical shifts due to the nitrogen atoms in TARF and Me_2TARI in $CHCl_3$ and of the corresponding compounds in aqueous solution are very similar. This indicates that the absence of the C(8) methyl group does not influence the particular role of the N(10) atom on polarization. This observation is also important for our studies on flavoproteins where only MeIMN is available for ^{15}N NMR studies to investigate all four nitrogen atoms of the prosthetic group.

The particular behavior of the N(10) atom in flavin is also supported by crystallographic data. Such data on oxidized

Table III: Relevant ^{13}C Chemical Shifts of 1,5-Dihydroflavins As Dependent on Substitution, Concentration, and Solvent

compd[a]	concn (mM)	solvent	^{13}C chemical shifts (ppm)[b] C(2)	C(4)	C(4a)	C(5a)	C(6)	C(7)	C(8)	C(9)	C(9a)	(10a)
5a	50	C^2HCl_3	152.2	158.5	103.8	135.2	122.8	132.7	131.3	114.5	138.2	145.6
5b	50	C^2HCl_3	151.5	157.7	111.8	137.7	123.7	134.3	129.8	115.6	131.2	138.4
5c	50	C^2HCl_3	151.7	159.0	113.3	141.6	122.2	133.8	130.7	119.7	137.4	146.7
5d	50	C^2HCl_3	152.4	158.2	98.1	129.0	127.6	132.5	135.1	115.8	134.9	147.9
5e	50	C^2HCl_3	152.2	158.3	105.8	133.0	126.7	133.2	135.3	119.3	138.6	150.8
5f	50	C^2HCl_3	150.4	158.4	93.3	124.9	128.3	132.4	134.4	113.8	134.3	148.5
5g	50	C^2HCl_3	150.4	157.6	95.3	125.7	127.0	132.5	135.2	116.6	132.6	148.1
$TARFH_2$	50	C^2HCl_3	150.6	157.0	105.2	136.0	116.1	133.6	129.0	118.0	128.2	137.1
$FMNH_2$	20	pH 5.0	151.1	157.2	103.1	134.4	117.1	134.3	130.4	117.4	130.4	144.3
[2,4,4a,10a-$^{13}C_4$]$FMNH_2$	0.3	pH 5.0	151.1	158.3	102.8							144.0
$FMNH^-$	40	pH 8.5	157.9	157.2	101.4	133.7	116.5	132.8	130.8	117.3	130.1	154.9
[2,4,4a,10a-$^{13}C_4$]$FMNH^-$	0.3	pH 8.5	158.2	157.7	101.4							155.5
$MeIMNH^-$	20	pH 8.5	158.1	157.3	101.3	135.0	115.1	136.6	122.0	116.0	130.9	155.4

[a] For the structures, see Figure 5. [b] Relative to Me_4Si.

flavin show that the N(10) atom is placed somewhat out of the molecular plane (Kierkegaard et al., 1971). In addition, theoretical calculations on the oxidized flavin molecule (Dixon et al., 1979) have shown that only 8.4 kJ/mol is required to bend the molecule slightly out of the molecular plane. Furthermore, the calculated π-electron densities for the N(3) and the N(10) atoms are 1.731 and 1.680 electrons, respectively (Palmer & Platenkamp, 1979). Considering that both nitrogen atoms possess pyrrole-type character, it would be expected that the N(3) atom should resonate at higher field than the N(10) atom. This is found for FMN in water, but not for TARF in chloroform.

Since the calculations have been performed for the coplanar structure, the result is not surprising. Consequently, the incorrect prediction of the chemical shift for the N(3) atom of TARF in chloroform can be attributed to the different structure of flavin in apolar solvents.

The shape of the fluorescence emission and light absorption spectra and the fluorescence quantum yield and fluorescence lifetime of flavin are strongly dependent on the polarity of the solvent (Visser & Müller, 1979; Eweg et al., 1979, 1980a). It is suggested that our NMR data are also related to these phenomena. For instance, the lower degree of resolution of the light absorption spectrum of flavin in aqueous than in organic solvents could be related to the existence of the mesomeric structures as presented in Figure 1.

1,5-Dihydroflavin. In the past few years reduced flavin has attracted considerable interest, especially because of its oxygen-activating property, a reaction of great biological relevance (Massey et al., 1969). The physical and chemical properties of reduced flavin have been studied (Ghisla et al., 1973; Dudley et al., 1964). These and crystallographic studies (Kierkegaard et al., 1971) revealed that the flavin molecule possesses a bent structure.

In principle, a combination of ^{13}C and ^{15}N NMR techniques should also yield detailed information on the solution structure of reduced flavin, as has been demonstrated above for the oxidized molecule. In the following analysis, we make use of published data and of a personal study of several model compounds. Owing to the higher complexity of the structure of reduced flavin, independent ^{13}C (Van Schagen & Müller, 1980, 1981) and ^{15}N NMR (Franken et al., 1984) studies on free and protein-bound reduced flavin did not allow development of a correlation between the chemical shifts and the structure of a particular flavin molecule. With the data now available, such a correlation appears to exist, which is presented here. For practical reasons (sensitivity, availability of compounds), ^{13}C NMR results were preferably collected and supplemented by a few ^{15}N NMR results. The data are presented in Tables

Table IV: ^{15}N Chemical Shifts of Reduced Flavins in Aqueous and Chloroform Solutions

compd	solvent	^{15}N chemical shift (ppm)[a] N(1)	N(3)	N(5)	N(10)
[1,3,5,10-$^{15}N_4$]Me_2TARIH_2	C^2HCl_3	116.7	145.8	60.4	72.2
[1,3,5,10-$^{15}N_4$]$MeIMNH_2$	pH 5.4	128.1	150.7	60.6	87.3
[1,3,5,10-$^{15}N_4$]$MeIMNH^-$	pH 8.5	186.9	150.7	60.6	97.2
[1,3,5-$^{15}N_3$]$FMNH^-$	pH 7.8	182.6	149.3	57.7	

[a] Relative to external NH_3.

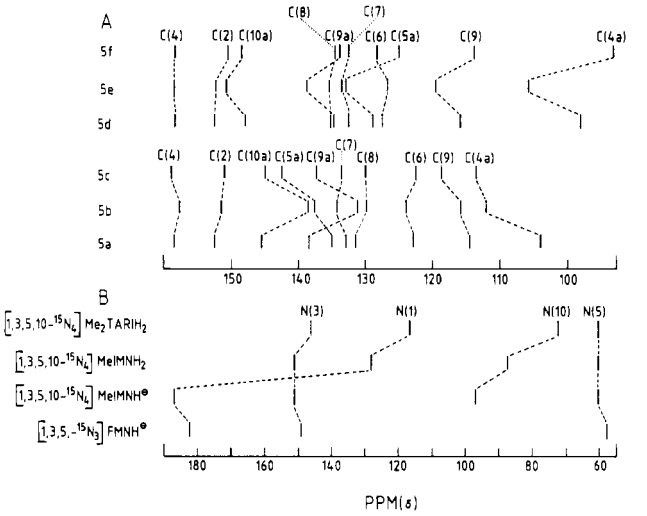
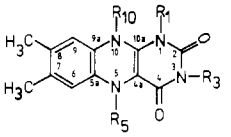


FIGURE 4: Correlation diagram of ^{13}C and ^{15}N NMR chemical shifts of reduced flavins.

III and IV. For convenience, some selected data are presented diagrammatically in Figure 4. The structures of the less common reduced flavins are given in Figure 5.

It is evident from Table III that subsequent substitution of the various N atoms of flavin influences certain ^{13}C resonance lines dramatically. For convenience, this is illustrated in Figure 4A for two sets of a few selected comparable compounds. For instance, it is noticeable that especially the quaternary atoms C(4a), C(5a), C(9a), and C(10a) of compounds **5a–c** undergo shifts from 0.8 to 9.5 ppm. Yet the only difference between these compounds remains in the substituent at N(1). It should be noticed that C(9a) and C(10a) on the one hand and C(4a) and C(5a) on the other hand show parallel shifts. The C(9) atom follows the shifts of the latter carbon pair. The observed shifts are clearly too large to be caused solely by the effect of substitution. The shifts must therefore be related to the conformation of reduced flavin, indicating π-electron density changes at the atoms in question (Van Schagen & Müller, 1980).



5a: $R_1=H, R_3=R_5=R_{10}=CH_3$

5b: $R_1=R_3=R_{10}=CH_3, R_5=H$

5c: $R_1=R_3=R_5=R_{10}=CH_3$

5d: $R_1=H, R_3=CH_3, R_5=COCH_3, R_{10}=(CH_2)_{10}CH_3$

5e: $R_1=R_3=R_{10}=CH_3, R_5=COCH_3$

5f: $R_1,R_{10}=CH_2-CH_2, R_3=CH_3, R_5=COCH_3$

5g: $R_1=R_3=CH_3, R_5=COCH_3, R_{10}=H$

FIGURE 5: Structures of less common reduced flavins, used in this study (cf. Tables III and IV).

In case of optimal folding of reduced flavin, the benzene and the pyrimidine subnuclei could be regarded as isolated ring systems. It would then be expected that substitution of the proton at N(1) by a methyl group would only influence the chemical shifts of the pyrimidine subnucleus. This is clearly not observed (Figure 4A). On the other hand, substitution of the hydrogen atom at N(3) by a methyl group (data not shown) does only slightly influence the chemical shifts ($\Delta\delta <$ 1 ppm). This substitution does not cause severe sterical hindrance. Thus, the ^{13}C NMR results strongly suggest that the observed shifts are caused by a conformational change due to sterical hindrance, i.e., peripositioned methyl substituents at N(1) and N(10).

In the following an attempt will be undertaken to analyze the chemical shift changes in terms of the conformation of the reduced flavin. To do this we have to consider first the electronic structure of the N(10) and N(5) atoms, which should yield valuable information for the analysis.

If the reduced flavin were planar, then both N(5) and (10) atoms would be pyrrole-type nitrogen atoms; i.e., both atoms are potential π-electron donors. In the folded state, however, both nitrogen atoms would be sp^3 hybridized, and the electron lone pair could hardly participate in mesomeric structures. This does not necessarily mean that both N atoms have to attain the same configuration; it is possible that the degree of hybridization is different for both N atoms. The degree of hybridization of the N atoms for a few available flavin derivatives can be estimated from ^{15}N NMR data (Figure 4B, Table IV). The N(1) and the N(3) atoms resonate at lower field than the N(5) and the N(10) atoms. The chemical shifts indicate that N(1) and N(3) are predominantly pyrrole-type, i.e., sp^2 type, whereas N(5) and N(10) are in the region of aniline-type N atoms, i.e., somewhere between sp^2 and sp^3 type. It has already been argued (Axenrod et al., 1971) that ^{15}N chemical shifts in aniline-type compounds reflect the participation of the nitrogen electron lone pair in the π-electron system. ^{13}C chemical shifts can also be used to monitor the degree of participation of the electron lone pair of the N(5) and N(10) atoms in the aromatic subsystems. Increasing the planarity at the N(10) atom, for example, results in π-electron donation via mesomeric structures, especially to C(4a) and C(5a) (cf. Figure 6), leading to an upfield shift of the latter atoms. On the other hand, increasing the planarity at the N(5) atom results in π-electron donation to C(10a) and C(9a). The carbon pairs C(4a) and C(5a) and C(9a) and C(10a) actually show parallel shifts, supporting our notion that the configuration of N(10) and N(5) is mainly reflected by the chemical shift of the mentioned carbon pairs. Compound **5c** contains methyl groups at positions 1, 3, 5, and 10, leading undoubtedly to a strong steric overlap between the methyl groups at N(10) and N(1) and a strong overlap between the N(5) methyl group and the carbonyl group at C(4). The downfield shifts of all four quaternary carbon atoms in compound **5c** as compared to those of compound **5b** clearly indicate the inhibited π-electron-donating characters of N(5) and N(10), suggesting a strong bending of the flavin molecule. Comparing compound **5a** with **5c**, it is seen that C(9a) and C(10a) show about the same chemical shifts, whereas C(5a) and C(4a) in compound **5a** are shifted to high field by 6.4 and 9.5 ppm, respectively. This indicates that the π-electron-donating character of the N(10) atom is increased in compound **5a** and that the configuration of N(5) remains about the same in both compounds. Compound **5b** exhibits opposite effects to compound **5c**. The π-electron-donating character of N(5) is strongly enhanced, whereas that of N(10) is decreased. These interpretations are in agreement with the expected steric effects in these flavins.

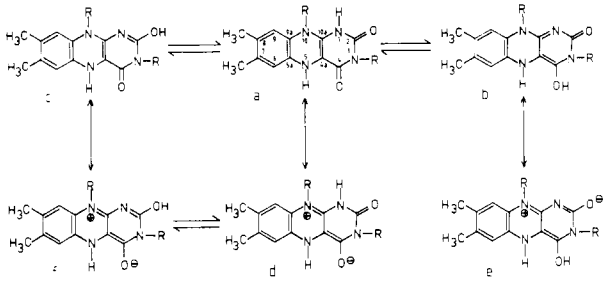


FIGURE 6: Possible mesomeric structures of reduced flavin as deduced from NMR results.

As evident from Figure 4A (Table III), the quaternary atoms in compounds **5d–g** are also very sensitive to structural variations. Although the four quaternary carbon atoms and C(9) now show almost parallel shifts, the two pairs of carbon atoms can still be distinguished by the relative value of the shifts.

If our assumption, that the hybridization of the N(5) and N(10) atoms is reflected especially by the ^{13}C chemical shifts of the C(4a), C(5a), C(9a), and C(10a) atoms is correct, then the calculated endocyclic angles for the two nitrogen atoms, from ^{13}C chemical shift values, should agree with those obtained by X-ray crystallography. These calculations should, however, be regarded with caution, since in these calculations we assume that only the change in the configuration of the nitrogen centers N(5) and N(10) is contributing to the observed shifts of the ^{13}C resonance lines, while minor additional effects cannot be fully excluded.

The endocyclic angle φ is calculated by using the following equation:

$$\varphi = \frac{\delta(C_{i,sp^3}) - \delta_{obsd}(C_i)}{\delta(C_{i,sp^3}) - \delta(C_{i,sp^2})} \times 10.5° + 109.5° \qquad (1)$$

where C_i is C(4a) or C(5a) for the calculation of the endocyclic angle of N(10) and C(9a) or C(10a) for the calculation of the endocyclic angle of N(5) and $\delta(C_{i,sp^3})$ and $\delta(C_{i,sp^2})$ are the corresponding limit chemical shifts of carbon atom C_i for a sp^3- and sp^2-hybridized nitrogen atom, respectively. The endocyclic angle for a sp^3-hybridized nitrogen atom is 109.5°. The difference in angles between a sp^2- and sp^3-hybridized nitrogen atom is 10.5°. The limiting values for $\delta(C_{i,sp^3})$ and $\delta(C_{i,sp^2})$ must be known to perform the calculations. Assuming that N(10) in compound **5f** approximates total sp^2 character, the endocyclic angle of N(10) in the various compounds is

Table V: Comparison of Endocyclic Angles of N(10) and N(5) Atoms in Reduced Flavins As Determined by Crystallographic and NMR Methods (for Details, See Text)

	endocyclic angles of nitrogen atom (deg)					
	N(10)			N(5)		
compd	X-ray	$\delta_{C(4a)}$	$\delta_{C(5a)}$	X-ray	$\delta_{C(10a)}$	$\delta_{C(9a)}$
5a		114.5	113.5		112.6	112.0
5b	111.9[a]	110.3	111.9	116.4[a]	*b*	*b*
5c			*b*		112.0	112.5
5d	116.8[c]	117.5	117.4	113.8[c]	111.4	114.1
5e	113.0[d]	113.4	114.9	112.0[d]	109.9	111.7
5f		*b*	*b*		111.1	114.4
5g	117.0[e]	118.9	119.5	114.8[e]	111.3	115.5
$TARFH_2$		113.8	113.0		117.1	118.3
$FMNH_2$		114.9[f]	114.4[f]		113.3[f]	116.9[f]

[a] Taken from Norrestam & von Glehn (1972). [b] Values used in the calculations, see text. [c] Taken from Norrestam et al. (1969). [d] Taken from Werner & Rönnquist (1970). [e] Taken from Leijonmarck & Werner (1971). [f] These values must be considered with great caution; for an explanation, see text.

calculated by using the limiting values of $\delta(C_{4a,sp^2}) = 93.3$ ppm and $\delta(C_{5a,sp^2}) = 124.9$ ppm (Table III, compound **5f**). For an approximately sp^3-hybridized N(10) atom, the chemical shifts due to C(4a) and C(5a) of compound **5c** (Figure 4A) are assumed to represent the needed limiting values, i.e., $\delta(C_{4a,sp^3}) = 113.3$ ppm and $\delta(C_{5a,sp^3}) = 141.6$ ppm, respectively. The endocyclic angles of the N(10) atom of various flavin derivatives, as determined from experimental ^{13}C chemical shift values, are presented in Table V, together with published crystallographic data. Table V demonstrates that the two sets of data agree surprisingly well, indicating that our method of determining the endocyclic angle of the N(10) atom in reduced flavin can be used reasonably safely. The minor differences between the two sets of data are probably caused by the fact that we have completely neglected possible effects on the chemical shifts of the atoms in question by substitution of nearby atoms. Apparently, such effects are only of minor importance.

The determination of the endocyclic angle of the N(5) atom is much more difficult because no safe indications are available demonstrating that a particular 1,5-dihydroflavin possesses a sp^2-hybridized N(5) atom. We therefore used the following modified procedure. It is known from crystallographic studies (Norrestam & Von Glehn, 1972) that the N(5) atom in compound **5b** possesses an endocyclic angle of 116.4°, i.e., the atom is about 65% sp^2 and 35% sp^3 hybridized. The degree of hybridization is an agreement with the $^1J(^{15}N-^1H)$ coupling constant of 87.5 Hz for a similar compound (Franken et al., 1984). Thus, the chemical shift values of the C(9a) and C(10a) atoms of compound **5b** (Table III) are used as reference chemical shifts for an endocyclic angle of 116.4° (eq 1). In addition, it was assumed that a change from sp^3 to sp^2 hybridization of N(5) causes similar chemical shift changes of C(9a) and C(10a), as a hybridization change of N(10) on the chemical shift of C(4a) and C(5a), as discussed above. Although somewhat arbitrary, this assumption is supported by the fact that the introduction of periovercrowding effects between a N(5) substituent and the C(4) carbonyl function causes chemical shift changes at the C(9a) and C(10a) centers comparable with analogous effects on the chemical shift changes at the C(4a) and the C(5a) centers by N(1) and N(10) substituents. In addition, the value of 109.5° in eq 1 was replaced by 116.4° for the calculations.

The calculated values are compared with crystallographic data in Table V. The endocyclic angles calculated from NMR results are in fair agreement with the crystallographic data. The good agreement supports the validity of our empirical approach, both for the N(5) and the N(10) atom. Therefore, the data provide a basis for the calculation of endocyclic angles of compounds that cannot be studied by crystallographic methods. We wish to emphasize that our calculations of the endocyclic angles of the N(5) and the N(10) atoms do not yield numerical values on the degree of folding of the reduced flavin molecule, although the endocyclic angles of the two atoms are related to the degree of folding of the molecule. Since the degree of hybridization of the two nitrogen atoms can be independently modulated, a fact also supported by crystallographic studies (Norrestam et al., 1969; Werner & Rönnquist, 1970; Leijonmarck & Werner, 1971; Norrestam & Von Glehn, 1972), the term "folding angle of the flavin molecule" should be avoided. In fact, the degree of hybridization of the nitrogen atoms describes the structure of the reduced flavin molecule more accurately than the angle of folding. Furthermore, it should be mentioned that ^{15}N chemical shifts of N(5) acetylated compounds cannot be used to calculate the N(5) endocyclic angle since this substitution will undoubtedly influence the ^{15}N chemical shift of the N(5) atom.

In Table V the calculated endocyclic angles for $TARFH_2$ are also given. The results indicate that the N(5) atom is considerably sp^2 hybridized. This result is in excellent agreement with the $^1J(^{15}N(5)-^1H)$ coupling constant of $TARFH_2$ (Franken et al., 1984). The N(10) atom, on the other hand, possesses less sp^2 character than the N(5) atom. The results indicate that $TARFH_2$ possesses a fairly planar conformation, more bent at the N(10) than the N(5) center.

The calculated endocyclic angles for $FMNH_2$ in aqueous solution are also presented in Table V. The calculated endocyclic angle for the N(5) atom, especially that calculated from the chemical shift of $^{13}C(10a)$, is smaller than that for $TARFH_2$. On the basis of published light absorption data (Dudley et al., 1964), it was expected that the endocyclic angle of the N(5) atom in $FMNH_2$ would be at least as large as that in $TARFH_2$. The reason for this apparent discrepancy will be explained in the following on the basis of the ^{13}C and ^{15}N chemical shifts of $FMNH_2$ (Tables III and IV). As compared to Me_2TARIH_2 in $CHCl_3$, the ^{15}N chemical shifts of $MeIMNH_2$ in aqueous solution appear, with the exception of that of the N(5) atom, at lower fields (Figure 4B, Table IV). The large downfield shift of the resonance line of N(10) indicates a drastic change to enhanced sp^2 character. The increased sp^2 character of N(10) is also reflected in the upfield shift of the resonances due to C(4a) and C(5a). This upfield shift is however less than would be expected on the basis of the downfield shift of the N(10) resonance. The N(5)H and N(3)H groups in reduced flavin form hydrogen bonds with the solvent in aqueous solution, leading to downfield shifts of the corresponding ^{15}N chemical shifts. This is clearly observed on the chemical shift of N(3). The small effect observed on the N(5) resonance suggests that the expected downfield shift is almost cancelled by an increase of the sp^3 character of N(5). This interpretation is supported by the downfield shift of the resonances due to C(9a) and C(10a), but the downfield shift observed for C(10a) is much larger than expected by the change of hybridization of the N(5) atom. Changing the solvent from $CHCl_3$ to aqueous solution also leads to an unexpectedly large downfield shift of the resonance line of N(1). This downfield shift of the resonance of the pyrrole-type N(1) atom is too large to originate solely from hydrogen-bond formation. The large downfield shift suggests a considerable increase in sp^2 character of the N(1) atom. The similar (in

magnitude) and parallel shifts of the resonances due to the N(1) and N(10) atoms indicate that both atoms can in some way act cooperatively. In contrast to the oxidized flavin, the chemical shifts due to C(2) and C(4) are only slightly affected when going from $CHCl_3$ to aqueous solution. This indicates that the change in solvent hardly increases the partial positive charge of the C(2) and C(4) centers. The explanation is that the π-electron density of the O(2α) and O(4α) centers is increased by a cooperative electron donation from N(10) and N(1), and even from N(3). As a consequence, O(2α) and O(4α) can no longer polarize C(2) and C(4) to such an extent as in oxidized flavin. It should be noted that the ^{13}C chemical shifts are only slightly dependent on concentration, although aggregation does occur as judged from the increase of line widths upon increasing the concentration.

In our opinion, the electronic structure of $FMNH_2$ can, on the basis of the ^{13}C and ^{15}N NMR data, best be described by the structures shown in Figure 6. The existance of the tautomeric structures b and c cannot be directly deduced from the NMR results, but we believe that they are important to understand the electronic structure of reduced flavin. Structures e and f are in full agreement with the parallel large downfield shifts of the resonances due to N(1) and N(10) and also explain the unexpected large downfield shift of the resonance of C(10a). The latter observation is also in agreement with structure d. All three mesomeric structures are in accord with the observation that the π-electron density at the C(4a) center is increased in $FMNH_2$, as compared to $TARFH_2$. On the other hand, the N(5) atom is forced to acquire more sp^3 character, as deduced from ^{15}N NMR data. This implies that π-electron density will be withdrawn from the centers C(10a), C(9a), C(6), and C(8). The downfield shifts of these atoms support this idea.

It becomes evident from the discussion above that our semiempirical approach to calculate the endocyclic angles of reduced flavin in aqueous solution only yields lower limits. In our calculations, we assumed that the change in hybridization of the N(5) and N(10) atoms will lead to a π-electron density increase or decrease at the centers C(9a) and C(10a) and C(4a) and C(5a), respectively. In apolar solutions where mesomeric structures, as presented in Figure 6, are not favored, the semiempirical approach yields reasonably good results. In highly polar solvents, where mesomeric structures play a role, the ^{15}N chemical shifts due to the N(5) and N(10) atoms are more reliable parameters to estimate the endocyclic angles. At any rate, it is estimated from the combination of ^{13}C and ^{15}N NMR results that in $FMNH_2$ the endocyclic angle for N(10) is about 116–118° and that for N(5) is slightly less.

The N(1)H group of reduced flavin deprotonates with a pK_a of 6.6 (Dudley et al., 1964; Van Schagen & Müller, 1981; Franken et al., 1984). The negative charge at N(1) leads to a strong downfield shift of the ^{15}N chemical shift of N(1) and the ^{13}C chemical shifts of the neighboring carbon atoms C(2) and C(10a). These shifts are similar to those observed in other heteroaromatic systems (Pugmire & Grant, 1968, 1971; Ewers et al., 1974; Tokuhiro & Frankel, 1969). The $^{13}C(2)$ resonance is shifted less downfield than the $^{13}C(10a)$ resonance, owing to the increased π-electron density at O(2α). It was expected that the negative charge at N(1) would increase the π-electron density at N(10), leading to an upfield shift of the resonance of N(10). In contrast, a downfield shift of 10 ppm is observed, indicating an increase of sp^2 character of N(10). The increased sp^2 character of N(10) is also reflected in the small upfield shift of the resonances due to C(4a), C(5a) and C(7). Deprotonation of reduced flavin influences the chemical shift of the N(5) resonance only slightly, the small upfield shift indicates a slight decrease of the predominant sp^2 character of the N(5) atom. These results indicate that the N(10) atom in ionized reduced flavin possesses almost full sp^2 character, while that of the N(5) atoms is somewhat less.

The analysis presented above shows that the conformation of reduced flavin is mainly governed by the polarity of the solvent and by steric hindrance introduced by N-substitution. In polar solvents, where mesomeric structures are stabilized, unsubstituted reduced flavin attains an almost planar structure. It has been suggested that the pyrazine subnucleus of reduced flavin possesses antiaromatic character causing a strong bending of the molecule (Tauscher et al., 1973). The mesomeric structures presented in Figure 6 show that the molecule can "escape" this antiaromaticity and attain a fairly coplanar structure. The previous interpretation of the light absorption spectra of reduced flavin (Dudley et al., 1964) should be reconsidered in view of our NMR results; i.e., the observed molar absorption coefficient at 450 nm for a particular flavin is most probably solely related to the degree of sp^2 hybridization of the N(5) atom of reduced flavin. Our results are also in agreement with theoretical calculations by Dixon et al. (1979), who showed that the reduced flavin molecule is only slightly bent and that only a relatively small activation energy is needed for the molecule to acquire a planar conformation. These results were recently supported by photoelectron spectroscopy (Eweg et al., 1980b).

Our results have several biological implications. It has been shown by crystallographic studies (Ludwig et al., 1976) that $FMNH^-$ is almost coplanar when bound to *Clostridium* MP flavodoxin. In *M. elsdenii* flavodoxin, which is closely related to *Clostridium* MP flavodoxin, the sp^2 character of the N(5) atom of the reduced prosthetic group is confirmed by the coupling constant (Franken et al., 1984). Simondsen & Tollin (1980) suggested that the planar structure of $FMNH^-$ in these proteins is mainly responsible for the altered redox potential of the couple semiquinone–fully reduced, as compared to free $FMNH^-$. Our results, however, indicate that free $FMNH^-$ already possesses an almost planar conformation. It is therefore unlikely that the redox potential in flavodoxin is governed mainly by the conformation of protein-bound $FMNH^-$. Probably other factors such as negative charges in the vicinity of the bound flavin play a much more important role. In other flavoproteins, the reduced prosthetic group could well attain a bent structure since this would cost only little energy as outlined above.

In this paper we have presented a detailed analysis for the interpretation of the chemical shifts of free flavin, which provides a good basic for NMR studies on flavoproteins. The easy manner in which the structure of reduced flavin is altered by the introduction of sterical hindrance or hydrogen bonds is striking. It is suggested that these effects are involved in directing the different pathways of flavin catalysis. Reduced flavin reacts with molecular oxygen at the C(4a) position, as was deduced by Ghisla et al. (1978) from NMR data on a luciferase. This reaction will be favored if the π-electron density at the C(4a) atom is enhanced. As we have seen, this can be accomplished by sp^2 hybridization of the N(10) atom but also by providing a hydrophobic-like environment where mesomeric structures are not favored. These aspects are currently under study in our laboratory.

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manuscript.

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