Structural and Dynamic Information on the Complex of *Megasphaera elsdenii* Apoflavodoxin and Riboflavin 5′-Phosphate. A Phosphorus-31 Nuclear Magnetic Resonance Study[†]

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ABSTRACT: It is shown that commercial FMN contains a considerable amount of the 4′, 3′, and 2′ isomers and other phosphorus-containing compounds. These impurities can easily be analyzed and quantified by the ^{31}P NMR technique. The phosphate group of FMN bound to *Megasphaera elsdenii* apoflavodoxin is probably in the dianionic form. Its chemical shift is almost independent of pH in the range 5.5–9.2 and of the redox state of the protein. The phosphate group of bound FMN is buried in the protein. Protons of the apoprotein and of bound water located in the vicinity of the phosphate group in native flavodoxin are not exchangeable with deuteron of the bulk solvent. These protons are, however, easily exchangeable in apoflavodoxin, as shown by reconstitution experiments. The distance between the phosphorus atom of bound FMN and the N(10) atom of the isoalloxazine moiety of FMN is calculated to be about 7.8 Å. This result is in good agreement with X-ray data published for the related flavodoxin from *Clostridium MP*. The electron exchange between the oxidized and semiquinone state of *M. elsdenii* flavodoxin is rather slow ($k_{exc'} \ll 2\ s^{-1}$) whereas that between the semiquinone and hydroquinone form is much more favored ($k_{exc'} \gg 100\ s^{-1}$). This indicates that the activation energy for the transition between the semiquinone and hydroquinone states must be smaller than that for the transition between the oxidized and semiquinone states. These results offer a reasonable explanation for the one-electron transfer reaction of flavodoxins in biological reactions.

Flavodoxins are proteins of small relative molecular mass functioning as electron carriers in low potential oxidation–reduction reactions (Mayhew & Ludwig, 1975). In these redox reactions the flavodoxins are often interchangeable with the iron–sulfur proteins ferredoxins. The flavodoxins contain riboflavin 5′-phosphate (FMN)[1] as prosthetic group. Flavodoxins can be reduced in two distinct one-electron steps with the formation of the relatively oxygen-stable flavosemiquinone as an intermediate (Mayhew & Massey, 1973). During electron transfer reactions, flavodoxins shuttle between the semiquinone and hydroquinone state (Mayhew & Ludwig, 1975, and references therein). The chemical and physical properties of the flavodoxins have been investigated in great detail (Mayhew & Ludwig, 1975).

Flavodoxin from *Megasphaera elsdenii* is easily available in large quantities. Its physical chemical properties are very similar to those of *Clostridium MP* flavodoxin for which three-dimensional structural data at high resolution are published (Smith et al., 1977). Because the latter flavodoxin is much more difficult to isolate in the large quantities required for certain physical techniques (e.g., nuclear magnetic resonance), *M. elsdenii* flavodoxin is often used as a substitute in such studies. In the past the binding of FMN to apoflavodoxin of *M. elsdenii* has been investigated by fluorescence and visible light absorption techniques (Mayhew & Massey, 1969) and by conventional kinetic (Gast et al., 1976) and temperature-jump techniques (Gast & Müller, 1978). In order to complement these studies and to gain, if possible, insight into the interactions between the apoenzyme and its prosthetic group on an atomic level, we set up a NMR program on *M. elsdenii* flavodoxin and related biomolecules. The aim of such studies is to elucidate the subtle, specific interactions between the constituents of a flavoprotein which are probably, as proposed earlier (Müller, 1972), responsible for the specific biological action of a certain flavoprotein.

Here we report on a ^{31}P NMR study on the binding of FMN by *M. elsdenii* apoflavodoxin. The results yielded information on the interaction of the phosphate group of the prosthetic group with the apoprotein, the distance between the phosphorus atom and the isoalloxazine moiety, and the rate of electron exchange between the molecules in the different redox states. In addition, it is shown that commercial FMN contains several isomeric compounds which can be identified by their chemical shifts and quantitated by the integrals of the corresponding resonance lines.

Materials and Methods

Flavodoxin from *Megasphaera elsdenii* was isolated and purified according to published procedures (Mayhew & Massey, 1969). FMN was obtained from Sigma (lot 44B-0820). 7,8-Dimethyl-N^{10}-(2-hydroxyethyl)isoalloxazine 2′-phosphate was synthesized as described earlier (Müller et al., 1973). Sodium dithionite was purchased from Merck, Darmstadt, West Germany.

For removal of the exchangeable protons in the "interior" of the flavodoxin, the apoflavodoxin was prepared according to the method of Wassink & Mayhew (1975). The apoflavodoxin was then dissolved in a minimal volume of a solution of 50 mM Tris-HCl in deuterium oxide (pH 8.0). This solution was dialyzed against the same buffer for 1 day at room temperature and then for 2 days at 4 °C. The buffer solution was exchanged 2 times against freshly prepared deuterated buffer solutions. After the last change, a 3-fold excess of FMN was added to the fresh buffer and equilibrated for 1 day at 4 °C. Excess of FMN was removed from reconstituted flavodoxin by dialysis against a solution of 50 mM Tris-HCl in H_2O (pH 8.0). The flavodoxin thus obtained was then concentrated by lyophilization.

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[1] Abbreviations: FMN, riboflavin 5′-phosphate; 4′-FMN, riboflavin 4′-phosphate; 3′-FMN, riboflavin 3′-phosphate; 2′-FMN, riboflavin 2′-phosphate; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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Reduction and reoxidation experiments were conducted by the addition of the desired amount of a dithionite solution to the anaerobic solution of flavodoxins or free FMN. Anaerobiosis was achieved by carefully flushing the solutions in the NMR tube with argon for about 20 min. The desired degree of reoxidation was obtained by injecting small volumes of air into the NMR tube containing the anaerobic flavohydroquinone solution followed by gentle mixing.

For relaxation measurements on solutions of free FMN, traces of paramagnetic metal ions were removed from the solutions by passing them through a small Chelex-100 (product of Bio-Rad) column. In all other cases a small amount of EDTA was added to the solution to be investigated.

^{31}P NMR measurements were recorded on a Varian XL 100-15 spectrometer operating at 40.5 MHz and equipped with a 16K Varian 620-L computer. A spectral width of 1000 Hz (4K data points) and an acquisition time of 2 s was used. All spectra were acquired under proton noise decoupling conditions unless otherwise stated. All chemical shift values were determined relative to the external standard 85% H_3PO_4. The spectrometer was locked on the deuterium resonance line of deuterium oxide contained in the buffer solution. All spectra were recorded at 26 ± 2 °C.

Wilmad 12-mm precision NMR tubes were used. The samples contained 1.5–3 mM flavodoxin in 150 mM Tris-HCl solutions of pH 8.0. Samples of free FMN contained 56 mM FMN in the same buffer (pH 8.2) unless otherwise stated. Depending on the kind of experiment, the buffer solutions were made up of 10–100% deuterium oxide. In pH titration experiments the desired pH value was adjusted in the NMR tube by addition of 0.1 M HCl or solid Tris. The pH meter readings were used without correction for isotope effects.

Spin–lattice (T_1) relaxation measurements were performed by using the inversion–recovery method (Vold et al., 1968) as described by Levy & Peat (1975). At least 10 spectra were recorded for the determination of one T_1 value. The experimental values were fitted to one exponential function by computer analysis (Sass & Ziessow, 1977).

Spin–spin (T_2) relaxation values were estimated from the line width of the experimental spectra.

Equilibrium ultracentrifugation was performed in a MSE analytical ultracentrifuge. Samples contained 0.5–3 mM flavodoxin in 150 mM Tris buffer of pH 8.0. Measurements were done at 26 °C. These conditions are the same as used in the NMR experiments.

Results and Discussion

^{31}P NMR of Free FMN. It has been shown that apoflavodoxin from *M. elsdenii* binds only those flavin derivatives which carry at the N(10) position a side chain of five carbon atoms and a terminal phosphate group (Mayhew, 1971; Gast & Müller, 1978) (Figure 1). Mayhew (1971) and Wassink & Mayhew (1975) demonstrated from binding studies of apoflavodoxin from *M. elsdenii* that commercial FMN contains impurities (20–30%) not binding to the apoflavodoxin. Pure FMN, however, can be obtained by affinity column chromatography using apoflavodoxin from *M. elsdenii* as an affinity label (Mayhew & Strating, 1975). More recently Scola-Nagelschneider & Hemmerich (1976) demonstrated from ^{1}H NMR data that riboflavin 4′-phosphate (4′-FMN) is the major byproduct of FMN purified by ion-exchange chromatography. It is known that the chemical shift of phosphate esters directly reflects the bond angles in the O–P–O grouping (Gorenstein, 1975). These bond angles are also dependent on the ionization state of the phosphate group in phosphate esters such as FMN. In addition hydrogen bonds and stereochemical effects also affect the ^{31}P chemical shifts, but to a lesser degree than the bond angle (Evans & Kaplan, 1979). The polarity of the environment of the phosphate ester does not, or at most only to a minor degree, influence the ^{31}P chemical shifts (Gorenstein et al., 1976). These facts make it possible to interpret ^{31}P NMR spectra accurately. Therefore, commercial FMN was also investigated with the aim to identify, if possible, the byproducts.

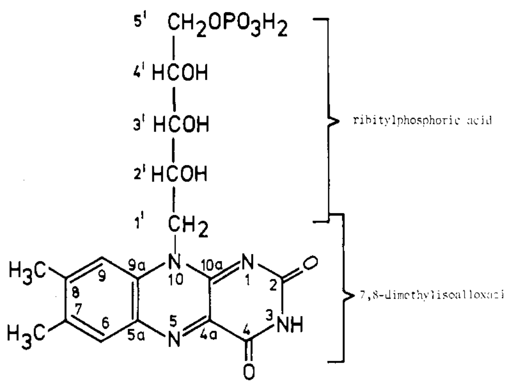

FIGURE 1: Structure of riboflavin 5′-phosphate (FMN).

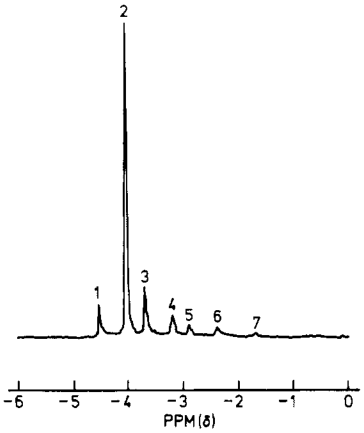

FIGURE 2: ^{31}P NMR spectrum of commercial FMN (56 mM) in 150 mM Tris-HCl, pH 7.0.

In Figure 2 the ^{31}P NMR spectrum of commercial FMN is shown. The pH value used here (pH 7.0) gives the best resolution of the resonance lines. From this spectrum it is obvious that besides FMN and 4′-FMN, which are the major components of the sample, other phosphorus-containing compounds are present (Table I). For characterization and facilitation of the assignment of the resonance lines of the major components in Figure 2, a pH titration study was performed. The minor components in Figure 2 (peaks 5–7) were not analyzed further. It was found that the phosphate groups exhibit a p*K* value of about 6 and that all four resonances in Figure 2 (peaks 1–4) show a downfield shift of 3.5–5 ppm on going from pH 4.0 to 9.0. This strongly indicates that all major resonances are due to phosphate esters. The small differences in their pK_a values reflect probably some stereochemical differences among these esters as is also obvious from the corresponding chemical shift values (Table I). For instance, the pK_a value of the phosphate ester corresponding to peak 4 of Figure 2 is higher than that of FMN (peak 2). This may be due to a weak interaction of the OH group of the phosphate ester with the isoalloxazine ring, i.e., N(1). To check this possibility the model compound 7,8-dimethyl-N^{10}-(2-hydroxyethyl)isoalloxazine 2′-phosphate was studied. This compound indeed shows a pK_a value identical with the phosphate ester at −3.17 ppm (Table I). Under identical conditions the chemical shift of the model compound is about

Table I: Analysis of Commercial FMN by ^{31}P NMR

peak in Figure 2	pK_a[a]	abundance (%)[b]	chemical shifts[c] at pH			assignment
			4.0	7.0	9.0	
1	5.7	~5	−0.15	−4.48	−4.92	3'-FMN
2	6.1	78	−0.90	−4.01	−4.70	FMN
3	6.1	~9	−0.07	−3.65	−4.33	4'-FMN
4	~6.3[d]	~4	0.20	−3.17	−3.90	2'-FMN
HIP[e]	6.3		0.10	−2.85	−3.83	

[a] The accuracy of the values is ±0.05 pH unit. The pK_a values were calculated by fitting pH vs. chemical shift curves. [b] Quantitatively determined by integration of ^{31}P NMR spectra where the delay between two accumulations was greater than $5T_1$. No exponential multiplication was applied in such experiments. [c] Chemical shifts (ppm) are reported relative to external H_3PO_4 (85%). [d] This value could not be determined with the same accuracy as the other reported values owing to the overlap with other peaks. [e] 7,8-Dimethyl-N^{10}-(2-hydroxyethyl)isoalloxazine 2'-phosphate.

0.3 ppm toward higher field. The downfield shift of the corresponding phosphate group in 2'-FMN is explained by the difference in substitution of the 2' position in the two molecules. On the basis of these results, peak 4 (Figure 2) can be assigned with great confidence to a 2'-phosphate ester of riboflavin.

The assignments of the resonance lines of the ^{31}P NMR spectrum of commercial FMN (Figure 2) as presented in Table I were confirmed by investigating a solution of half-reduced FMN. It is known that such solutions contain about 4% flavosemiquinone at pH 7 (Müller et al., 1971). This intramolecular paramagnetic label should yield information with respect to the distance between the various phosphate esters and the isoalloxazine ring system by the mechanism of dipolar broadening of the resonance lines. We observed that in spectra of such solutions peak 1 and peak 4 were no longer detectable because of broadening, and peak 2 was hardly broadened, whereas peak 3 showed a greater degree of broadening than peak 2. This indicates that the distance between the phosphate ester group and the isoalloxazine ring system increases in the series: peaks 4 and 1, peak 3, and peak 2. These results support the assignments given in Table I.

The ^{31}P chemical shift of FMN reported in this paper is in agreement with values published by Favaudon et al. (1980) and Edmondson & James (1979). In addition it was found that NaCl and urea in concentrations up to 1 M did not influence the ^{31}P chemical shift of FMN (pH 8.2). This is in accordance with the conclusion of Gorenstein et al. (1976) from results on other phosphate esters that ^{31}P chemical shifts are only little influenced by the environment.

It is known that FMN in aqueous solution aggregates; i.e., stacking of the isoalloxazine ring system occurs. Sarma et al. (1968) investigated this phenomenon by ^1H NMR. We extended this study with ^1H NMR to lower concentrations than previously used. Our results are fully consistent with those of Sarma et al. (1968). It was, however, found that stacking of FMN already occurs at concentrations as low as 0.3 mM (100 mM KP_i, pH 6.5). In fact it was demonstrated by light absorption difference spectroscopy that monomeric FMN is only found at concentrations lower than 50 μM (Müller et al., 1973). Moreover ^{31}P T_1 measurements indicate that the mobility of the ribityl phosphate side chain of FMN decreases with increasing concentrations of FMN, i.e., increasing stacking. For concentrations of 3.5, 14.2, and 59.3 mM FMN in 50 mM Tris, pH 8.2, we found the following T_1 values: 4.7, 4.3 and 3.9 s, respectively. These T_1 values are in the same range as those of adenosine 5'-phosphate measured at 40.5 MHz (Nanda et al., 1980).

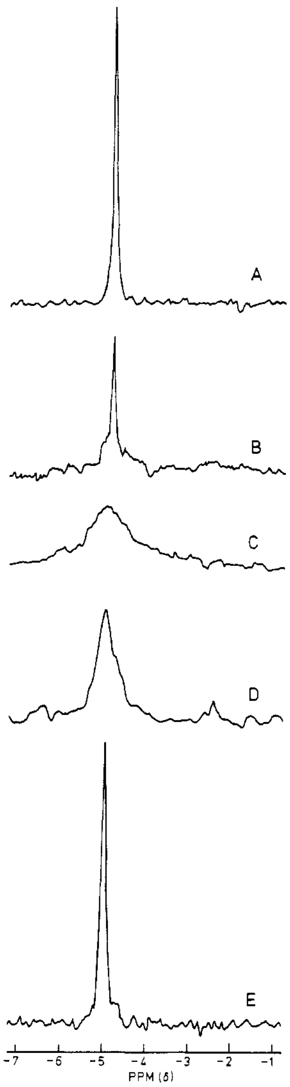

FIGURE 3: ^{31}P NMR spectra of *M. elsdenii* flavodoxin (3 mM) in the oxidized, the semiquinone, and the hydroquinone states in 150 mM Tris-HCl, pH 8.2. (A) Oxidized state (line broadening 1 Hz). (B) Mixture of about 30% oxidized and about 70% semiquinone form (line broadening 1 Hz). (C) Semiquinone form (line broadening 3 Hz). (D) Mixture of about 2% semiquinone and about 98% hydroquinone form (line broadening 3 Hz). (E) Hydroquinone form (line broadening 1 Hz).

^{31}P NMR on M. elsdenii Flavodoxin. The ^{31}P NMR spectrum of *M. elsdenii* flavodoxin is shown in Figure 3A. The spectrum exhibits only one phosphorus resonance at −4.80 ppm due to bound FMN in contrast to other flavodoxins containing more than one phosphate group (Edmondson & James, 1979). Free FMN in the dianionic form shows a chemical shift of −4.70 ppm. The chemical shift of protein-bound FMN suggests therefore that the phosphate group of FMN is bound to the protein in the dianionic form. The small downfield shift of protein-bound FMN as compared to that of free FMN is probably due to steric effects or strain. Such a downfield shift due to strain has been observed in alkaline phosphatase (Bock & Sheard, 1975).

The ^{31}P chemical shift of *M. elsdenii* flavodoxin is independent of the pH in the range 6.0–9.2. Below pH 6.0 a small upfield shift is observed which amounts to 0.3 ppm at pH 5.5. At pH values below 5.5, the solubility of the flavodoxin decreases [p*I* is about 4 (Gast et al., 1976)] preventing studies at lower pH values. The results indicate that the phosphate group of protein-bound FMN is deeply buried in the apoenzyme and unaccessible to bulk solvent.

The proton-coupled ^{31}P NMR spectrum of free FMN shows a triplet due to coupling of the 5′-CH$_2$ group with the 5′-phosphorus atom (data not shown). The vicinal coupling constant is about 7.2 Hz. The proton-coupled spectrum of *M. elsdenii* flavodoxin does not show a spitting of the phosphorus resonance line, but the width of the resonance line is about 5.5 Hz. The proton-decoupled spectrum, on the other hand, exhibits a line width of 2.3 ± 0.2 Hz. These observations can be analyzed by a Karplus-like relation (Cozzone & Jardetzky, 1976) in terms of the structure of the ribityl phosphate bonding and indicate a gauche–gauche conformation. Similar results were reported by Favaudon et al. (1980) for flavodoxin from *D. vulgaris* and *D. gigas*. The latter results and those presented here are in agreement with three-dimensional data on *Clostridium MP* flavodoxin (Burnett et al., 1974).

The influence of reduction of an anaerobic solution of *M. elsdenii* flavodoxin on the ^{31}P NMR spectrum is shown in Figure 3. During the addition of the first electron to oxidized flavodoxin, yielding quantitatively the flavosemiquinone (Mayhew, 1978), the resonance line due to oxidized flavodoxin is decreased and broadened. In the semiquinone form only a broad peak ($\delta \sim -4.9$ ppm) is observed. Further reduction of the flavosemiquinone yielding finally the flavohydroquinone does not alter the NMR spectrum until almost full reduction is achieved. The ^{31}P NMR spectrum of the flavohydroquinone exhibits a sharp resonance line at −4.9 ppm. The observed spectral changes are fully reversible upon stepwise reoxidation of the solution of reduced flavodoxin. The broadening of the resonance line of the phosphate group of bound FMN must be due to the flavin radical since other effects, such as drastic conformational changes of the protein, can be excluded by three-dimensional data obtained for the related flavodoxin from *Clostridium MP* (Andersen et al., 1972). The small difference between the ^{31}P chemical shifts of the three redox states of flavodoxin indicates that the binding interaction between the phosphate group of FMN and the apoprotein is not perturbed by redox reactions of the isoalloxazine moiety. This conclusion is in agreement with X-ray data on *Clostridium MP* flavodoxin (Andersen et al., 1972). ^{31}P NMR data similar to that described above were found for *D. vulgaris* and *D. gigas* flavodoxin by Favaudon et al. (1980).

Analysis of the line width observed during oxidation–reduction experiments yields information with respect to the rate of electron transfer between flavodoxin molecules of different redox state. By analogy to chemical exchange reactions (Dwek, 1973; McLaughlin & Leigh, 1973), the perturbation of T_1 and T_2 of the phosphorus resonance can be correlated with the rate of transfer of one electron between two molecules of flavodoxins. Reduction of the oxidized flavodoxin to the semiquinone state does not affect the resonance line of the oxidized flavodoxin, but it is superimposed on the broad line of the semiquinone form (Figure 3A–C). Such a situation is characterized by a slow exchange reaction. The electron transfer reaction can be described by the equilibrium reaction

$$\mathrm{Fl}_{1(\mathrm{ox})} + \mathrm{Fl}_2\cdot \rightleftarrows \mathrm{Fl}_1\cdot + \mathrm{Fl}_{2(\mathrm{ox})} \qquad (1)$$

where $\mathrm{Fl}_{1(\mathrm{ox})}$ and $\mathrm{Fl}_2\cdot$ are two flavodoxin molecules in the oxidized and semiquinone (Fl·) state, respectively. The electron-transfer reaction is a pure first-order process. The experimental data were analyzed according to McLaughlin & Leigh (1973). Since no broadening of the resonance line of the oxidized flavodoxin, is observed, i.e., T_2 is not affected, by reduction to the semiquinone form, the limit of the rate of electron exchange is calculated to be $\ll 2$ s^{-1}. James et al. (1973) calculated from ^{1}H NMR data a value for $k_{\mathrm{exch}} \ll 50$ s^{-1}. This latter value is much larger than our value, but the experimental data of James et al. (1973) did not allow a more accurate calculation of k_{exch}.

In going from the semiquinone to the hydroquinone form of flavodoxin, no superimposed lines are observed in the ^{31}P NMR spectrum. Even in the presence of only a very small concentration of flavosemiquinone in the solution studied only a broad resonance line is observed. In fact the spectrum shown in Figure 3D was obtained by admission of a small volume of air to the solution of the flavodoxin hydroquinone, yielding a small concentration of flavosemiquinone (about 1–2%). The fact that no sharp line could be observed indicates that we are dealing here with a fast-exchange reaction. Analysis of these data according to McLaughlin & Leigh (1973) yields a lower limit of the rate of electron exchange of $\gg 100$ s^{-1}. The small difference between the ^{31}P chemical shifts of the oxidized and semiquinone form of flavodoxin, on the one hand, and that between the semiquinone and hydroquinone form, on the other hand, does not allow a more accurate analysis of the rates of electron transfer. Nevertheless, the experimental results show clearly that under our experimental conditions a large difference exists in the rate of electron transfer between molecules of oxidized and semiquinone flavodoxin and that between molecules of semiquinone and hydroquinone flavodoxin. This is the first direct proof that fast electron transfer occurs under equilibrium conditions between semiquinone and hydroquinone flavodoxin molecules. James et al. (1973) also reported that the rate of electron transfer between semiquinone and hydroquinone molecules is the same as that between the oxidized and semiquinone forms, i.e., $\ll 50$ s^{-1}. This is in contradiction with our results and is caused by the severe limitations of the analysis of the ^{1}H NMR data.

The kinetics of the two step one-electron reduction of *M. elsdenii* flavodoxin by dithionite was investigated by Mayhew & Massey (1973). These authors found that the rate of reduction of the semiquinone to the hydroquinone form is at least 2 orders of magnitude larger than that of the reduction of the oxidized to the semiquinone state. On the other hand, the potential of the semiquinone–hydroquinone redox couple is more negative than that of the quinone–semiquinone redox couple, suggesting that the latter redox reaction should be more favored energetically than the former one. The kinetic data of Mayhew & Massey (1973) and our own data strongly indicate that the activation energy for the reduction of the semiquinone and the electron exchange between semiquinone and hydroquinone is much smaller than that for the quinone–semiquinone couple. These observations may be explained by a protein conformational change occurring during the quinone–semiquinone transition and not during the semiquinone–hydroquinone transition, resulting in a higher transition energy for the first transition. The X-ray results of Andersen et al. (1972) support this suggestion. The formation or breakage of specific interactions between the prosthetic group and the apoprotein may also cause small local conformational (configurational?) changes of some group(s). The problem is therefore of kinetical rather than thermodynamic origin. Our electron-exchange studies now also offer an explanation for the fact that the comproportionation reaction, i.e., $\mathrm{Fl}_{\mathrm{ox}} + \mathrm{Fl}_{\mathrm{red}} \rightleftarrows 2\mathrm{Fl}\cdot$, in flavodoxin is not favored (Mayhew & Massey, 1973). From a kinetic point of view, taking into account our results, the electron-exchange reaction between quinone and hydroquinone must be orders of magnitude smaller than that calculated for the quinone–semiquinone transition.

The biological function of flavodoxins is to transfer one electron at a time to other redox proteins (e.g., hydrogenase) shuttling thereby between the semiquinone and hydroquinone state (Mayhew & Ludwig, 1975, and references therein). As can be calculated from recently published results of Van Dijk & Veeger (1981), the turnover number of *M. elsdenii* flavodoxin in a reaction with hydrogenase can be as high as 10^5 s^{-1}. The data of Mayhew & Massey (1973) and our own data allow some rationalization of these biologically important reactions.

The strong broadening of the phosphorus resonance in the NMR spectrum of flavodoxin in the semiquinone form (Figure 3C) allows us to calculate the distance between the isoalloxazine ring and the phosphorus atom of bound FMN. Since the electron-nuclear hyperfine coupling on the phosphorus atom can be neglected (no spin density of the radical on this nucleus), we have to consider only the dipolar part of the original Solomon–Bloembergen (Solomon, 1955; Bloembergen, 1957) equation.

The rotational correlation time of flavodoxin *M. elsdenii* was calculated according to the Stokes–Einstein equation, taking into account the solvation of the molecule (Tanford, 1961). The rotational correlation time was calculated to be 5×10^{-9} s at 26 °C (temperature of NMR experiments). Analytical ultracentrifugation experiments were carried out in order to check if the concentrations (3 mM) used in the NMR experiments lead to association of flavodoxin molecules. No aggregation could be detected, so the calculated τ_c value is a good approximation. The T_{2p} (paramagnetic spin–spin relaxation) value was calculated from the line width of spectra of the flavodoxin. From Figure 3B a line width of 43 Hz is calculated. The line width in the spectrum of oxidized flavodoxin yields the diamagnetic contribution to the line width, i.e., field inhomogeneity, $^1H–^{31}P$ dipole–dipole interaction, and chemical shift anisotropy. In the semiquinone form these contributions are approximately the same. This procedure yields a line width of about 37 Hz (taking into account the exponential line broadening) for the paramagnetic contribution, from which a T_{2p} $[=(\pi\Delta\nu_{1/2})^{-1}]$ of 8.6×10^{-3} s is calculated. With the aid of these values the distance between the isoalloxazine radical and the phosphorus nucleus of FMN was calculated to be 8.8 Å (0.88 nm). The highest spin density in flavin radicals is located in the pyrazine subnucleus of the isoalloxazine molecule (Müller et al., 1971). If we assume for convenience that the average effective location of the free electron is at about 1-Å distanced from the N(10) atom of flavin, i.e., centered in the pyrazine subnucleus, then the calculated distance between the N(10) atom and the phosphorus nucleus of FMN is about 7.8 Å. This is in good agreement with the distance between the two atoms (8.5 Å) as obtained by X-ray studies for the *Clostridium MP* flavodoxin (Burnett et al., 1974). The rather good agreement between the two independent methods indicates that the side chain of FMN is strongly bound, with little or no internal freedom. If the side chain had possessed some internal mobility, we would have been forced to take into account an additional τ_c value for the internal mobility which would influence the calculated value for the distance considerably.

According to the three-dimensional structure of *Clostridium MP* flavodoxin (Burnett et al., 1974), no charged groups are located in the immediate neighborhood of the phosphate group of FMN. There are, however, various polar amino acid residues in the vicinity of the phosphate group, i.e., four hydroxyamino acid residues and five backbone NH protons. In addition, one molecule of water is possibly also in the vicinity of the phosphate group. For investigation of the environment of the phosphate group, a comparative relaxation study between native flavodoxin and flavodoxin reconstituted in deuterium oxide (cf. Materials and Methods) was performed. T_1 and T_2 measurements should make it possible to elucidate the influence of labile protons in the vicinity of the phosphate group on the relaxation times. Figure 4 shows spin–lattice relaxation experiments performed with flavodoxin reconstituted in deuterium oxide. Fitting of the data by one exponential curve yields a T_1 value of 1.17 s. In Table II the calculated T_1 and T_2 values are presented. From the T_2 values of Table II it is obvious that the line width of the phosphorus resonance is different in the two preparations studied. The chemical and the physical properties of the two preparations are identical, so it must be concluded that the difference in line width must originate from the influence of labile protons on the T_2 relaxation.

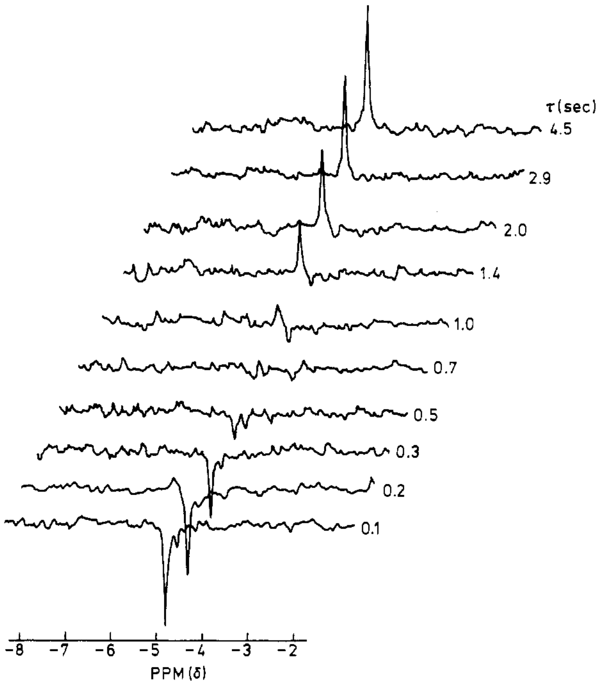

FIGURE 4: Partially relaxed ^{31}P NMR spectra at 40.5 MHz of *M. elsdenii* flavodoxin (3 mM) in 150 mM Tris-HCl, pH 8.2, obtained by the $180°–\tau–90°$ pulse sequence and Fourier transformation. The labile protons were exchanged prior to the experiments (cf. Materials and Methods).

Table II: Calculated T_1 and T_2 Relaxation Times of Oxidized *M. elsdenii* Flavodoxin

protein preparation	T_1[a] (s)	T_2[b] (s)
native protein	0.57 ± 0.06	0.14 ± 0.02
protein reconstituted in deuterium oxide[c]	1.17 ± 0.12	0.21 ± 0.03

[a] Determined by the inversion-recovery technique (accuracy estimated). [b] Determined from the line width (accuracy estimated). [c] Cf. Materials and Methods.

For studies on a possible back exchange of deuterons in reconstituted protein, a sample was dissolved in a mixture of $H_2O/^2H_2O$ (9:1 v/v) in 150 mM Tris, pH 8.0, and the line width followed with time. It was found that up to 20 h the line width remained constant, i.e., no back exchange occurred. A similar experiment (10 h) with the hydroquinone form of flavodoxin yielded the same result. This means that the exchange reaction, if it occurs at all, must be very slow. Also these results indicate that the phosphate group of FMN interacts very strongly with the apoprotein and that the exchange of FMN molecules in flavodoxin is a very slow process.

For oxidized, native flavodoxin the spin–lattice relaxation can be described by

$$\frac{1}{T_{1(\text{total})}} = \frac{1}{T_{1(^1\text{H,exch})}} + \frac{1}{T_{1(^1\text{H,nonexch})}} + \frac{1}{T_{1(\text{rest})}} \qquad (2)$$

where $T_{1(^1\text{H,exch})}$ is the relaxation by labile protons in native flavodoxin, $T_{1(^1\text{H,nonexch})}$ is the relaxation by nonexchangeable protons in flavodoxin reconstituted in deuterium oxide, and $T_{1(\text{rest})}$ is the relaxation by all other mechanisms. For the reconstituted flavodoxin the relaxation by labile protons is eliminated whereas the relaxation by deuterons can be neglected because of the low gyromagnetic ratio. The relaxation by labile protons can therefore be expressed as

$$\frac{1}{T_{1(^1\text{H,exch})}} = \frac{1}{T_{1(\text{native flavod})}} - \frac{1}{T_{1(\text{reconst flavod})}} \qquad (3)$$

Using the values given in Table II and eq 3 yields a value of 1.11 ± 0.3 s for the relaxation by labile protons $(T_{1(^1\text{H,exch})}$. With the aid of eq 4 (Abragam, 1961) the calculated value

$$\frac{1}{T_{1(^1\text{H,exch})}} = \left(\frac{n\hbar^2\gamma_H^2\gamma_P^2}{10r_{PH}^6}\right) \times \left[\frac{\tau_c}{1 + (\omega_H - \omega_P)^2\tau_c^2} + \frac{3\tau_c}{1 + \omega_P^2\tau_c^2} + \frac{6\tau_c}{1 + (\omega_H + \omega_P)^2\tau_c^2}\right] \qquad (4)$$

can be correlated with the number of labile protons and their distance from the phosphorus atom of FMN. n is the number of protons separated by a distance r_{PH} from the phosphorus nucleus, γ_H and γ_P are the gyromagnetic ratios of the proton and phosphorus nucleus, respectively, ω_H and ω_P are the corresponding Larmor precession frequencies, τ_c is the rotational correlation time, and h is Plank's constant. It should be mentioned that eq 4 does not allow us to determine the number of protons accurately, but only an estimate can be made. Moreover the distance of the protons from the phosphorus atom is assumed to be the same for all labile protons, but in reality this distance may be different for different labile protons. This is important to notice since because of the sixth power dependence, the distance between protons and the phosphorus atom is of great influence on T_1. For the distance r_{PH} a value of 2.75 Å was assumed. With these values the number of labile protons was calculated to be five (eq 4). Considering the approximate value of r_{PH} the calculated value of five protons is in fair agreement with crystallographic data (Burnett et al., 1974) on the flavodoxin from *Clostridium MP.* This result confirms the conclusion drawn above with respect to the strong interaction of the phosphate group of FMN with the apoflavodoxin from *M. elsdenii.*

With the results described above we can finally test if the decrease of the line width observed in reconstituted flavodoxin as compared to that in native flavodoxin is in agreement with the T_1 data (Table II). For the analysis of the T_2 experiment, equations analogous to eq 2 and 3 can be written. In this way we obtain a value of 0.42 ± 0.15 s for the spin–spin relaxation by labile protons. For analysis of this value, the following equation is applied (Abragam, 1961):

$$\frac{1}{T_2} = \left(\frac{n\hbar^2\gamma_H^2\gamma_P^2}{20r_{PH}^6}\right)\left[4\tau_c + \frac{\tau_c}{1 + (\omega_H - \omega_P)^2\tau_c^2} + \frac{3\tau_c}{1 + \omega_P^2\tau_c^2} + \frac{6\tau_c}{1 + \omega_H^2\tau_c^2} + \frac{6\tau_c}{1 + (\omega_H + \omega_P)^2\tau_c^2}\right] \qquad (5)$$

The symbols in this equation are the same as in eq 4. When $n = 5$ and $r_{PH} = 2.75$ Å, a theoretical value of 0.6 s for the spin–spin relaxation by labile protons is calculated. This value is in fair agreement with the experimental value of 0.42 s This means, therefore, that the results of the analysis of T_2 support the data of the analysis of T_1. From this it can be concluded that labile protons interact via hydrogen bonding with the phosphate group of protein-bound FMN, but the results do not allow a precise determination of the number of protons involved in the hydrogen bonding and their distance to the phosphorus nucleus. The agreement between our results and X-ray data also indicates that the ribityl phosphate group of FMN is strongly bound to the *M. elsdenii* apoflavodoxin and possesses no measurable internal mobility.

Finally it is hoped that the ^{31}P NMR technique, in combination with other physical methods, will provide a deeper insight into the structure–function relationship of flavoproteins.

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