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A Comparative Carbon-13, Nitrogen-15, and Phosphorus-31 Nuclear Magnetic Resonance Study on the Flavodoxins from *Clostridium MP*, *Megasphaera elsdenii*, and *Azotobacter vinelandii*†

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ABSTRACT: The flavodoxins from *Megasphaera elsdenii*, *Clostridium MP*, and *Azotobacter vinelandii* were studied by ^{13}C, ^{15}N, and ^{31}P NMR techniques by using various selectivity enriched oxidized riboflavin 5′-phosphate (FMN) derivatives. It is shown that the π electron distribution in protein-bound flavin differs from that of free flavin and depends also on the apoflavoprotein used. In the oxidized state *Clostridium MP* and *M. elsdenii* flavodoxins are very similar with respect to specific hydrogen bond interaction between FMN and the apoprotein and the electronic structure of flavin. *A. vinelandii* flavodoxin differs from these flavodoxins in both respects, but it also differs from *Desulfovibrio vulgaris* flavodoxin. The similarities between *A. vinelandii* and *D. vulgaris* flavodoxins are greater than the similarities with the other two flavodoxins. The differences in the π electron distribution in the FMN of reduced flavodoxins from *A. vinelandii* and *D. vulgaris* are even greater, but the hydrogen bond patterns between the reduced flavins and the apoflavodoxins are very similar. In the reduced state all flavodoxins studied contain an ionized prosthetic group and the isoalloxazine ring is in a planar conformation. The results are compared with existing three-dimensional data and discussed with respect to the various possible mesomeric structures in protein-bound FMN. The results are also discussed in light of the proposed hypothesis that specific hydrogen bonding to the protein-bound flavin determines the specific biological activity of a particular flavoprotein.

Flavodoxins are a group of relatively small flavoproteins (14 000–23 000 Da) consisting of one polypeptide chain and containing a single molecule of noncovalently bound riboflavin 5′-phosphate (FMN)[1] (Mayhew & Ludwig, 1975).

The flavin coenzyme can exist in three redox states, i.e., oxidized, one electron reduced or semiquinone, and two

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[1] Abbreviations: FMN, $FMNH_2$, and $FMNH^-$, riboflavin 5′-phosphate in the oxidized, two-electron-reduced neutral, and anionic state, respectively; TARF and $TARFH_2$, tetraacetylriboflavin in the oxidized and two-electron-reduced state, respectively; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; Me_4Si, tetramethylsilane; OYE, "old yellow enzyme"; *C.MP*, *Clostridium MP*; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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electron reduced or hydroquinone. During in vivo redox reactions the flavodoxins probably function only as one-electron carriers, shuttling between the hydroquinone and the semiquinone states. In vitro the flavin molecule shuttles between the oxidized, semiquinone, and hydroquinone states. The redox potential of the different redox states is strongly altered as compared with free FMN. The redox potential for the semiquinone-reduced couple is profoundly modified, i.e., from −124 mV in free FMN to about −400 or even −500 mV in the flavodoxins (Mayhew & Ludwig, 1975; Anderson, 1983).

Knowledge of the interaction between FMN and apoflavodoxin should help to unravel the different contributions to these alterations in redox potential. It has been suggested (Massey & Hemmerich, 1980) that the energy barrier for the transition from the bent, i.e., bent along the N(5)–N(10) axis of the flavin molecule, to the planar conformation in the two-electron-reduced state provides a means of regulating the redox potential of protein-bound flavin. Crystallographic studies on *Clostridium MP* flavodoxin seemed to support this idea (Burnett et al., 1974; Smith et al., 1977). An almost coplanar reduced isoalloxazine ring of the prosthetic group was found in the crystal structure. Recently Moonen et al. (1984a) showed by ^{13}C NMR techniques that the activation barrier for the transition from the bent to the planar conformation is very much lower than originally proposed (Tauscher et al., 1973). Furthermore, Moonen et al. (1984b) also showed that free reduced flavin has an almost fully sp^2 hybridized N(10) atom and a N(5) atom with an endocyclic angle of 115–117°, indicating its predominant sp^2 character. This implies that free reduced flavin in water is intrinsically almost planar. Therefore, the observed change in redox potential in the semiquinone–hydroquinone transition in flavodoxins can no longer be explained by a constrained planar configuration. Instead it was proposed that charge–charge interactions are probably more important in the regulation of redox potentials of not only flavodoxins but also flavoproteins in general (Moonen et al., 1984c). Another important factor might be the specific hydrogen bonding between the apoflavoprotein and its prosthetic group, as suggested by Müller (1972).

One of the most powerful and versatile tools for studying molecular and submolecular interactions in a protein is the nuclear magnetic resonance (NMR) technique. It is possible to monitor conformational and electronic aspects of the bound flavin in a detailed way by using specifically ^{13}C- and ^{15}N-enriched flavins, as has been shown in recent ^{13}C and ^{15}N NMR studies on *Desulfovibrio vulgaris* flavodoxin (Vervoort et al., 1984, 1985) and *Megasphaera elsdenii* flavodoxin (Van Schagen & Müller, 1981; Franken et al., 1984). These studies not only revealed the specific hydrogen-bonding network between the prosthetic group and the apoprotein but also showed that there exist marked differences in electronic structure between free and bound flavin.

^{31}P NMR have been done on the flavodoxins from *M. elsdenii* (Moonen & Müller, 1982), *Azotobacter vinelandii* (Edmondson & James, 1979), and *D. vulgaris* (Favaudon et al., 1980). These studies revealed information not only on the phosphate binding site of FMN (Moonen & Müller, 1982) but also on the rate of electron transfer between the semiquinone and reduced state in *M. elsdenii* flavodoxin (Moonen & Müller, 1984a) and on a covalently bound disubstituted monophosphate in *A. vinelandii* flavodoxin (Edmondson & James, 1979). In order to complement these studies and to unravel the protein active site, a two-dimensional ^1H NMR project has been started, and it has been shown that in spite of the relatively large molecular mass of *M. elsdenii* flavodoxin the desired information can be obtained (Moonen & Müller, 1984a,b; Moonen et al., 1984d). This paper shows that subtle differences in interaction between the apoflavodoxins from *M. elsdenii*, *Clostridium MP*, and *A. vinelandii* and FMN reveal themselves by different ^{13}C, ^{15}N, and ^{31}P NMR chemical shifts. All carbon and nitrogen atoms of the isoalloxazine moiety of protein-bound FMN have been investigated and assigned. The results obtained with these three flavodoxins are compared with the results obtained on *D. vulgaris* flavodoxin (Vervoort et al., 1985) and a further FMN-containing protein, "old yellow enzyme" from yeast (Beinert et al., 1985a,b).

Materials and Methods

FMN and other flavin derivatives selectively enriched with ^{13}C at positions 2, 4, 4a, and 10a were prepared as described previously (Van Schagen & Müller, 1981). The synthesis of ^{15}N-enriched flavins was described elsewhere (Franken et al., 1984; Müller et al., 1983). The preparation of flavins enriched in the xylene ring of FMN will be reported elsewhere (Bacher and Sedlmaier, unpublished results). The enrichment of the isotopes was 90–95 atom % for C(2), C(4), C(4a), C(10a), and the four nitrogen atoms and 45–78% for the carbon atoms in the xylene moiety of flavin.

The flavodoxins were isolated and purified according to published procedures by Mayhew and Massey (1969) (*Clostridium MP* and *M. elsdenii* flavodoxin) and Hinkson and Bulen 1967) (*A. vinelandii* OP flavodoxin). The apoproteins were prepared by the trichloroacetic acid precipitation method (Wassink & Mayhew, 1975). The reconstitution of the apoflavodoxins with ^{13}C- or ^{15}N-labeled prosthetic groups was carried out in neutral buffered solutions at 4 °C. Excess of flavin was removed on a Bio-Gel P-6DG column. The protein–FMN complex was then concentrated by either ultrafiltration (Amicon) or by means of lyophilization.

The NMR measurements were performed on a Bruker CXP 300 spectrometer operating at 30.4 MHz for ^{15}N NMR, at 75.6 MHz for ^{13}C NMR, and at 121.0 MHz for ^{31}P NMR measurements. Some ^{13}C and ^{31}P NMR measurements were done on a Bruker WH 200 spectrometer operating at 50.3 MHz for ^{13}C NMR and at 81.0 MHz for ^{31}P NMR measurements. Wilmad 10-mm precision NMR tubes were used for ^{13}C and ^{31}P NMR measurements and some ^{15}N NMR measurements. Wilmad 15-mm precision NMR tubes were usually used for ^{15}N NMR measurements. The sample volume was 1.6 mL in 10-mm tubes and 3.5 mL in 15-mm tubes, both containing 10% ^2H_2O for locking the magnetic field. The samples contained 1–5 mM flavodoxin in 100 mM potassium pyrophosphate, pH 8. Broad-band decoupling of 0.5–1.0 W was used for ^{13}C, ^{15}N, and ^{31}P NMR measurements, unless otherwise stated.

Distortionless enhancement by polarization transfer (DEPT) spectra were recorded by using the method of Doddrell et al. (1982), optimized for the $^1J(^{15}N-^1H)$ coupling constants as reported by Franken et al. (1984). Dioxane (3 μL) served as an internal standard for ^{13}C NMR measurements. Chemical shifts are reported relative to Me_4Si[1] [δ(dioxane) − $\delta(Me_4Si)$ = 67.84 ppm].

Pure $CH_3{}^{15}NO_2$ was used as an external reference for ^{15}N NMR using a coaxial cylindrical capillary as recommended by Witanowski et al. (1981). Chemical shifts are reported relative to liquid NH_3 at 25 °C [$\delta(CH_3NO_2)$ − $\delta(NH_3)$ = 381.9 ppm for the magnetic field parallel to the sample tube (Witanowski et al., 1981)]. Values are reported as true shieldings, i.e., corrected for bulk volume susceptibilities. ^{31}P chemical shifts were determined relative to an external

Table I: ^{31}P Chemical Shifts of Various Flavodoxins in the Oxidized and Reduced State[a]

flavodoxin	^{31}P chemical shifts (ppm) in redox state	
	oxidized	reduced
free FMN	5.1	5.1
C.MP	5.7	5.8
M. elsdenii	5.3; 4.8[b]	5.4; 4.9[b]
D. vulgaris	5.4; 5.0[c]	5.5; 5.0[c]
A. vinelandii	6.3, 0.9; 5.6[d], 0.8[d]	6.4, 1.0; 5.4[d], 0.4[d]

[a] The spectra were taken in 100 mM Tris-HCl, pH 8.0. Chemical shifts are relative to 85% H_3PO_4. Positive chemical shifts are downfield from 85% H_3PO_4. [b] From Moonen and Müller (1982). [c] From Favaudon et al. (1980). [d] From Edmondson and James (1979).

standard of 85% phosphoric acid. The accuracy of the reported values is about 0.1 ppm for ^{13}C and ^{31}P NMR and about 0.3 ppm for ^{15}N NMR chemical shift values.

Quadrature phase detection was used. The instrumental settings were as follows: 30° pulse, 1-s repetition time, 8K data points. For determination of the nuclear Overhauser effects (NOEs)[1] a repetition time of 10 s was used. The temperature of the samples was 26 ± 2 °C for *M. elsdenii* and *Clostridium MP* flavodoxin and 20 ± 2 °C for *A. vinelandii* flavodoxin. Reduction of the samples was conducted by the addition of the desired amount of a dithionite solution to the anaerobic solution. Anaerobic conditions were achieved by carefully flushing the solution in the NMR tube with argon for about 10 min. The NMR tube was sealed with a rubber cap (suba seal).

Results and Discussion

^{31}P NMR Studies in the Oxidized and Reduced State. Table I shows the effect of redox state on the ^{31}P chemical shifts of flavodoxins. The chemical shifts reported in this study appear to be different from those reported previously (Moonen & Müller, 1982; Favaudon et al., 1980; Edmondson & James, 1979). However, the spectra measured previously were recorded on an NMR instrument, equipped with a conventional electromagnet where the magnetic field is perpendicular to the sample tube. We performed our measurements on an NMR apparatus equipped with a superconducting magnet where the external magnetic field is parallel to the sample tube. When the correction for the differences in magnetic susceptibility is applied (Gadian, 1982), some differences remain for the chemical shift of the phosphate resonances reported previously and those given in Table I, but the differences are very small. Yet, the chemical shift of the covalently bound phosphorus residue in oxidized *A. vinelandii* flavodoxin still differs by about 0.6 ppm from that reported previously (Edmondson & James, 1979), after correction for differences in magnetic susceptibility. The chemical shifts reported here indicate that in all flavodoxins the phosphate group of FMN is bound in the dianionic form. This interpretation is supported by kinetic data of MacKnight et al. (1973), who showed that the FMN phosphate can bind to the apoprotein of *A. vinelandii* flavodoxin as either the phosphate dianion or the phosphate monoanion. The interpretation is also supported by the fact that the ^{31}P resonance of free FMN is found at 5.1 ppm in the dianionic form (pH 9.0). All ^{31}P resonances in the flavodoxins are downfield-shifted from that of free FMN. These downfield shifts can be interpreted as (small) distortions of the O–P–O bond angles (Gorenstein, 1975). The largest distortion is observed in *A. vinelandii* flavodoxin.

On reduction of the proteins the ^{31}P chemical shifts undergo a slight downfield shift. This indicates that no large change occurs in the phosphate–apoprotein interaction on two-electron reduction of the protein.

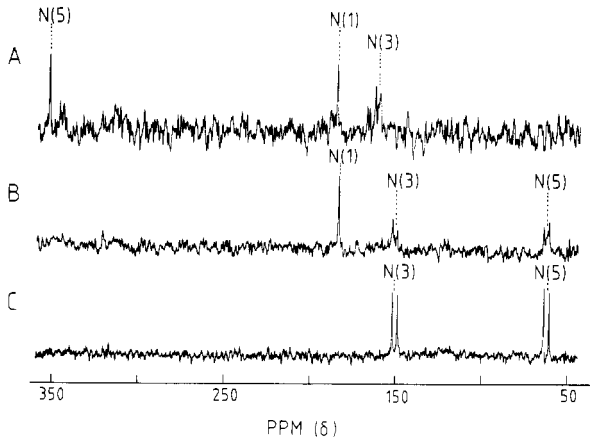

FIGURE 1: ^{15}N NMR spectra of *Clostridium MP* apoflavodoxin reconstituted with [1,3,5-$^{15}N_3$]FMN in the oxidized state (A) (55 800 acquisitions) and in the reduced state (B and C) in 100 mM potassium pyrophosphate, pH 8.0. Spectrum B (119 800 acquisitions) was taken under proton-coupled conditions, and spectrum C (54 300 acquisitions) was obtained by applying the DEPT pulse sequence.

All ^{31}P resonance lines of the flavodoxins in the semiquinone state are strongly broadened. The line width increases in the order *M. elsdenii* (Moonen & Müller, 1982), *D. vulgaris* (Favaudon et al., 1980), and *A. vinelandii* (Edmondson & James, 1982) flavodoxin. Although no calculations and no additional experiments have been done, the results suggest that the distance between the isoalloxazine moiety and the phosphate moiety of FMN is somewhat shorter in *A. vinelandii* flavodoxin than in *M. elsdenii* flavodoxin.

It has recently been shown theoretically, by using the crystallographic data on *Clostridium MP* flavodoxin, that the ribityl 5′-phosphate moiety of FMN acquires a certain conformation for optimal binding (Vinayaka & Rao, 1984). In this context our results indicate that this conformation obviously differs somewhat from flavodoxin to flavodoxin and that therefore the theoretical calculations (Vinayaka & Rao, 1984) obtained for one flavodoxin should not be generalized.

^{13}C and ^{15}N NMR Studies in the Oxidized State. The ^{15}N chemical shifts of flavin form the central basis for a detailed interpretation of the ^{13}C chemical shifts (Moonen et al., 1984b). Therefore the ^{15}N chemical shifts will be discussed first. For a thorough understanding of the discussion it is helpful to notice the following. The nitrogen atoms in heterocyclic aromatic compounds are generally categorized as pyridine-like or β-type and pyrrole-like or α-type atoms (Witanowski et al., 1981). The ^{15}N chemical shifts of the former are very sensitive to hydrogen-bonding interactions (upfield shift) whereas those of the latter are less sensitive and show a small downfield shift on hydrogen bonding. In oxidized flavin the N(1) and N(5) atoms represent β-type nitrogen atoms. The N(3) and N(10) atoms in the oxidized state and all four nitrogen atoms in the reduced state of flavin are of the α type. The chemical shifts of free flavin in a polar solvent (FMN) and in an apolar solvent (TARF) are used as a reference set in the interpretation of all chemical shifts of protein-bound flavin.

A typical ^{15}N NMR spectrum of flavodoxins in the oxidized state is shown in Figure 1A for clostridial flavodoxin. The ^{15}N chemical shifts of the flavodoxins are collected in Table II and for convenience presented diagrammatically in Figure 2. The ^{15}N chemical shifts reported here agree very well with those reported earlier by Franken et al. (1984). However, it should be noted that Franken et al. (1984) reported the ^{15}N chemical shifts relative to $^{15}NH_4NO_3$. From the results it can be concluded that *M. elsdenii* and *Clostridium MP* apofla-

Table II: ^{13}C and ^{15}N Chemical Shifts of Various Selectively Enriched Free and Protein-Bound FMN Derivatives[a]

	Oxidized ^{13}C and ^{15}N chemical shifts (ppm) in						
atom	*A.v.*	*D.v.*[b]	*M.e.*	*C.MP*	OYE[c]	FMN[b]	TARF[d]
C(2)	159.6	159.7	159.8	159.8	160.6	159.8	155.2
C(4)	161.7	162.4	162.4	162.3	164.2	163.7	159.8
C(4a)	135.7	134.3	135.6	135.5	137.1	136.2	135.6
C(5a)	136.9	137.4	138.4	138.5	135.2	136.4	134.6
C(6)	132.6	132.5	133.0	133.1		131.8	132.8
C(7)	141.2	142.0	141.4	141.4	141.2	140.4	136.6
C(7α)	20.4	20.5	20.6	20.5	19.3	19.9	19.4
C(8)	152.2	154.0	153.0	153.0	151.8	151.7	147.5
C(8α)	21.9	23.3	22.1	22.0	21.9	22.2	21.4
C(9)	117.9	117.2	117.5	117.4	117.7	118.3	115.5
C(9a)	131.5	131.9	132.9	132.9	131.7	133.5	131.2
C(10a)	153.5	152.3	151.5	151.4	152.9	152.1	149.1
N(1)	186.4	188.0	185.0	184.5	194.3	190.8	199.9
N(3)	160.2	159.9	160.9	161.1	164.1	160.5	159.8
N(5)	341.4	341.1	349.3	351.5	319.4	334.7	344.3
N(10)[e]	161.5	165.6	165.6	164.8	161.5	164.6	150.2

	Reduced ^{13}C and ^{15}N chemical shifts (ppm) in							
atom	*A.v.*	*D.v.*[b]	*M.e.*	*C.MP.*	OYE[c]	$FMNH^-$[b]	$FMNH_2$[d]	$TARFH_2$[d]
C(2)	158.3	157.5	156.9	156.6	159.4	158.2	151.1	150.6
C(4)	155.2	154.0	154.8	154.8	163.0	157.7	157.2	157.0
C(4a)	102.6	102.7	103.5	103.7	95.3	101.4	103.1	105.2
C(5a)	135.5	134.6	136.3	136.5	133.9	134.2	134.4	136.0
C(6)	113.8	114.5	112.4	112.4		117.3	117.1	116.1
C(7)	130.4	130.7	131.1	131.3	131.8	133.0	134.3	133.6
C(7α)	19.8	19.4	19.6	19.6	18.3	19.0	19.1	19.1
C(8)	125.5	126.7	125.9	125.6	128.5	130.3	130.4	129.0
C(8α)	19.3	20.3	19.2	19.1	19.5	19.4	19.4	19.3
C(9)	115.2	114.7	115.3	114.8	117.0	116.8	117.4	118.0
C(9a)	131.2	129.1	131.8	132.1	131.8	130.9	130.4	128.2
C(10a)	155.2	155.0	154.5	154.1	157.7	155.5	144.3	137.1
N(1)	182.0	186.6	183.4	182.8	187.4	182.6	128.0	116.7
N(3)	150.0	148.3	149.7	150.1	153.2	149.3	149.7	145.8
N(5)	61.7	62.1	61.3	61.9	48.6	57.7	58.0	60.4
N(10)[e]	96.7	98.4	98.3	97.7	97.6	97.2	87.3	72.2

[a] The spectra were taken in 100 mM potassium pyrophosphate, pH 8.0. ^{13}C chemical shifts are relative to Me_4Si. ^{15}N chemical shifts are relative to liquid NH_3. Values for old yellow enzyme are taken from Beinert et al. (1985a,b). Values for TARF and $TARFH_2$ were determined in $CHCl_3$. Abbreviations: *A.v.*, *A. vinelandii*; *D.v.*, *D. vulgaris*; *M.e.*, *M. elsdenii*; *C.MP*, *Clostridium MP*; OYE, old yellow enzyme. [b] From Vervoort et al. (1985). [c] From Beinert et al. (1985a,b). [d] From Moonen et al. (1984b). [e] [1,3,5,10-$^{15}N_4$]7-Methyl-10-ribitylisoalloxazine 5′-phosphate.

vodoxins do not form a hydrogen bond with the N(5) atom of FMN. This conclusion follows from the fact that the ^{15}N chemical shifts due to the N(5) atom of FMN in these two flavodoxins appear even at lower field than that of TARF in an apolar solvent ($CHCl_3$) (Table II, Figure 2). The shifts for the N(5) atom of FMN in *D. vulgaris* (Vervoort et al., 1985) and *A. vinelandii* flavodoxin appear at an intermediate field between that of TARF and FMN, indicating the formation of a weak hydrogen bond between the apoflavodoxins and the N(5) atom of protein-bound FMN. The fact that the ^{15}N chemical shifts of N(5) in the former flavodoxins are downfield from that of free FMN also suggests that the π electron density at the N(5) atom of protein-bound FMN is lower than that in free oxidized flavin.

The ^{15}N chemical shift of N(1) of protein-bound FMN appears at higher field than that of free FMN (Table II, Figure 2). From this it is concluded that all four apoflavodoxins form strong hydrogen bonds with the N(1) atom of FMN. These hydrogen bonds are stronger in *Clostridium MP* and *M. elsdenii* flavodoxin than in *A. vinelandii* and *D. vulgaris* flavodoxins.

The pyrrole-type nitrogens N(3) and N(10) of apoflavodoxin-bound FMN show smaller differences than the pyridine-type nitrogen N(1) and N(5). The ^{15}N chemical shifts indicate that the N(3)H group of FMN forms a strong hydrogen bond with the *Clostridium MP* and *M. elsdenii* apoflavodoxins. This hydrogen bond interaction is weaker in *A. vinelandii* and *D. vulgaris* flavodoxins (Table II, Figure 2).

As previously described (Moonen et al., 1984b), the N(10) atom in free oxidized flavin shows an unexpected large downfield shift on going from apolar to polar solvents. Since this pyrrole-like nitrogen atom cannot form a hydrogen bond, the downfield shift of its resonance had to be explained as an increase in sp^2 hybridization. This increase in hybridization only occurs when the polarization of the isoalloxazine ring is stabilized by hydrogen bond formation with C(2α) and C(4α). In keeping with this interpretation, which is also supported by ^{13}C NMR data (see below), *M. elsdenii* and *D. vulgaris* flavodoxins show an increased degree of sp^2 hybridization as compared with that of free FMN and *Clostridium MP* flavodoxin, whereas that of *A. vinelandii* flavodoxin is considerably decreased with respect to that of free FMN. Therefore, the isoalloxazine ring of protein-bound FMN is somewhat less planar in *A. vinelandii* flavodoxin with respect to the N(10) atom than in the other three flavodoxins studied.

The $^1J[^{15}N(3)-^1H]$ coupling constants of the protein-bound FMN have been determined by using the DEPT pulse sequence of Doddrell et al. (1982), optimized for a coupling constant of 90 Hz (Franken et al., 1984). This technique yields theoretically a 10-fold increase in the signal to noise ratio in the ^{15}N NMR spectra (see also below, Figure 1C). This allowed us to determine the coupling constants rather easily.

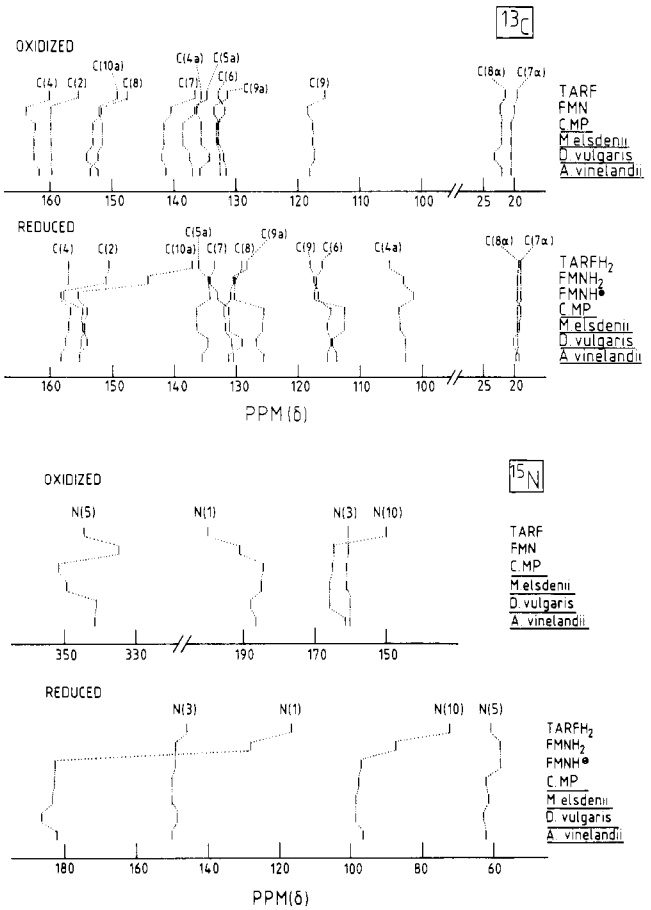

FIGURE 2: Correlation diagram of ^{13}C and ^{15}N chemical shifts of free flavin and flavin bound to *Clostridium MP*, *M. elsdenii*, *D. vulgaris*, and *A. vinelandii* apoflavodoxins in the oxidized state and in the reduced state.

Table III: ^{15}N–^{1}H Coupling Constants of Free and Protein-Bound Flavins in the Oxidized and Reduced State[a]

redox state	flavodoxin	coupling constants (Hz) $^{1}J[^{15}N(3)–^{1}H]$	$^{1}J[^{15}N(5)–^{1}H]$
oxidized	*C.MP* flavodoxin	90.3	
	M. elsdenii flavodoxin	88.2[b]	
	D. vulgaris flavodoxin	90.6[c]	
	A. vinelandii flavodoxin	90.6	
	TARF	92.7[b]	
reduced	*C.MP* flavodoxin	91.6	94.0
	M. elsdenii flavodoxin	93.1[b]	92.1[b]
	D. vulgaris flavodoxin	89.8[c]	86.2[c]
	A. vinelandii flavodoxin	89.7	90.3
	$TARFH_2$	93.1[b]	87.5[b]

[a] The accuracy of the coupling constants is ±1.2 Hz. Tetraacetylriboflavin (TARF) was measured in $CHCl_3$. The flavodoxins were measured in 100 mM potassium pyrophosphate, pH 8.0. [b] From Franken et al. (1984). [c] From Vervoort et al. (1985).

The data are given in Table III and compared with published values for *D. vulgaris* and *M. elsdenii* flavodoxins and TARF. The $^{1}J[^{15}N(3)–^{1}H]$ coupling constant is a few hertz smaller in the flavodoxins than in TARF. The fact that the coupling constant can be observed in these proteins indicates that the exchange of the N(3)–H protons is slow on the NMR time scale. Apparently the solvent has no access to this position in the proteins; i.e., the lifetime of the interaction between N(3) and its covalently attached proton is $\gg$10 ms.

The ^{1}J coupling constants between ^{15}N and ^{1}H nuclei are almost completely influenced by the Fermi contact term (Bourn & Randall, 1964). On the basis of this fact a semi-empirical relationship between the experimental $^{1}J(^{15}N–^{1}H)$ coupling constants and the hybridization of the nucleus under investigation was deduced (Bourn & Randall, 1964; Binsch et al., 1964). This relationship yields values of about 72 Hz for sp^3 hybridized nitrogen atoms and of about 93 Hz for sp^2 hybridized nitrogen atoms. From this it can be concluded that the N(3) atom of FMN is highly sp^2 hybridized in the flavodoxins. However, it should be kept in mind that the magnitude of the $^{1}J(^{15}N–^{1}H)$ constant is also dependent on the distance; i.e., hydrogen bonding to N(3)H will increase the bond distance and thereby apparently decrease the value of the coupling constant. This fact is probably reflected in the somewhat decreased coupling constants determined for the flavodoxins, except that for *M. elsdenii* flavodoxin. The value for *M. elsdenii* flavodoxin was determined in the absence of the DEPT pulse sequence (Franken et al., 1984) leading to a decreased signal to noise ratio and therefore to a less accurate determination of the coupling constant. This could explain the apparent discrepancy.

The nuclear Overhauser effect (NOE) of, e.g., N(H) or C(H) groups registers the increase of intensity of the resonance line of the nucleus under investigation while the proton is decoupled. The NOE is, among other factors, dependent on the rotational correlation time of the molecule under study. Such measurements can therefore yield some insight into the mobility of protein-bound flavin. We have determined the NOE of the N(3)H group of FMN bound to *M. elsdenii* and *D. vulgaris* apoflavodoxins under proton decoupling conditions. The values determined for both proteins are −0.4. Free FMN has a NOE value of −4.6 (Franken et al., 1984), which is very close to the theoretical maximum. The results on the two flavodoxins suggest that FMN is tightly bound and probably possesses little internal motion. This conclusion is in agreement with an earlier investigation in which the quaternary carbon atoms of FMN bound to *M. elsdenii* apoflavodoxin were studied (Moonen & Müller, 1983).

The ^{13}C chemical shifts of a molecule are related to the π electron density of the corresponding atoms (Stothers, 1972). It has been shown that such a correlation also exists in oxidized and reduced free flavin (Van Schagen & Müller, 1980). Therefore, the ^{13}C chemical shifts observed in protein-bound flavin can reliably be interpreted in terms of π electron changes with respect to free flavin.

The ^{13}C chemical shifts of the flavodoxin from *M. elsdenii*, *Clostridium MP*, and *A. vinelandii* are given in Table II. For comparison the ^{13}C chemical shifts of FMN (in H_2O), TARF (in $CHCl_3$), and old yellow enzyme (Beinert et al., 1985b) are also given. Old yellow enzyme is an FMN-containing protein. In contrast to the flavodoxins this enzyme is probably involved in the catalysis of oxidation or reduction of an organic molecule, although its precise biological function is not yet known. This makes a comparison with the electron-transfer flavoproteins interesting.

Some typical ^{13}C NMR spectra of *Clostridium MP* and *M. elsdenii* flavodoxin are shown in Figures 3A and 4A,B. The ^{13}C chemical shifts are schematically presented in Figure 2 (see also Table II). As is already obvious from the ^{15}N chemical shifts, the flavodoxins from *Clostridium MP* and *M. elsdenii* show very similar ^{13}C chemical shifts. This could be expected from the fact that they exhibit very similar chemical and physical properties (Mayhew & Ludwig, 1975). The ^{13}C chemical shifts of these two flavodoxins differ from those of *A. vinelandii* and *D. vulgaris* flavodoxins and also differ from each other.

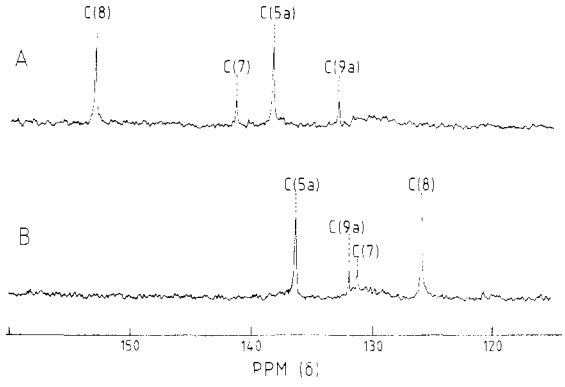

FIGURE 3: ^{13}C NMR spectra of *M. elsdenii* apoflavodoxin reconstituted with a mixture of ^{13}C-enriched FMN containing 80% [8,5a-$^{13}C_2$]FMN and 20% [7,9a-$^{13}C_2$]FMN in the oxidized state (A) (105 000 acquistions) and in the reduced state (B) (109 000 acquisitions) in 100 mM potassium pyrophosphate, pH 8.0.

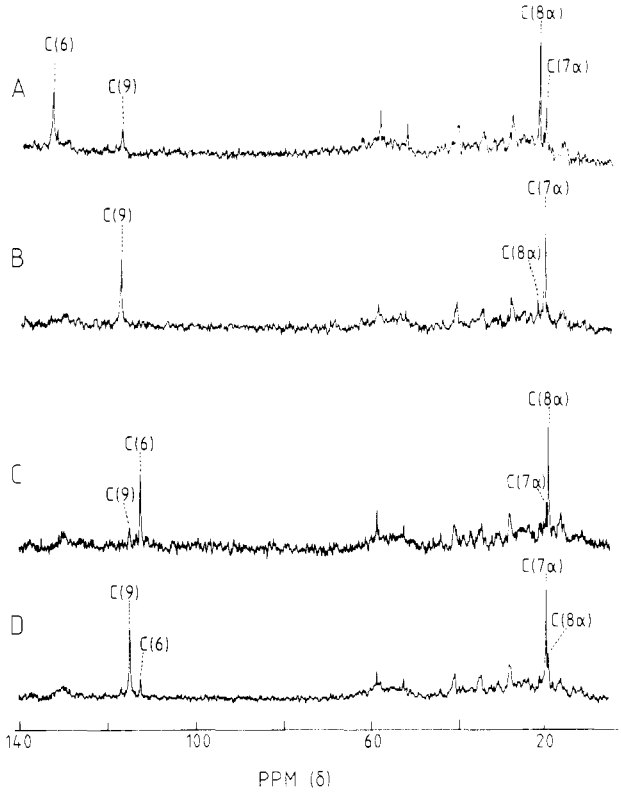

FIGURE 4: ^{13}C NMR spectra of *Clostridium MP* apoflavodoxin reconstituted with various mixtures of ^{13}C-enriched FMN in the oxidized state (A and B) and in the reduced state (C and D) in 100 mM potassium pyrophosphate, pH 8.0: (A) 80% [6,8α-$^{13}C_2$]FMN and 20% [7α,9-$^{13}C_2$]FMN (11 200 acquisitions) in the oxidized state; (B) 80% [7α,9-$^{13}C_2$]FMN and 20% [6,8α-$^{13}C_2$]FMN (5800 acquisitions) in the oxidized state; (C) 80% [6,8α-$^{13}C_2$]FMN and 20% [7α,9-$^{13}C_2$]FMN (5200 acquisitions) in the reduced state; (D) 80% [7α,9-$^{13}C_2$]FMN and 20% [6,8α-$^{13}C_2$]FMN (17 000 acquisitions) in the reduced state.

The ^{13}C chemical shifts due to C(2) of FMN in all four flavodoxins (Table II, Figure 2) are very similar and resemble that of FMN in aqueous solution but differ from that of TARF in $CHCl_3$. This indicates that a rather strong hydrogen bond exists between C(2α) and the apoflavodoxins. These hydrogen bondings seem to be of similar strength in all four flavodoxins as deduced from the ^{13}C chemical shifts. It must be noticed, however, that the strength of the hydrogen bond formed between the N(1) atom of FMN and the apoprotein counteracts the ^{13}C chemical shifts of the neighboring carbon atoms, i.e., leading to an upfield shift of the resonance due to C(2) and C(10a). Therefore it must be concluded that the hydrogen bond with C(2α) is stronger in *Clostridium MP* and *M. elsdenii* flavodoxins than in *A. vinelandii* and *D. vulgaris* flavodoxins but that in all four flavodoxins the hydrogen bonds are stronger than that of C(2α) of FMN with the solvent water. These results also demonstrate that the electronic structure of the flavin molecule is very complex and that an environmental influence at one particular atom also affects other atoms in the molecule as was already demonstrated for free flavin by the coherent anti-Stokes Raman spectroscopy technique (Müller et al., 1983).

The ^{13}C chemical shifts of C(4) in flavodoxins are upfield-shifted as compared with that in FMN but are downfield from that of TARF in $CHCl_3$. This indicates that the π electron density at C(4) in the flavodoxins is increased with respect to that of FMN and decreased with regard to that of TARF, indicating weak hydrogen bonding with C(4α) in *Clostridium MP*, *M. elsdenii*, and *D. vulgaris* flavodoxins and an even weaker hydrogen bond in *A. vinelandii* flavodoxin.

According to results previously described for free flavins (Moonen et al., 1984b) a strong hydrogen bond with C(2α) will influence the π electron density on C(8), C(6), N(5), C(9a), and C(10a) through conjugative effects leading to a highly polarized flavin molecule. To stabilize the resulting mesomeric structure [for structures, see Moonen et al., (1984b)], a solvent possessing a high permittivity is needed. Therefore, the strong hydrogen bond formed between C(2α) of FMN and the apoprotein in the flavodoxins should lead to a downfield shift of the resonances due to the atoms mentioned above. This is indeed observed for C(8), C(8α), and C(6), but not for the C(9a) and C(10a), which show a shift in the opposite direction. The unexpected trend of the ^{13}C chemical shift due to C(10a) can probably be ascribed to the fact that it is under the influence of the neighboring nitrogen atoms N(1) and N(10); i.e., a hydrogen bond to N(1) will shift the resonance due to C(10a) as discussed above for C(2). This interpretation seems to be in agreement with the further upfield shift observed for N(1) in flavodoxins as compared with free FMN (Figure 2). From these results it can be concluded that FMN in *D. vulgaris* flavodoxin is most polarized in the direction from C(8), C(6), C(9a), N(5), C(10a), to C(2α), that in *M. elsdenii* and *Clostridium MP* flavodoxins somewhat less, and that in *A. vinelandii* flavodoxin the least. The stabilization of the mesomeric structure is a combined effect of hydrogen bonding with C(2α) and a high permittivity at the dimethylbenzene edge of protein-bound flavin, which is known to be accessible to solvent from crystallographic work (Smith et al., 1977, 1983; Watenpaugh et al., 1972). As a consequence it could also be concluded from our NMR results that of the four flavodoxins studied the FMN in *A. vinelandii* flavodoxin is the least accessible to bulk solvent and that in *D. vulgaris* flavodoxin is the most accessible.

The ^{13}C chemical shifts due to C(9a) and C(10a) of FMN in *D. vulgaris* and *A. vinelandii* flavodoxins deserve some further comments since they do not show the shifts expected on the basis of the results for free flavin (Moonen et al., 1984b). We have recently suggested (Vervoort et al., 1985) that the slight upfield shift observed for C(9a) in *D. vulgaris* flavodoxin is a result of ring current effects of Trp-60 (Watenpaugh et al., 1972, 1973, 1975). However, there is no equivalent aromatic amino acid residue in *A. vinelandii* flavodoxin (Dubourdieu & Fox, 1977), except perhaps Tyr-102, unless the folding of the active site is completely different from the other flavodoxins, which does not seem likely (Smith et al., 1977, 1983). On the other hand, even if the N(10) atom

Table IV: Direct ^{13}C–^{13}C Coupling Constants of Free and Protein-Bound [4,4a,10a-$^{13}C_3$]Flavin in the Oxidized and Reduced State[a]

redox state	compd	coupling constants (Hz) ^1J[^{13}C-(4)–^{13}C-(4a)]	^1J[^{13}C-(4a)–^{13}C-(10a)]
oxidized	TARF[a]	75.5	53.3
	FMN[a]	75.4	55.9
	C.MP flavodoxin	76.3	56.2
	M. elsdenii flavodoxin	76.5	56.5
	D. vulgaris flavodoxin[b]	76.9	57.3
	A. vinelandii flavodoxin	76.3	56.5
reduced	$TARFH_2$[a]	79.1	84.5
	$FMNH_2$[a]	82.0	81.6
	$FMNH^-$[a]	84.0	74.4
	C.MP flavodoxin	85.5	73.2
	M. elsdenii flavodoxin	85.2	73.2
	D. vulgaris flavodoxin[b]	86.7	72.8
	A. vinelandii flavodoxin[c]	87.9	72.0

[a] The accuracy of the coupling constants is ±0.5 Hz. [b] From Vervoort et al. (1985). [c] The accuracy of the coupling constants is 1.0 Hz.

would be under the influence of ring current effects from an aromatic amino acid residue, the chemical shifts of the two atoms in question could not be explained satisfactorily. Therefore, we propose that both atoms are shifted upfield by the partial positive charge (α effect) developed by the increased sp^2 hybridization of N(10). This interpretation would explain fully the C(10a) shifts in both proteins; i.e., in *D. vulgaris* flavodoxin the hydrogen bond to N(1) is weaker than in the other flavodoxins, leading to a downfield shift, and in *A. vinelandii* flavodoxin the sp^2 hybridization of N(10) is relatively strongly decreased by comparison with the other flavodoxins, yielding a larger downfield shift. Since the C(9a) atom is under the influence of the mentioned effect of N(10) and is also involved when the molecule is polarized, the shift of C(9a) is more difficult to interpret in detail but seems consistent with the above-given interpretation.

An increase in sp^2 hybridization of N(10) in free flavin leads to a downfield shift of the resonances due to C(9), C(7), C(7α), and C(5a) due to the delocalization of the partial positive charge on N(10) (Moonen et al., 1984b). This is also observed with the flavodoxins, and the downfield shifts indeed parallel that of N(10) (Figure 2, Table II), except for C(9). The relative upfield shift of C(5a) in *D. vulgaris* and in *A. vinelandii* flavodoxins compared to *M. elsdenii* and *Clostridium MP* flavodoxins probably reflects the hydrogen bonding to N(5) in the first two proteins, the strength of which is less than in FMN (Figure 2).

When the degree of hybridization of the N(10) atom of FMN is increased, π electrons are delocalized onto C(4a) and C(4α), when C(4α) is capable of forming a hydrogen bond. As discussed above, only a weak, if any, hydrogen bond with C(4α) is present in the flavodoxins. Therefore, the π electron density is transferred mainly to C(4a) in the flavodoxins, leading to an upfield shift of the resonance due to C(4a) as compared with that in free FMN (Figure 2). It can be seen (Figure 2) that, as expected, the chemical shift of C(4a) parallels that of N(10), but in the opposite direction. These effects are very obvious in *A. vinelandii* flavodoxin where the N(10) atom exhibits a lower sp^2 hybridization than in the other flavodoxins. As a consequence, the resonance due to C(4a) is shifted downfield, owing to the release of π electron density at this atom. At the same time the resonances due to C(7) and C(5a) shift upfield because of the decrease of the positive charge delocalized to these atoms upon an increase of sp^2 hybridization of N(10). The relative upfield shift for C(4a)

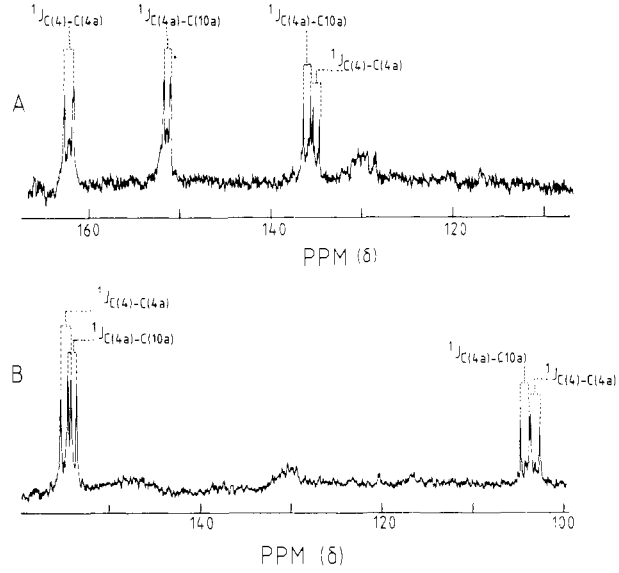

FIGURE 5: ^{13}C NMR spectra of *Clostridium MP* apoflavodoxin reconstituted with [4,4a,10a-$^{13}C_3$]FMN in the oxidized state (A) (35 400 acquisitions) and in the reduced state (B) (54 100 acquisitions) in 100 mM potassium pyrophosphate, pH 8.0.

in *A. vinelandii* and *D. vulgaris* flavodoxins compared to *M. elsdenii* and *Clostridium MP* flavodoxins also probably reflects the weak hydrogen bond to N(5) in the former two.

The one-bond coupling constants ^1J[^{13}C(4a)–^{13}C(4)] and ^1J[^{13}C(4a)–^{13}C(10a)] are given in Table IV (Figure 5). It is generally accepted that the direct ^{13}C–^{13}C coupling constants reflect the s character of the orbitals making up the bond (Stothers, 1972). The coupling constant for ^1J[^{13}C(4)–^{13}C-(4a)] is larger than that for ^1J[^{13}C(4a)–^{13}C(10a)], suggesting that the former bond possesses a considerably higher s character than the latter one, but the difference between free and protein-bound flavins is small. The coupling constant of ^1J-[^{13}C(4a)–^{13}C(10a)] shows a larger variation and seems to reflect the degree of polarization of the flavin molecule since it increases in the order TARF, FMN, and protein-bound flavin (see also above).

The NOEs of the hydrogen-bearing carbon atoms C(6) and C(9) of FMN in *M. elsdenii* and *D. vulgaris* flavodoxins have been estimated by broad-band decoupling. The values found are 1.2 ± 0.2, indicating that little, if any, enhancement is observed. These results are in agreement with those obtained on the N(3)H group (see above) and support the observation that flavin is rather tightly bound to these apoflavodoxins.

^{13}C and ^{15}N NMR Studies in the Reduced State. A detailed interpretation of the chemical shifts observed for the reduced compounds will not be given here because the fundamental concept is the same as described above for the oxidized compounds. The discussion will be confined to the most useful findings.

It is most convenient to discuss again first the ^{15}N chemical shifts. All nitrogen atoms in the reduced state of flavin are of the pyrrole type. The chemical shifts of N(1) of FMN bound to all of the apoflavodoxins are very similar to that of $FMNH^-$ (Figure 2, Table II). This already indicates that the prosthetic group in reduced flavodoxins is ionized, information that cannot be obtained unambiguously by other physical techniques, although a similar conclusion has been reached for *A. vinelandii* (Edmondson & Tollin, 1971) and *M. elsdenii* (Ghisla et al., 1974) flavodoxins by other techniques. The ionization of $FMNH_2$ in the flavodoxins is also supported by the fact that no N(1)–H coupling is observed. The ionization of N(1) is also reflected by the large downfield shift of the

resonance due to the neighboring carbon atoms C(2) and C(10a) (Figure 2, Table II). These downfield shifts are caused by the negative charge (field effect) on N(1) (Van Schagen & Müller, 1981). The chemical shifts of N(1) of $FMNH^-$ in the flavodoxins are independent of pH in the range from 6.0 to 8.5. This implies that the pK_a value of the N(1) atom of flavin in flavodoxins is decreased compared with that of free $FMNH_2$.

The N(3) chemical shifts of apoflavodoxin-bound $FMNH^-$ appear either downfield (*Clostridium MP*, *M. elsdenii*, and *A. vinelandii*) or upfield (*D. vulgaris*) as compared with that of $FMNH^-$ (Figures 1 and 2, Table II). This suggests that a hydrogen bond is present in the first group of flavodoxins and also in *D. vulgaris* flavodoxin (Vervoort et al., 1985), although in this case the bond is weak.

The resonance of N(5) of $FMNH^-$ in all flavodoxins is shifted downfield with respect to that of free $FMNH^-$, suggesting that a strong hydrogen bond is formed with N(5).

The N(10) chemical shifts in the flavodoxins appear at lower field than in $FMNH^-$, except that in *A. vinelandii* flavodoxin. This indicates that the sp^2 character of the N(10) atom is somewhat increased in *M. elsdenii*, *Clostridium MP*, and *D. vulgaris* flavodoxins and decreased in *A. vinelandii* flavodoxin.

Flavin in the reduced state is an electron-rich molecule. On two-electron reduction the ^{13}C chemical shifts of apoflavodoxin-bound flavin are different from those of $FMNH^-$. An upfield shift is observed for the resonances of all sp^2 carbon atoms in flavodoxins, except those of C(4a), C(5a), and C(9a). The downfield shift of the latter carbon atoms indicates that both N(10) and N(5) nitrogen atoms possess an increased sp^2 hybridization that delocalizes the partial positive charge onto these carbon atoms and increases the π electron density at C(8) and C(6) on the one side, and C(7) and C(9) on the other. The fact that C(4) is also strongly upfield shifted even by comparison with that in $TARFH_2$ in an apolar solvent strongly suggests that some π electron density from N(5) is delocalized onto this atom. These facts make it difficult to derive from the ^{13}C chemical shifts of C(2) and C(4) whether or not these two atoms are involved in hydrogen bonding, because counteracting effects mask the influence of hydrogen bonding. In *D. vulgaris* flavodoxin the further upfield shift of the resonance of C(4), which is accompanied by a downfield shift of the resonances due to C(6) and C(8), and an upfield shift of those of C(5a) and C(9a), suggests that π electron density is redistributed from C(8) and C(6) onto C(4) and C(9a) [from N(5)] and from N(10) onto C(5a) and C(4a) due to a further increase of the sp^2 hybridization of N(10) (Figure 2).

The $^{15}N-^1H$ coupling constants of N(3)H and N(5)H (Figure 1) indicate that both atoms have a high degree of sp^2 character (Table III) (Levy & Lichter, 1979; Binsch et al., 1964), exceeding that of N(5)H in $TARFH_2$. The fact that these coupling constants can be observed indicates that the two N–H groups are not accessible to bulk solvent in the flavodoxins; i.e., the proton exchange reaction must be slow, $\nu \ll 100$ Hz. The somewhat lower value for the N(5)H coupling for *D. vulgaris* flavodoxin has recently been explained by the formation of a strong hydrogen bond with this group, increasing the bonding distance between N(5) and its proton.

The $^{13}C-^{13}C$ coupling constants for the $^{13}C(4a)-^{13}C(4)$ and $^{13}C(4a)-^{13}C(10a)$ bonds are given in Table IV (Figure 5). From these results it follows that the s character (Stothers, 1972) of the C(4)–C(4a) bond increases on going from free to protein-bound flavin and that the s character of the C(4a)–C(10a) bond is decreasing. These coupling constants were also used to assign unambiguously the resonance of the C(4) and C(10a) atoms. In addition, it should be noted that the previously published C(13)–C(13) coupling constants for *M. elsdenii* flavodoxin (Van Schagen & Müller, 1981) deviate somewhat from the values given in Table IV. This can be ascribed to a lower signal to noise ratio of the spectra obtained in the previous work.

The magnitudes of the NOEs for N(3)H and N(5)H in *D. vulgaris* and *M. elsdenii* flavodoxins were determined to be −0.40 and −0.57, respectively. Moreover, the NOEs for C(6)H and C(9)H were the same as those found for the oxidized proteins. These results strongly suggest that $FMNH^-$ bound to apoflavodoxins is rigidly bound, as found for the oxidized proteins. It should be noted, however, that we had to use rather high concentration of proteins to obtain these results and that the results should not be extrapolated to very dilute solutions. On the other hand, the flavodoxin concentrations found in the corresponding bacteria are rather high, approaching the concentrations used in the NMR measurements. Therefore, the above statement should be of biological relevance.

The above results demonstrate that the electronic structure of flavin is altered on binding to apoflavoproteins. The electronic structures of the flavins in *M. elsdenii* and *Clostridium MP* flavodoxin are very similar, but they differ from those in *A. vinelandii* and *D. vulgaris* flavodoxins. Even the latter two flavodoxins show distinct differences. On the other hand it is also obvious that there is a greater similarity among the flavodoxins than between the flavodoxins and old yellow enzyme (Table II).

It has been proposed that specific interaction between oxidized flavin and a particular apoflavoprotein determines the biological reaction of a certain flavoprotein (Müller, 1972; Massey & Hemmerich, 1980). These hypotheses involve specific hydrogen bonding to N(1) and/or C(2α), and to N(5). In a previous paper (Eweg et al., 1982) it has been shown that hydrogen bonding to N(1) of flavin has a pronounced effect on the electronic system of the molecule. The present NMR results reveal this effect in more detail and show in addition that a hydrogen bond to the carbonyl function in position 4 of flavin plays an important role in the stabilization of mesomeric structures involving the lone electron pair of the N(10) atom [see also Moonen et al. (1984b)]. As outlined in this paper, the strength of hydrogen bonding to this carbonyl function determines the π electron density at C(4a), in both the oxidized and the reduced state. On the other hand, the strength of hydrogen bonding to C(2α) of the flavin molecule determines the degree of polarization of the molecule in the direction of C(8), C(6), N(5), C(10a), to C(2).

Our NMR results also indicate that the above-mentioned hypothesis cannot fully explain the different biological functions of the flavoproteins, because we already observe different hydrogen-bonding patterns within the family of flavodoxins in the oxidized state (Figure 6). This observation is not too surprising considering the fact that flavodoxins shuttle between the semiquinone and the hydroquinone state in the electron-transfer reactions. In the reduced state the hydrogen-bonding patterns in the flavodoxins are very similar, but the hydrogen-bonding interaction with the N(1) atom of flavin is very weak or even completely absent. Therefore, it is suggested that the above-mentioned hypothesis should be refined in such a way that only the redox states involved in the catalysis of a particular flavoprotein should be taken into consideration; i.e., for flavoprotein oxidases the oxidized state and for flavoprotein dehydrogenases the reduced state have to be considered mainly. However, in order to evaluate the general validity of these hypotheses, more comparative data on other

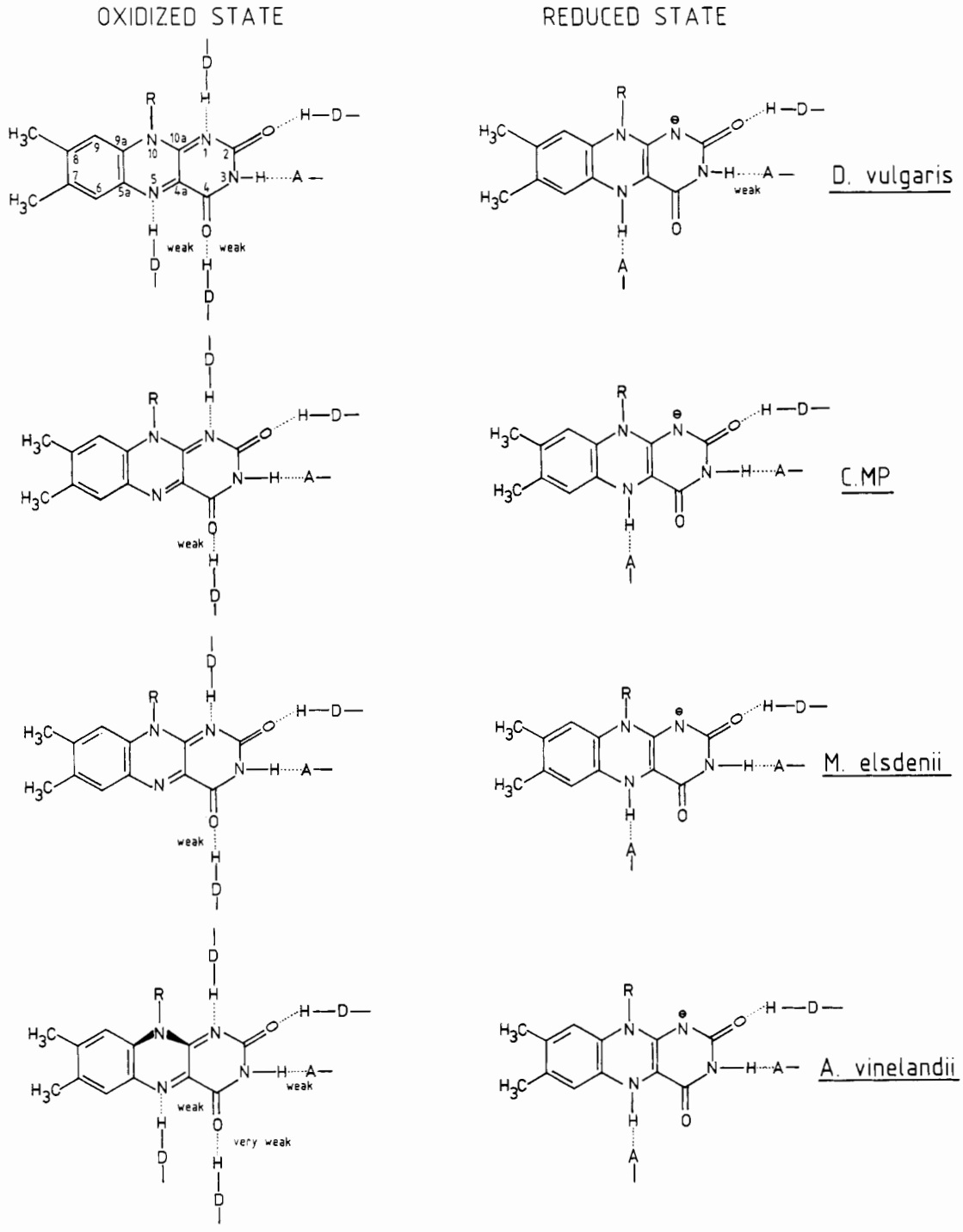

FIGURE 6: Hydrogen-bonding pattern between apoflavodoxin and FMN in the oxidized and reduced state as deduced from the ^{13}C and ^{15}N chemical shifts. A = hydrogen bond acceptor; D = hydrogen bond donor from the apoprotein.

flavoproteins are needed. Such a program is now under active investigation.

The higher complexity of the electronic structure of protein-bound flavin by comparison with free flavin also makes it very difficult to deduce the endocyclic angles of N(10) and N(5) from the NMR data, as has been done with free flavins (Moonen et al., 1984b). However, in the protein-bound state this information can be obtained from the ^{15}N–^{1}H coupling constant; this coupling cannot be observed in free flavin in aqueous solution.

It has been suggested, on the basis of mainly circular dichroism and UV spectra, that flavodoxins can be divided into a pasteurianum type (*M. elsdenii*, *Clostridium MP*) and a rubrum type (*A. vinelandii*, *D. vulgaris*) flavodoxin (D'Anna & Tollin, 1972). Our results indicate that caution should be used in classifying the flavodoxins from *A. vinelandii* and *D. vulgaris* together (D'Anna & Tollin, 1972) because their NMR properties show considerable variation. Their circular dichroism spectra are similar, but such spectra cannot reval directly the electronic structure of flavin in great detail.

The three-dimensional structures of *Clostridium MP* (Burnett et al., 1974; Ludwig et al., 1976; Smith et al., 1977, 1983) and *D. vulgaris* flavodoxins have been determined. The flavin in *D. vulgaris* flavodoxin is flanked by Tyr-98 on one side and by Trp-60 on the other side. Tyr-98 is nearly coplanar with the flavin ring and located above the pyrazine moiety (central ring) of flavin. Trp-60 overlaps primarily with the benzene subnucleus of FMN, covering the C(8α), C(8), C(9),

and C(9a) atoms of flavin, but it is not coplanar with the flavin. There is an angle of 45° between the normals of the two planes. *Clostridium MP* flavodoxin has a methionine in place of tryptophan on the one side of the flavin and tryptophan in place of tyrosine on the opposite site. The tryptophan residue overlaps partially with the benzene subnucleus of flavin. The role of these amino acid residues in the particular function of the flavodoxins is not yet known. Considering the differences in the electronic structure of the two flavodoxins (Figure 2), which are rather large in the reduced state, it is suggested that these residues play a role in the particular π electron density distribution in the two flavodoxins and in the stabilization of the resulting mesomeric structures.

The hydrogen bonding between FMN and apoflavodoxin from *D. vulgaris* as revealed by NMR results is in agreement with the "first model" proposed from X-ray data (Watenpaugh et al., 1973). The hydrogen-bonding patterns of the four flavodoxins studied, as deduced from our NMR data, are presented schematically in Figure 6. It is interesting to note that *Clostridium MP* and *M. elsdenii* flavodoxins do not form a hydrogen bond with the N(5) atom in the oxidized state. In the reduced state a strong hydrogen bond is formed. These results are in agreement with crystallographic studies on *Clostridium MP* flavodoxin (Smith et al., 1977) showing that the peptide backbone at Glu-17 undergoes a conformational change on reduction that facilitates the interaction of the carbonyl group of Glu-17 with the proton at N(5) of flavin. A similar situation exists in *M. elsdenii* flavodoxin as deduced from the NMR results. Another common feature seems to be the absence of hydrogen bonding with C(4α) of flavin in all four flavodoxins. However, it should be kept in mind that the complexity of the electronic structure of flavin in reduced flavodoxins did not allow us to deduce with certainty the absolute absence of any hydrogen-bonding interactions. Nevertheless, it has been found in *Clostridium MP* flavodoxin (Smith et al., 1977) that O(17) from Glu-17 is very close to the C(4α) atom of FMN. It has been suggested that this configuration must be associated with an unfavorable Coulombic interaction, *i.e.*, repulsive. This may have some chemical consequences for the electron redistribution in reduced flavodoxins leading to the destabilization of certain mesomeric structures observed with free reduced flavin (Moonen et al., 1984b). Therefore, we believe that the unusual chemical shifts due to C(4) in the flavodoxins also reflect this effect.

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Registry No. FMN, 146-17-8; FMNH, 5666-16-0; $FMNH_2$, 5666-16-0; TARF, 752-13-6; $TARFH_2$, 18717-85-6.

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Metabolic Pathways for Ketone Body Production. ^{13}C NMR Spectroscopy of Rat Liver in Vivo Using ^{13}C-Multilabeled Fatty Acids†

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ABSTRACT: The hormonal regulation of ketogenesis in the liver of living rat has been studied noninvasively with ^{13}C nuclear magnetic resonance. The protocol involved the use of a surface coil that was placed on the skin of the rat, directly over the normal location of the liver. Signals from superficial tissue were suppressed with a 180° pulse at the center of the coil. A resolution of 0.6 ppm was obtained in the ^{13}C NMR spectra at 20.1 MHz, which was equal to or better than that observed in experiments where the liver was surgically exposed and surrounded with radiofrequency coil. The spatial selection for the liver was better than 90%, with extrahepatic adipose tissue contributing only a very small amount of signal. The metabolic activities of the liver were investigated by infusion of ^{13}C-labeled butyrate in the jugular vein of the anesthetized rat. The rate of butyrate infusion was chosen to be close to the maximum oxidative capacity of the rat liver, and the ^{13}C signal intensities were enhanced by using doubly labeled [1,3-^{13}C]butyrate as a substrate. Different ^{13}C NMR spectra and hence different metabolites were observed depending on the hormonal state of the animal. In the *fasted* rat, the most intense ^{13}C signal came from the end product of the Krebs cycle, namely, HCO_3, with additional resonances from glutamine and glutamate. Weak resonances of the ketone bodies 3-hydroxybutyrate and acetoacetate could also be detected and allowed an evaluation of the "redox state" of the in vivo liver. In contrast, 3-hydroxybutyrate was clearly the most important metabolite in the liver of *diabetic* animals, with a weak resonance of HCO_3 as the only other detectable metabolite under in vivo conditions. The ^{13}C NMR studies demonstrate that even when rates of acetyl-CoA production are high, the disposal of this compound is not identical in fasted and diabetic animals. This supports previous suggestions that the redox state of the mitochondrion represents the most important factor in regulation. For a given metabolic state of the animal, different signal intensities were obtained depending on whether butyrate was labeled at C-1, C-3, or C-1,3. From the ratios of incorporation of ^{13}C label into the carbons of 3-hydroxybutyrate, it could be estimated that a large fraction of butyrate evaded β-oxidation to acetyl-CoA but was converted directly to acetoacetyl-CoA. ^{13}C-Labeled glucose could be detected in vivo in the liver of diabetic rats.

A variety of physiological or pathological conditions leads to the enhanced production of acetoacetate and 3-hydroxybutyrate in the body. The two four-carbon organic acids are synthesized by β-oxidation from free acids delivered to the liver, which is the predominant site of ketone body production. The output of ketone bodies depends, however, not only on the rate of fatty acid input but also on the capacity of the liver to generate acetyl-CoA and to dispose of this compound through nonketogenic pathways (McGarry & Foster, 1980). For the same input level of free fatty acids marked differences in the rate of ketone body production have been found de-

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