THE REGULATION OF THE STRUCTURE AND FUNCTION OF FLAVIN (VITAMIN B_2) ON BINDING TO APOFLAVOPROTEINS AND ITS BIOLOGICAL IMPLICATIONS

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Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
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van de Landbouwhogeschool te Wageningen.

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STELLINGEN

1. De 'omvattende reconstructie', het streven naar 'numeriek rendement' en 'modulegewijze opbouw' voor het geplande wetenschappelijk onderwijs (concept beleidsnota Beiaard) ontkent het wetenschappelijk onderwijs als een culturele waarde, die onlosmakelijk verbonden is met de identiteit van onze cultuur.

2. De individuele vrijheid, die als een normatieve kracht in het denken te voorschijn treedt, is een fundamentele verworvenheid in onze cultuur. In dit licht moeten we het afstemmen van het wetenschappelijk onderwijs op de arbeidsmarkt als een inbreuk op de individuele vrijheid zien.

3. Nu de invloed van de religie in Nederland als leverancier van normen en waarden steeds minder wordt, is het van des te groter belang om aandacht te schenken aan de zin en de ontwikkeling van normen en waarden.

4. In discussies over de legitimatie van wetenschappelijk onderzoek spelen de kwalificaties 'toegepast' of 'fundamenteel' onderzoek een belangrijke rol (zie bijv. de nota 'Biochemie Over Leven'). Deze kwalificaties dienen achterwege gelaten te worden, zolang deze terminologie gebrekkig gedefinieerd en daardoor subjectief is.
   'Biochemie Over Leven', Staatsuitgeverij, 's-Gravenhage, 1982.

5. In het zoeken naar een verklaring voor het 'afstemmechanisme' van de functie van flavine co-enzymen door apoflavoproteïnen is de rol van de modulatie van de aktiveringsenergie in de verschillende redoxovergangen onderschat.

6. Aangezien röntgenkristallografische gegevens van gereduceerde flavoëiwitten wat betreft het isoalloxazine passend worden gemaakt met slechts één variabele, i.e. de 'vlinderhoek', zijn de conclusies omtrent de werkelijke conformatie van de isoalloxazinering twijfelachtig.

7. De algemene aanvaarding van het C(4a) peroxyflavine als een essentieel intermediair in de activatie van zuurstof door flavines is voor een belangrijk deel te danken aan het feit, dat deze verbinding door Ghisla et al. d.m.v. ^{13}C NMR is waargenomen in het enzym luciferase. De door de auteurs aan het C(4a) peroxyflavine toegekende piek representeert dit intermediair echter niet.
   Ghisla, C., Hastings, J.W., Favaudon, V. and Lhoste, J.-M.
   Proc.Natl.Acad.Sci. USA 75, 5860-5863, 1978.

8. Hoewel reeds lang bekend is, dat C(4a) peroxyflavine een geschikt hydroxyleringsreagens is voor electronenrijke verbindingen, concludeert Visser uit enkel de structuur van het geoxydeerde enzym-substraat complex van p-hydroxybenzoaathydroxylase, dat het enzymatische reactiemechanisme geen analogon in de organische chemie kan hebben. De omgekeerde conclusie i.e. de reactie heeft een analogon en daarom is het model onjuist, moet waarschijnlijker worden geacht.

   Visser, C.M., Eur.J.Biochem. 135, 543-548, 1983

9. De resultaten van Tauscher et al., overigens grotendeels onjuist, hebben voor het onderzoek naar de modulatie van redoxpotentialen van eiwitgebonden flavines een werking gehad, die in biochemische termen omschreven kan worden als een 'dead-end' of 'suicide' inhibitie.

   Tauscher, L., Ghisla, S. and Hemmerich, P., Helv.Chim.Acta 56, 630-649, 1973.

10. Schakende promovendi moeten zich realiseren dat hun stelling voor een opponent wel eens mat zou kunnen zijn.

Chrit Moonen
The regulation of the structure and function of flavin (vitamin B_2) on binding to apoflavoproteins and its biological implications
16 december 1983, Wageningen

LANDBOUWHOGESCHOOL
WAGENINGEN

"With the role of flavins in bacterial bioluminescence as precedent, we can anticipate that flavins may continue to provide their own spotlight on their roles in redox biochemistry"

Christopher Walsh

The work described in this thesis has in part been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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Voorwoord

Ondanks het (te) individualistische karakter van het promotieonderzoek, kan ik terugzien op perioden van uitstekende samenwerking, waarvoor ik met genoegen mijn dank uitspreek. Bij voorbaat excuseer ik mij, indien ik mensen mocht vergeten.

Mijn promotor, Franz Müller, voor het eindeloze geduld, waarmee hij mijn ideeën aanhoorde en corrigeerde. Hij betekende meer voor mij, dan de term „promotor" aanduidt.

Willem van Berkel en Willy van den Berg isoleerden en analyzeerden voor mij de eiwitten, zodat ze aan de belangrijkste criteria voldeden: 1) het moet geel zijn; 2) het moet veel zijn.

Jacques Vervoort en Robert Wijnands, die mij met allerhande werkzaamheden hebben geholpen en mede de ontspannen sfeer op lab VI bepaalden.

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Martin Boumans en Bery Sachteleben voor het tekenwerk.

Dit onderzoek zou vrijwel onmogelijk geweest zijn zonder NMR machines van uitstekende kwaliteit. Van onschatbare waarde hierbij waren Adrie de Jager voor de Bruker CXP 300 en Varian XL100, Klaas Dijkstra voor de Bruker HX360, Pieter van Dael en Cees Haasnoot voor de Bruker WM500 en Joost Lohman voor de Bruker WM250.

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Chapters 2,3,4 have been published separately.

Chapters 5,6,7,8,10,11 will be published separately.

Chapter 2: C.T.W. Moonen and F. Müller, Biochemistry, 1982, 21, 408-414.
Chapter 3: C.T.W. Moonen, P.J. Hore, F. Müller, R. Kaptein and S.G. Mayhew, FEBS Letters, 1983, 149, 141-146.
Chapter 4: C.T.W. Moonen and F. Müller, Eur.J.Biochem., 1983, 133, 463-470.
Chapter 5: C.T.W. Moonen, J. Vervoort and F. Müller, submitted to Biochemistry.
Chapter 6: C.T.W. Moonen, J. Vervoort and F. Müller, submitted to Biochemistry.
Chapter 7: C.T.W. Moonen and F. Müller, submitted to Eur. J. Biochem.
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Chapter 10: C.T.W. Moonen and F. Müller, submitted to Eur. J. Biochem.
Chapter 11: C.T.W. Moonen, R.M. Scheek, R. Boelens and F. Müller, submitted to Biochemistry.

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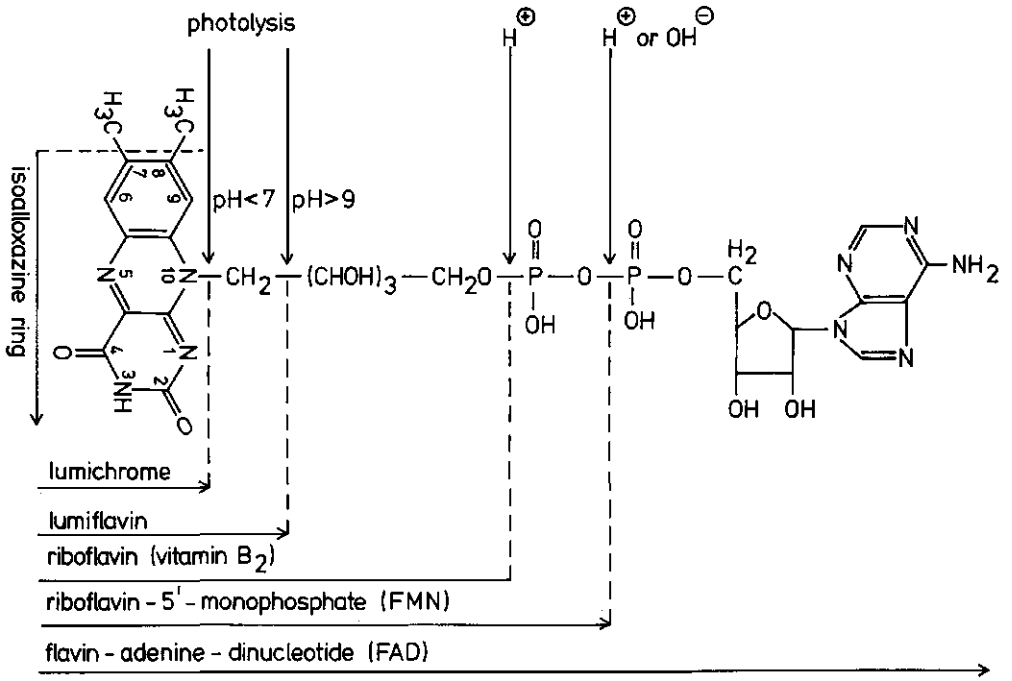
Chapter 1: General Introduction

At the crossroad of one- and two-electron redox biochemistry: the flavin coenzymes.

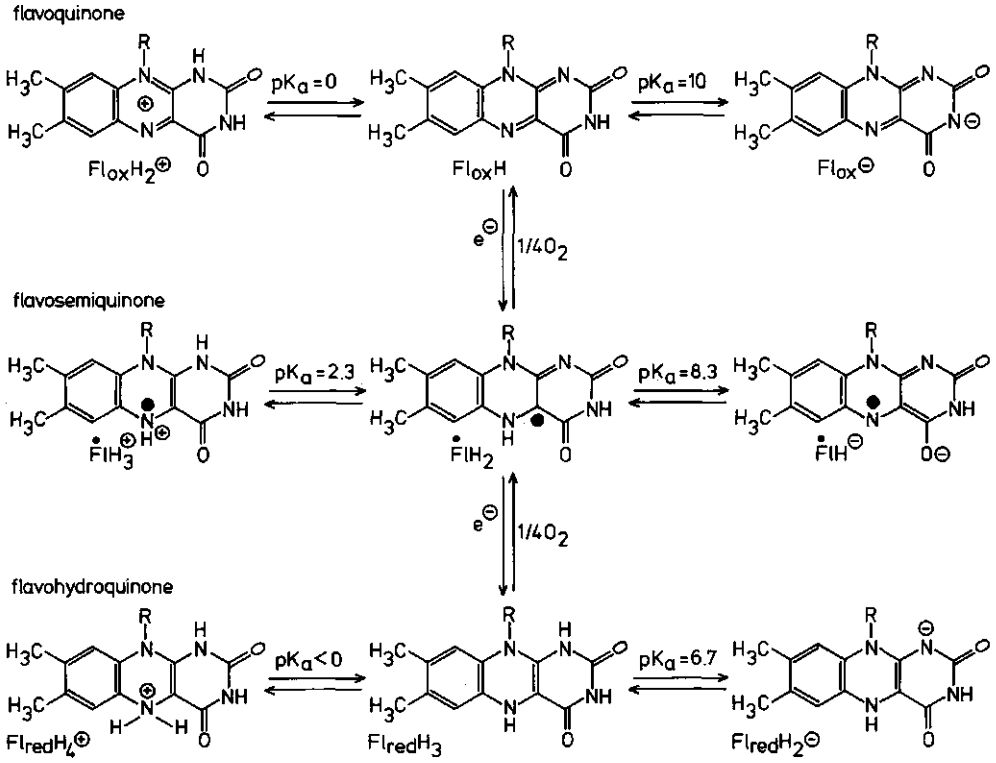
The term „flavin" refers to the yellow chromophore of a class of respiratory enzymes, occurring widely in animals and plants, i.e. the flavoproteins. The yellow chromophore is the tricyclic isoalloxazine molecule. Although various flavin coenzymes exist (for example, riboflavin (vitamin B_2), FMN, FAD) the isoalloxazine ring system is the functional part of the prosthetic group. In Scheme 1 the most common flavin coenzymes are shown including the internationally accepted numbering system.



Scheme 1

The most prominent features of the biochemical properties of flavin are the redox properties. As indicated in the title of this chapter, flavins can undergo both facile two- and one-electron redox transitions, which makes fla-

vins unique among all known redox coenzymes. The basis of this lies in the chemical properties of flavin as outlined in Scheme 2. The possible kationic and anionic forms of each state are added in Scheme 2, but it should be noted that only the neutral and anionic species are of biological relevance.



Scheme 2

Another prominent feature of flavin coenzymes is the high reactivity for reductive oxygen metabolism. This property is used in many different reactions catalyzed by flavoproteins. Especially mentioned is the light generating reaction catalyzed by luciferase, to which the remarkable statement of Cristopher Walsh refers on the first page of this dissertation.

Another feature distinguishes flavin coenzymes from, for example, nicotinamide coenzymes. This is the fact, that flavin coenzymes are always complexed with apoproteins. Thus flavin forms very strong, but generally non-covalent,

complexes with apoflavoproteins. Since the flavin possesses a different electronic structure and possibly also a different conformation in the three redox states, the affinity of the apoprotein for flavin generally differs for the three redox states. This implies that the redox potential of the protein-bound flavin is modified by the binding mode of a particular flavoprotein.

It is not surprising that flavin is often used in nature in a wide variety of biochemical reactions, since the required chemical properties are combined in one molecule. In Table 1 a classification of the various flavoproteins is given on the basis of the nature of the electron donor and acceptor. Other classifications have been proposed which are not presented because of their complexity and of doubtful use in biochemistry.

Table 1. A possible classification of flavoproteins.

Category	Comments	Example
1) Dehydrogenases	Acceptors are often 1 e^- acceptors such as cytochromes or nonheme-iron-sulfur clusters	lipoamide dehydrogenase
2) Oxidases	Acceptor is O_2, which is reduced by $2e^-$ to H_2O_2	D-amino acid oxidase
3) Oxidases-decarboxylases	Acceptor is O_2, which is reduced by $4e^-$ to H_2O	L-lactate oxidase
4) Monooxygenases (hydroxylases)	Acceptor is O_2: one oxygen ends up in H_2O and the other in the the hydroxylated product	p-hydroxybenzoate hydroxylase
5) Dioxygenases	Acceptor is O_2; both oxygen atoms end up in the product	2 nitropropane dioxygenase
6) Metalloflavoproteins	Acceptor may be O_2. Bound transition-metal ions (Fe^{II}, Fe^{III} or Mo^{VI}) are required. May be dehydrogenases or oxidases	xanthine oxidase
7) Flavodoxins	$1e^-$ transfer proteins. Acceptor may be nitrogenase	flavodoxin

For more detailed information the reader is referred to some recent review articles (Walsh, 1980; Müller, 1982; Bruice, 1980; Massey and Hemmerich, 1980), to the introduction of the following chapters and to the references given therein.

The striking versatility of flavin bound to different apoflavoproteins has led to the hypothesis that upon binding to apoflavoproteins the chemical and physical properties are modified in such a way that the coenzyme is „tuned" to its actual function in a particular flavoprotein (Müller, 1972). Although this hypothesis appears to be generally accepted at the moment, relatively little is known about the actual mechanism. The present knowledge stems mainly from kinetic data on flavoproteins (see Massey and Hemmerich, 1980 and references therein), model studies (Bruice, 1980) and crystallographic data on a few flavoproteins (Wierenga et al., 1979 and references therein). How impressive these studies may be, they do not offer yet adequate insight to explain the observed properties of flavoproteins, as can be deduced from the following.

i) From the chemistry of model compounds it is learned that the reactivity of flavins changes with a change in electron density at a particular atom in the flavin. However, nothing is known about how these changes in electron density are induced in flavoproteins.

ii) Crystallographic studies revealed the detailed structure of, for example, flavodoxin from Clostridium MP (Ludwig et al., 1976) and p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens (Wierenga et al., 1979). However, ambiguities still exist in the action mechanisms of both proteins (Chapters 7 and 9).

iii) Although it is known that redox potential modulation is a very important feature of apoflavoproteins (Walsh, 1980), the mechanism is still badly understood. Simondsen and Tollin (1980) argued that conformational effects exerted by the apoflavoprotein on the flavin are the main factors governing the redox potential modulation. This model is, however, too simplified as shown in Chapter 9.

Therefore, to arrive at a more detailed explanation of the tuning mechanism of protein-bound flavin, a method is required allowing a detailed insight into the molecular and submolecular structure of flavin, i.e. to obtain information on changes in hydrogen bond formation and electron densities. Two suitable techniques are available at the moment, i.e., Raman spectroscopy and Nuclear

Magnetic Resonance (NMR) spectroscopy. Although promising, Raman data appear at the moment to be difficult to interpret (Müller et al., 1983). The NMR technique is the most versatile technique and allows the observation of π electron density at specific carbon and nitrogen atoms of the flavin ring.
In addition, phosphorus and proton NMR can be used to monitor the structure of the flavin side chain and of the protein, respectively. Moreover, dynamic information can be obtained from NMR measurements. NMR studies on biomolecules are, however, hampered by the fact, that the method is in principle an insensitive method. Tremendous advances in NMR methodology and instrumentation in the last decade have brought the method closer to a meaningful application to biochemical problems. Unfortunately, natural abundance carbon and nitrogen contain only 1.1% and 0.4% of the NMR sensitive ^{13}C and ^{15}N isotope, respectively. This problem can be circumvented by isotopic enrichment, which is often used in such studies. For an introduction into the NMR technique the reader is referred to textbooks of Wüthrich (1976), Jardetzky and Roberts (1981) and Dwek (1973).

The aim of this research project was to investigate the particular mechanism of tuning the properties of the flavin coenzyme by apoflavoproteins. Two different approaches were used in this thesis.

1) A study of the electronic and conformational structure of the protein-bound flavin.
2) A study of the protein to investigate the interaction between the flavin coenzyme and a particular apoflavoprotein.

ad 1. The basis of this approach lies in the chemical synthesis of coenzymes in which certain carbon or nitrogen atoms are replaced („enriched") by the ^{13}C or ^{15}N isotopes, respectively. After removing the natural coenzyme, the protein can be reconstituted with the „enriched" coenzyme. In this way one obtains a flavoprotein, which does not differ in any chemical properties from the original flavoprotein, but which contains a built-in monitor in the form of the particular ^{13}C or ^{15}N atom. This approach allows to obtain a detailed view of one single atom of the flavin coenzyme

in the various redox states. In some favorable cases even the changes of the atom during the catalytic cycle can be followed.

The specific information obtained in this way must be „translated" into hydrogen bonds, structure, electron density and mobility. The data obtained on free flavin resulted in a semi-empirical theory which seems to be a valuable tool to interpret data obtained on flavoproteins.

ad 2. ^{31}P NMR is a relatively simple method, which gives direct information on the interaction of apoflavoprotein and the phosphate group(s) of the flavin coenzyme. Apart from ^{31}P NMR, ^{1}H NMR is a extremely powerful method for the elucidation of the structure of flavoproteins and for a description of the interaction between flavin and apoflavoprotein. However, ^{1}H NMR suffers from the lack of resolution as the flavoproteins contain an overwhelming number of hydrogen atoms. However, the recent, fast developing techniques in the field of ^{1}H NMR of biomolecules are very promising for an application to the smallest flavoproteins, the flavodoxins. In this thesis especially time-resolved photochemically induced dynamic nuclear polarization (CIDNP) and two-dimensional ^{1}H NMR spectroscopy were applied. The problem of the resolution was partially circumvented by making use of the paramagnetic semiquinone state. The information, collected by these methods is very specific and approaches information obtained by x-ray crystallography.

This thesis respresents by no means an exhaustion of the potentialities of the method in the field of flavoprotein biochemistry. On the contrary, from the still advancing methods and instruments it can be expected that the Nuclear Magnetic Resonance technique will continue to offer new insights into this field.

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Chapter 2

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

Chrit T. W. Moonen and Franz Müller*

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 ($\tau >> 0.5$ s) whereas that between the semiquinone and hydroquinone form is much more favored ($\tau << 0.01$ s). 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

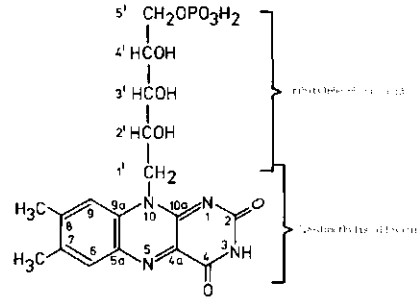

FIGURE 1: Structure of riboflavin 5′-phosphate (FMN).

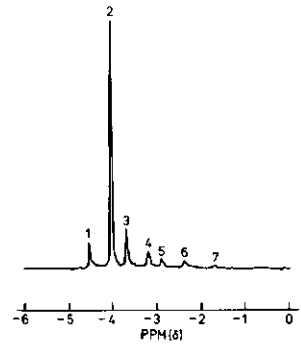

FIGURE 2: ^{31}P NMR spectrum of commercial FMN (56 mM) in 150 mM Tris-HCl, pH 7.0.

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.

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 pK 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 4.0	7.0	9.0	assignment
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).

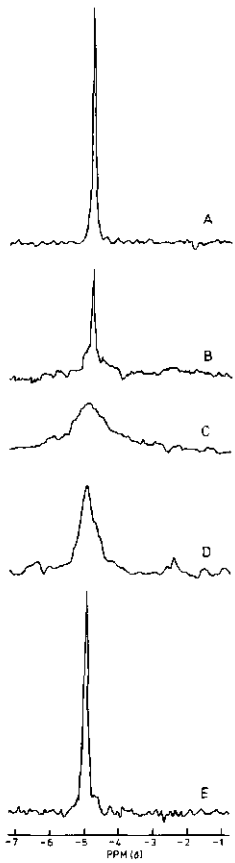

FIGURE 3: ^{31}P NMR spectra of *M. elsdenii* flavodoxin (3 mM) in the oxidized, the semiquinone, and the hydroquinone states in 150 mM Tris-HCl, pH 8.2. (A) Oxidized state (line broadening 1 Hz). (B) Mixture of about 30% oxidized and about 70% semiquinone form (line broadening 1 Hz). (C) Semiquinone form (line broadening 3 Hz). (D) Mixture of about 20% semiquinone and about 80% 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

$$Fl_{1(ox)} + Fl_{2}\cdot \rightleftarrows Fl_{1}\cdot + Fl_{2(ox)} \qquad (1)$$

where $Fl_{1(ox)}$ and $Fl_{2}\cdot$ are two flavodoxin molecules in the oxidized and semiquinone (Fl·) state, respectively. The electron-transfer reaction is a second-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 lifetime τ is calculated to be >>0.5 s. James et al. (1973) calculated from ^{1}H NMR data a value for τ >>0.02 s. This latter value is much smaller 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 20 %). 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 lifetime τ of <<0.01 s. 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 lifetime for the transfer between semiquinone and hydroquinone molecules is the same as that between the oxidized and semiquinone forms, i.e. >>0.02 s. 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., $Fl_{ox} + Fl_{red} \rightleftarrows 2Fl\cdot$, 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.

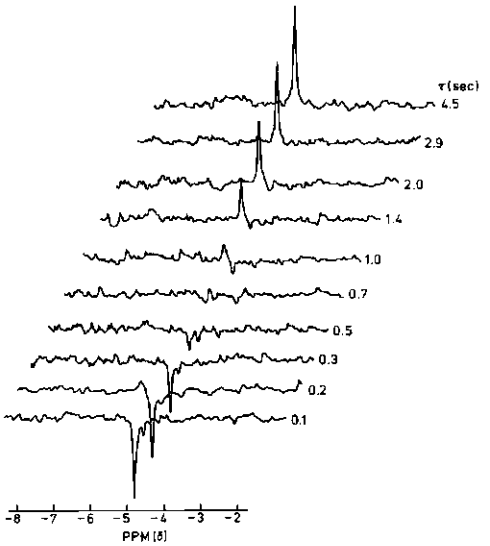

FIGURE 4: Partially relaxed ^{31}P NMR spectra at 40.5 MHz of *M. elsdenii* flavodoxin (3 mM) in 150 mM Tris-HCl, pH 8.2, obtained by the 180°–τ–90° pulse sequence and Fourier transformation. The labile protons were exchanged prior to the experiments (cf. Materials and Methods).

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

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

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

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

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

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

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):

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

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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A photo-CIDNP study of the active sites of *Megasphaera elsdenii* and *Clostridium MP* flavodoxins

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Megasphaera elsdenii and *Clostridium MP* flavodoxins have been investigated by photo-CIDNP techniques. Using time-resolved spectroscopy and external dyes carrying different charges it was possible to assign unambiguously the resonance lines in the NMR-spectra to tyrosine, tryptophan and methionine residues in the two proteins. The results show that Trp-91 in *M.elsdenii* and Trp-90 in *Cl.MP* flavodoxin are strongly immobilized and placed directly above the benzene subnucleus of the prosthetic group. The data further indicate that the active sites of the two flavodoxins are extremely similar.

Flavodoxin Megasphaera elsdenii Clostridium MP *Photo-CIDNP* *NMR*

1. INTRODUCTION

The flavodoxins are a class of small proteins (M_r 15000–23000) which contain the prosthetic group riboflavin 5'-phosphate (FMN) and function as electron carriers in biological reactions [1]. Chemical modification [2,3], X-ray crystallography [4] and fluorescence quenching [3] studies have shown that a number of aromatic amino acid residues are located in the neighbourhood of the flavin binding site and that these play an important role in the interaction between prosthetic group and apoenzyme. Here, we report a photo-CIDNP investigation of these aromatic residues in two closely related flavodoxins from the bacteria *Megasphaera elsdenii* and *Clostridium MP*.

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Abbreviations: photo-CIDNP, photochemically induced dynamic nuclear polarization; NMR, nuclear magnetic resonance; flavin I, 3-*N*-carboxymethyllumiflavin; flavin II, 3-*N*-ethylaminolumiflavin; FID, free induction decay

The photo-CIDNP method [5,6] is based on the generation of nuclear spin polarization in a reversible reaction between the protein and a photo-excited dye. When accessible to the dye, the side chains of histidine, tryptophan and tyrosine residues can be polarized resulting in selective enhancements in the ^{1}H NMR spectrum of the protein. A light-minus-dark difference technique leads to a photo-CIDNP spectrum containing only resonances from polarized nuclei [7].

An earlier investigation of the *M.elsdenii* and *Clostridium MP* flavodoxins showed that several tyrosine and tryptophan residues are polarizable [8]. Here, we extend this work to compare the active sites of the two flavodoxins, exploiting the dependence of CIDNP intensities on the timing of the experiment and on the nature of the dye to arrive at unambiguous NMR assignments as well as indications of the mobilities and exposure of the polarizable residues. Our results demonstrate that the active sites of the two proteins are extremely similar. As the spectra presented here are qualitatively much better than published for *M.elsdenii* flavodoxin in [8] chemical shifts could be determined much more accurately. This is the reason for

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the minor differences between chemical shifts reported in [8] and those presented here.

2. MATERIALS AND METHODS

Megasphaera elsdenii and *Clostridium MP* flavodoxins were isolated and purified as in [9]. NMR samples consisted of 1 mM protein with 100 mM potassium phosphate buffer in D_2O at pH 8.0. 3-*N*-Carboxymethyllumiflavin (flavin I) and 3-*N*-ethylaminolumiflavin (flavin II) were the dyes used for CIDNP generation. All spectra were recorded at 360 MHz on a Bruker HX-360 NMR spectrometer [5,7]. Ten free induction decays were accumulated for each spectrum. Chemical shifts are quoted with respect to trimethylsilylproprionate (TSP).

3. RESULTS AND DISCUSSION

Several photo-CIDNP spectra of *M.elsdenii* flavodoxin, obtained with the positively charged dye (flavin II), are presented in fig. 1. Two tyrosines and two tryptophans are polarized, the assignments coming directly from the characteristic CIDNP phases [5] and the fact that the molecule contains no histidine [10]. The distinction between resonances from the two Trp residues was achieved

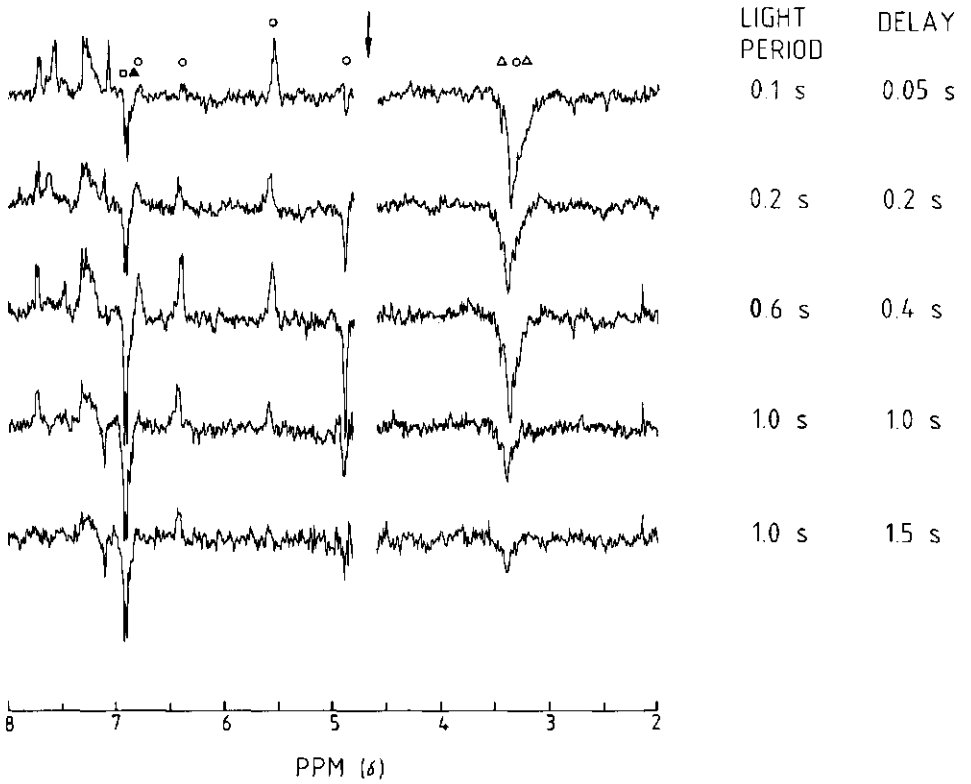

Fig. 1. Photo-CIDNP spectra of *M.elsdenii* flavodoxin with 0.5 mM flavin II as a function of the light period and delay time, as indicated: Trp I (○); Trp II (△); Tyr I (□); Tyr II (▲). The HDO resonance at 4.8 ppm (arrow) has been omitted.

by comparison of spectra (not shown) generated with the negatively charged dye (flavin I). With this dye, the lines of Trp I (○,●) decreased by ~50% relative to those of Trp II (△). These assignments are supported and completed by considering cross relaxation effects, as follows.

Of the Trp sidechain protons it is known that only H-2, H-4, H-6 and H-β are appreciably polarized in photoreactions with flavins. Any substantial CIDNP effects observed for H-5, H-7 and H-α arise by dipolar cross relaxation with directly polarized protons and are thus said to be cross- (or indirectly)-polarized [11]. The two types of polarization are easily distinguished by their time dependence [11]. With a short light flash and short delay before FID acquisition (upper spectrum in fig. 1) the indirect signals are very weak while with longer light and delay times they become stronger relative to their directly enhanced counterparts. For example, the resonances at 4.88, 6.41 and 6.80 ppm (fig. 1) are strongly cross-polarized whereas those at 5.57, 7.09 and 7.61 ppm are directly polarized. Such arguments combined with the line multiplicities enable us to assign all but the very weakest resonances visible in fig. 1 as summarized in table 1.

Further information can be extracted from fig. 1. First we note that cross relaxation is much less extensive in Trp II than in Trp I: indeed the H-α, H-5 and H-7 resonances of the former were too weak to be identified with certainty. This, together with the relative narrowness of its resonances, suggests that of the two, Trp II is considerably more mobile. Similarly, the two Tyr residues which exhibit relatively long relaxation times must also enjoy a fair degree of mobility.

The cross relaxation effects in Trp I are rather pronounced especially for H-α and H-2 both of which receive polarization from the emissively enhanced β protons. Indeed the effect is sufficiently great for H-2 to cause it to change phase to emission with increasing light and delay periods. This observation, although not unexpected theoretically [11], has not been seen before and implies that Trp I is strongly immobilized in *M.elsdenii* flavodoxin. To obtain some estimate of the dihedral angles [12], χ_1 and χ_2, which also influence cross polarization between the β protons and H-α and H-2, respectively, we simulated the evolution of the CIDNP intensities expected for a tumbling tryptophan residue with a rotational correlation time of 5 ns [13]. Of the various pairs of χ_1, χ_2 for which the calculation was performed, our experimental results agree most closely with $\chi_1 = 60°$ and $\chi_2 = \pm 90°$. The value for χ_1 is consistent with the failure to detect coupling for the H-α resonance (i.e., $J < 6$ Hz) indicating that H-α is approximately *gauche* with respect to both β protons.

Table 1

Assignments of resonances observed in the photo-CIDNP spectra of *Clostridium MP* and *M.elsdenii* flavodoxins

	M.elsdenii Trp-91	*Clostridium MP* Trp-90
Trp I		
H-β_A	3.38	3.34
H-β_B	3.38	3.34
H-α	4.88	4.98
H-2	7.09	7.07
H-4	7.61	7.55
H-5	6.80	6.80
H-6	5.57	5.62
H-7	6.41	6.35
Trp II	Trp-7	Trp-6
H-β_A	3.45	3.52
H-β_B	3.31	3.11
H-2	7.31	7.18
H-4	7.72	7.55
H-5	?	6.99
H-6	7.27	7.12
H-7	?	7.43
Tyr I	Tyr-6 or Tyr-89	Tyr-5 or Tyr-88
H-3, H-5	6.90	6.65
Tyr II	Tyr-6 or Tyr-89	Tyr-5 or Tyr-88
H-3, H-5	6.88	6.58

Fig. 2 shows photo-CIDNP spectra of *Clostridium MP* flavodoxin recorded with flavin II (2A) and flavin I (2B,2C) as external dyes. Most of the resonances were assigned (table 1) by the above methods with resolution enhancement [14] to determine the multiplet patterns. The unusual chemical shifts and the cross-polarization effects for Trp I here are very similar to *M.elsdenii;* however, it is clear that the charge of the dye plays a more important role in the *Clostridium MP* pro-

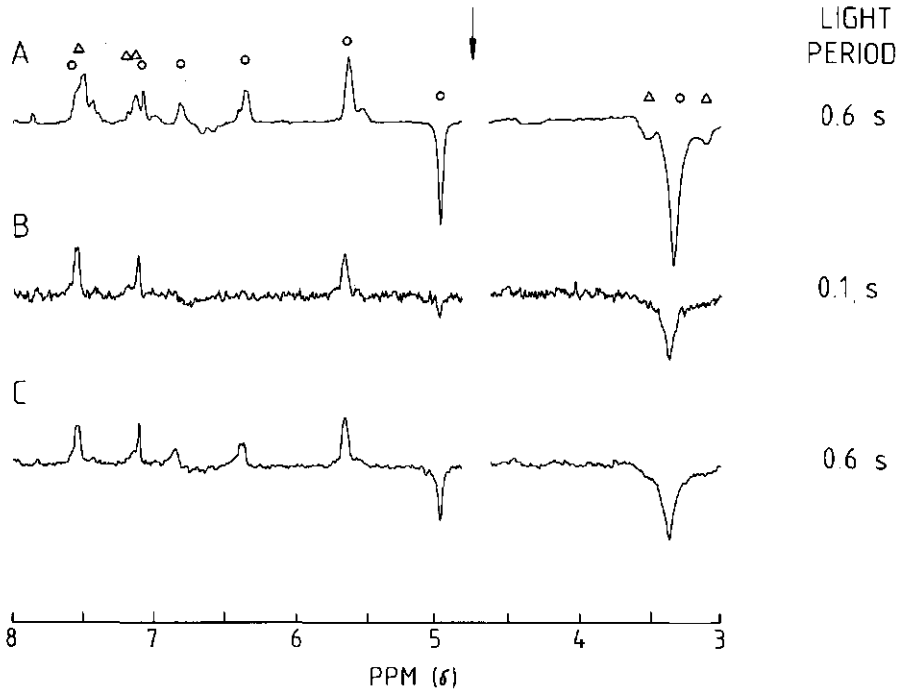

Fig. 2. Photo-CIDNP spectra of *Clostridium MP* flavodoxin with 0.5 mM flavin II (A) and 0.5 mM flavin I (B,C). A delay of 0.05 s was used with light periods as indicated. Notation as in fig. 1.

tein. With the negative flavin the enhancements of Trp I are much weaker while Trp II is completely absent.

We now consider the assignment of the observed resonances to particular amino acid residues in the primary sequences of the two proteins. *M.elsdenii* flavodoxin [10] contains four tryptophans at positions 7, 91, 96 and 100 whereas *Clostridium MP* [15] has three at 6, 90 and 95. The last three are invariant in the two proteins.

The solution to this problem comes from the dramatic upfield shifts (1.6 ppm for H-6 and 1.1 ppm for H-7) from the random coil positions experienced by some of the Trp I protons. Almost certainly due to ring current effects, such shifts require the affected protons to be 0.3–0.4 nm directly above the plane of a neighbouring aromatic ring [16]. Inspection of the crystal structure of *Clostridium MP* flavodoxin [4] shows Trp-90 to be the most obvious candidate. This residue is completely exposed to the solvent on one side and very close to the ring system of the protein-bound flavin on the other. The observed chemical shift of the individual protons of Trp I are in excellent qualitative agreement with this conclusion. Moreover reduction of the flavodoxin to its paramagnetic semiquinone form broadens the resonance of Trp I [17] providing independent support for the correctness of this assignment.

The similarity in behaviour and chemical shifts exhibited by Trp II in the two flavodoxins points to an invariant residue. The X-ray data for *Clostridium MP* flavodoxin indicate that Trp-6 is partially exposed but that Trp-95 is buried in the

interior of the protein and therefore probably inaccessible to either flavin dye. We therefore tentatively assign Trp II to Trp-6 in *Clostridium MP* and Trp-7 in *M.elsdenii* flavodoxin. This conclusion is supported by the observed accessibility of Trp II to the oppositely charged flavin dyes. In *Clostridium MP* flavodoxin, the carboxylate group of Glu-65 is close to Trp-6 and would facilitate the approach of flavin II but hinder that of flavin I. In *M.elsdenii* flavodoxin, where we do not observe such a strong dependence on the charge of the dye, this Glu is replaced by a valine.

A further interesting feature of fig. 1 is the sharp, indirectly polarized line at 2.15 ppm. A corresponding (although somewhat broader) resonance from *Clostridium MP* flavodoxin was found at 2.02 ppm. Showing a dependence on the charge of the dye similar to Trp II, it appears to receive its polarization from that residue, while its linewidth and position strongly suggest a methionine ϵ-methyl group. We assign this resonance tentatively to Met-56 in *Clostridium MP* flavodoxin (Met-57 in *M. elsdenii*) which has ϵ-protons <0.3 nm from Trp-6(7) in the crystal structure.

Turning to the tyrosine residues (positions 6 and 89 in *M.elsdenii* flavodoxin and 5 and 88 for *Clostridium MP*), the two directly polarized emissive doublets in the spectra of fig. 1 must arise from the H-3,5 protons of the two tyrosines, although one cannot say which is which. Interestingly these tyrosines in *Clostridium MP* flavodoxins are only weakly polarized by flavin II (at 6.65 and 6.58 ppm) and hardly at all by flavin I. This observation could be interpreted as evidence either for a lower accessibility of these residues or for some hydrogen bonding interaction of their hydroxyl groups in the *Clostridium MP* protein.

Finally, a number of as yet unidentified lines can be seen in the photo-CIDNP spectra of the two proteins. In fig. 1 weak absorptive resonances are visible at 7.56, 7.63, 7.46, 7.21, 7.18 ppm and in fig. 2 at 7.87 and 5.54 ppm. Some of these may arise from a third, weakly enhanced tryptophan, from the H-5 or H-7 protons of Trp II in the case of *M.elsdenii* flavodoxin or from other residues cross polarized from Trp I or Trp II.

The most important conclusions to come out of this study involve the active site residue Trp 89 (90). It shows almost identical behaviour in the two flavodoxins and enjoys little internal mobility. In *M.elsdenii* flavodoxin the isoalloxazine ring is also immobilized [18]. The question now arises as to what role this rigid isoalloxazine–tryptopohan complex plays in the electron transfer function of these flavoproteins.

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Chapter 4

On the Mobility of Riboflavin 5′-Phosphate in *Megasphaera elsdenii* Flavodoxin as Studied by ^{13}C-Nuclear-Magnetic-Resonance Relaxation

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The mobility of the isoalloxazine ring of the prosthetic group of *Megasphaera elsdenii* flavodoxin was investigated by a ^{13}C relaxation study of the non-protonated ring atoms 2, 4, 4a and 10a. In this study a selectively enriched (> 90% ^{13}C) prosthetic group was bound to the apoprotein. T_1 and T_2 values were determined at two magnetic field strengths, i.e. 8.46 T (90.5 MHz) and 5.88 T (62.8 MHz). Values of nuclear Overhauser effects (NOE) were determined at 5.88 T. It is shown that both the dipole-dipole interaction and the chemical shift anisotropy are important relaxation sources for all the carbon atoms investigated. The results are in agreement with a spectral density function of the isoalloxazine ring in which only the overall reorientational motion of the protein is accounted for. From this it is concluded that the isoalloxazine ring is tightly associated with the apoprotein. The protein-bound isoalloxazine ring does not exhibit large fluctuations on the nanosecond time scale, although small amplitude fluctuations cannot be excluded. This information was obtained by a combination of field-dependent T_1 and NOE measurements. T_2 values are in agreement with these results. On the basis of the dipolar part of the overall T_1 values, the distance between the carbon investigated and the nearest proton was calculated and found to be in fair agreement with the crystallographic results of the related flavodoxin from *Clostridium MP*.

In addition, it is shown that, based on the chemical shift anisotropy as a relaxation source, information on the internal mobility is difficult to obtain. The main reason for this is the low precision in the determination of the chemical shift anisotropy tensor.

Internal motions in proteins have recently attracted considerable interest since the realisation that such motions play an important role in cooperative and allosteric effects. Moreover, the internal mobility in enzymes probably contributes to the catalytic effects of biomolecules [1]. Theoretical calculations by Karplus and McCammon [2,3] indicate that such motions can occur on the nanosecond – picosecond time scale despite the close-packed structure of native proteins. Such an internal mobility in the nanosecond time range has recently been demonstrated in the flavoprotein lipoamide dehydrogenase [4].

Flavoproteins occur widely in nature and catalyze a variety of biological reactions. The physical and chemical properties of the flavin prosthetic group are perturbed upon binding to a particular apoflavoprotein. These observations and model studies led to the postulation [5] that the molecular basis for the specificity of a particular flavoprotein could be a specific interaction between the apoprotein and the prosthetic group; i.e. hydrogen bond formation between some amino acid residues and certain atoms of the prosthetic group. That such hydrogen bonds do influence the orbital structure of the flavin molecule has recently been demonstrated by Eweg et al. [6], both by photoelectron spectroscopy and from theoretical calculations. Furthermore, it cannot be excluded that the prosthetic group in flavoproteins possesses an internal mobility which might be related to the particular function of a flavoprotein.

Abbreviations. FMN, riboflavin 5′-phosphate; Ac_4rF, tetracetylriboflavin; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; CSA, chemical shift anisotropy.

Flavodoxins are proteins of small relative molecular mass (M_r 15000–23000) and function as electron carriers in biological reactions. The prosthetic group of flavodoxins is riboflavin 5′-monophosphate (FMN). The one-electron redox potential of FMN is altered by complex formation with apoflavodoxins [7]. Hydrogen bound formation between particular atoms of FMN and the apoprotein of *Megasphaera elsdenii* and *Azotobacter vinelandii* flavodoxins has recently been demonstrated by ^{13}C nuclear magnetic resonance (NMR) [8]. The question now arises whether the prosthetic group in flavodoxin is completely immobilized or still possesses some motional freedom. This uncertainty is related to the fact that the detailed mechanism of electron transfer is still poorly understood. It is possible that the degree of mobility of the prosthetic group in flavodoxins plays a role in the kinetics of electron transfer reactions.

Moonen and Müller [9], using the ^{31}P NMR technique have recently demonstrated that the phosphate group of the protein-bound prosthetic group in *M. elsdenii* flavodoxin is tightly bound without any detectable internal mobility on the nanosecond time scale. Owing to the fact that the fluorescence emission of the prosthetic group in *M. elsdenii* flavodoxin is completely quenched, the mobility of the isoalloxazine moiety cannot be probed by nanosecond laser fluorescence techniques, i.e. time dependence of anisotropy. ^{13}C relaxation measurements can also provide the desired information, however, and have been used to study the internal mobility of parts of proteins [10–12]. In these studies only hydrogen-carrying carbon atoms have been investigated. This has the advantage that one deals with a simple and well-defined

relaxation mechanism, i.e. the ^{13}C-^{1}H dipole-dipole relaxation mechanism.

With flavins, the situation is more complex because only C6 and C9 of the isoalloxazine ring carry protons and these positions are very difficult to enrich with ^{13}C by chemical synthesis. We have therefore studied the flavins which are enriched at positions C2, C4, C4a and C10a. Since the atoms in question are devoid of protons, analysis of our data had to include consideration of other possible relaxation mechanisms [13] as well.

The results show that it is possible, despite the complexity of our system, to obtain information on the mobility of the protein-bound prosthetic group by the combination of the following measurements: (a) spin-lattice relaxation at different magnetic field strengths, (b) nuclear Overhauser effects and (c) solid-state NMR to determine the principal elements of the chemical shift tensor.

MATERIALS AND METHODS

Flavodoxin from *Megasphaera elsdenii* (Lc1) was isolated and purified according to published procedures [14]. Apoflavodoxin was prepared as described by Wassink and Mayhew [15]. The holoprotein was reconstituted at pH 7–8 by addition of an excess of flavin to the apoprotein followed by exhaustive dialysis of the solution.

Tetraacetylriboflavin (Ac_4rF) was prepared from riboflavin as published earlier [16]. [2-^{13}C]FMN, [4a-^{13}C]FMN and [4,10a-$^{13}C_2$]FMN were synthesized as described by van Schagen and Müller [8]. [4a-^{13}C, 5-^{15}N]riboflavin was synthesized from *N*-D-ribityl-3,4-dimethylaniline [17] by coupling with the diazonium salt of aniline using sodium [^{15}N]-nitrite in the diazotation step. The resulting *N*-ribityl-2-[^{15}N]phenylazo-4,5-dimethylbenzene was condensed with [5-^{13}C]barbituric acid as described previously [8].

^{13}C NMR measurements were performed at 25.2 MHz on a Varian XL-100-15 NMR instrument equipped with a 16K 620 L computer, at 62.8 MHz on a Bruker WM 250 NMR spectrometer equipped with an Aspect 2000 computer and at 90.5 MHz on a Bruker HX 360 NMR apparatus equipped with a BNC 12 computer. All measurements were performed with quadrature detection and quadrature phase cycling, except for the measurements at 25.2 MHz, which were performed with single detection.

Proteins were dissolved in a mixture of $^{2}H_2O/H_2O$ (1:9, by vol.) and buffered with sodium phosphate (100 mM, pH 7–8). The final concentration of flavodoxin in the NMR tube was 2–3 mM. Ac_4rF was dissolved in $C^{2}HCl_3$ to a final concentration of 10 mM. FMN was dissolved in a mixture of $^{2}H_2O/H_2O$ (1:9, by vol.) or as otherwise stated. The solution was buffered with 100 mM sodium phosphate (pH 6.5). The final concentration of FMN was about 10 mM. In all experiments the temperature was kept at 26 ± 2 °C. All samples were degassed.

At 25.2 MHz 12-mm and at 62.8 MHz and 90.5 MHz 10-mm Wilmad precision tubes were used, respectively.

Spin-lattice (T_1) relaxation measurements were performed using the inversion-recovery method [18] as described by Levy and Peat [19]. At least 10 spectra were recorded for the determination of one T_1 value. The power of the broad-band decoupling was 2 W, except at 90.5 MHz; at this frequency, severe heating occurred at 2 W decoupling power and 1.2 W power was therefore applied. The experimental values were fitted to a single exponential function by computer analysis [20].

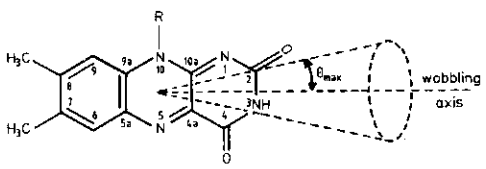
Nuclear Overhauser effect (NOE) values for the free flavins were obtained by integration of the resonance lines and comparing the values with and without proton decoupling. The waiting time in these experiments was at least four times T_1 and the observation pulse was 90° as described by Lyerla and Levy [21]. NOE measurements for flavodoxin required long time averaging. For this reason, these measurements were performed with the help of a microprogram, in which alternate scans were obtained under continuous proton decoupling and under proton decoupling applied only during the detection period. Here again, waiting times of at least four times T_1 were used. Subsequent subtraction of the free induction decays yielded the NOE values.

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

Powder spectra were obtained on a Bruker CXP 300 NMR spectrometer. A special solid-state probe with a saddle coil was used. No proton decoupling was applied. A 90° pulse (5 µs) was applied followed by a receiver dead time of 0.5 µs and direct detection; 512 points were accumulated per free induction decay. Repetition times for the experiment are given in Results and Discussion. About 200 mg powdered riboflavin was used for the powder spectra and 20–200 scans were accumulated depending on the repetition time. Benzene was used as a reference. The benzene sample was also used to homogenize the magnetic field.

RESULTS AND DISCUSSION

The aim of this study is to obtain information on the internal mobility of the prosthetic group in *Megasphaera elsdenii* flavodoxin using ^{13}C nuclear magnetic resonance relaxation. The description of the motional freedom is expressed by the spectral density function in the relaxation equations [22]. A precise knowledge of the spectral density function is necessary for the evaluation of the internal mobility. Different models have been proposed for the internal mobility [10, 11, 23]. The 'wobbling in cone' model, first proposed by Kinosita et al. [24], presents a reasonable description of the complex motions possible in proteins although one may argue that the internal motional freedom cannot be fully described by these restricted stochastic intramolecular motions. A possible internal motion of protein-bound flavin is illustrated in Fig. 1. The half-angle θ_{max} and the rotational coefficient τ_w determine the internal motion, which is superimposed on the rotational motion of the whole protein. The choice of the



a: R=CH_2-(CHOH)$_3$-$CH_2OPO_3H_2$
b: R=CH_2-(CHOCOCH$_3$)$_3$-CH_2OCOCH_3

Fig. 1. *The structure of (a) riboflavin 5'-phosphate and (b) tetraacetylriboflavin.* A model for the internal mobility of FMN bound to *M. elsdenii* flavodoxin is presented in dotted lines. The formalism of the 'wobbling in cone' model is used [24]. The internal mobility is limited by a cone with the half-angle θ_{max}, the frequency of the motion being expressed by the rotational coefficient τ_w. No full rotation around the wobbling axis is allowed. For further explanation see text

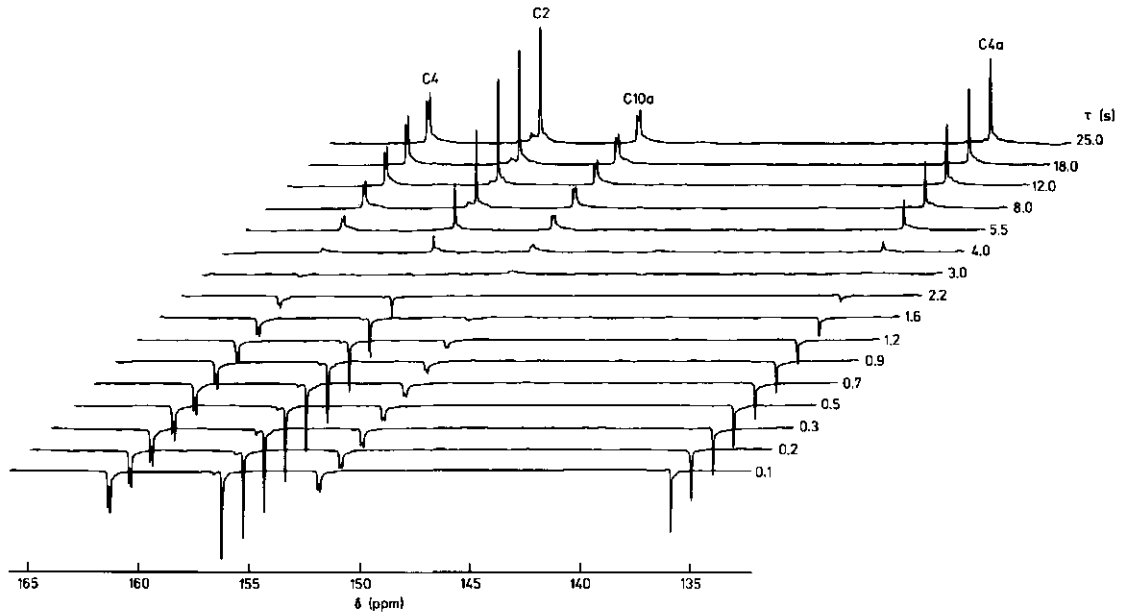


Fig. 2. *Partially relaxed ^{13}C NMR spectra of a mixture of equal concentrations of $Ac_4[2\text{-}^{13}C]rF$, $Ac_4[4a\text{-}^{13}C]rF$ and $Ac_4[4,10a\text{-}^{13}C_2]rF$ in C^2HCl_3 measured at 90.5 MHz obtained by a 180°-τ-90° pulse sequence and Fourier transformation*

wobbling axis is arbitrary, but does not influence our final results within the limited accuracy of the model. In the case of an internal motion similar to that described in Fig. 1, Richarz et al. [10], following the 'wobbling in cone' model, arrived at this spectral density function:

$$J(\omega) = S\,\frac{\tau_c}{1+(\omega\tau_c)^2} + (1-S)\,\frac{\tau_c'}{1+(\omega\tau_c')^2} \qquad (1)$$

where $S = {}^1/_4\cos^2\theta_{max}\,(1+\cos\theta_{max})^2$ and $(\tau_c')^{-1} = \tau_c^{-1} + \tau_w^{-1}\,(0.29\sin^2\theta_{max})^{-1}$. τ_c is the rotational correlation coefficient describing the isotropic reorientational motion of the whole protein. If $\tau_w \to \infty$ or $\theta_{max} \to 0$, Eqn (1) becomes the normal spectral density function for an isotropic, rotational motion [22].

The ^{13}C atoms (2, 4, 4a, 10a) studied in this paper are all non-protonated (cf. Fig. 1). From a theoretical point of view [25] the following relaxation mechanisms have to be considered for these carbon atoms: (a) the ^{13}C-^1H dipole-dipole interaction, (b) the ^{13}C-^{14}N dipole-dipole interaction and (c) the chemical shift anisotropy (CSA). The reader is referred to relevant textbooks [22, 26] for the general equations of the relaxation by dipole-dipole interaction and chemical shift anisotropy. These two relaxation mechanisms have a different dependence on the spectral density function and the magnetic field strength. In order to obtain information on the spectral density function, it is therefore necessary to separate the relative contributions of the different relaxation mechanisms. One possibility is to evaluate the anisotropy of the chemical shifts of protein-bound FMN by analyzing the anisotropy in free FMN. ^{13}C NMR measurements of FMN, free in aqueous solution and bound to *M. elsdenii* flavodoxin, showed that, despite certain differences due to specific hydrogen bonding interactions, the chemical shifts do not differ much [8]. This strongly suggests that the anisotropy of the chemical shifts in free FMN is indeed roughly the same as in protein-bound FMN. A disadvantage in using free FMN is the formation of aggregates in aqueous solutions [9]. We have therefore also studied tetraacetylriboflavin (Ac_4rF) in chloroform, which does not form aggregates in this solvent [27]. The change of solvent (water to chloroform) causes certain differences in polarization of the aromatic ring as evidenced by ^{13}C NMR [8], but the chemical shifts do not differ much on an absolute scale. Thus, the use of free FMN in aqueous solutions and Ac_4rF in chloroform is allowed (with some caution) for an evaluation of the anisotropy of the chemical shifts of protein-bound FMN.

Free-Flavins in Solution

Theoretically it is possible to determine the chemical shift anisotropy using field-dependent relaxation measurements [28–30]. Representative results of relaxation measurements are presented in Fig. 2. The ^{13}C relaxation data of Ac_4rF in chloroform are collected in Table 1. The overall T_1 values of Ac_4rF at 25.2 MHz have already been estimated by Ghisla et al. [31] and are in fair agreement with our data. In the motional narrowing limit ($\omega\tau_c \ll 1$) the relaxation by ^{13}C-^1H dipole-dipole interaction ($T_1^{DD\text{-}H}$) can be calculated from the overall T_1 and NOE values [32]. From Table 1 it can be concluded that the $T_1^{DD\text{-}H}$ values are independent of the magnetic field strength. This can be taken as proof that we are dealing with the motional narrowing limit in both magnetic field strengths [32]. In the motional narrowing limit T_1^{CSA} is the only relaxation parameter influenced by the magnetic field strength [28]. Thus, from a simple comparison of

Table 1. *The dependence on the magnetic field strength of the overall spin-lattice relaxation times (T_1), nuclear Overhauser effects (NOE) and the calculated relaxation due to dipole-dipole interaction (T_1^{DD-H}) of some ^{13}C atoms of tetraacetylriboflavin in C^2HCl_3*
The precision of the T_1 values is ± 5%, that of the NOE values is ± 0.05, and that of T_1^{DD-H} is dependent on the NOE value (see text for further explanations)

^{13}C atom	Frequency	T_1	NOE	T_1^{DD-H}
	MHz	s		s
C2	90.5	5.1	1.45	22.4
C4	90.5	5.8	1.48	23.9
C4a	90.5	5.7	< 1.10	> 50
C10a	90.5	4.7	1.10	46.3
C2	25.2	11.3	1.89	25.3
C4	25.2	12.1	2.06	22.6
C4a	25.2	35.9	< 1.10	≫ 50
C10a	25.2	16.4	1.69	47.2

Table 2. *The relaxation due to chemical shift anisotropy (T_1^{CSA}) of some ^{13}C atoms in tetraacetylriboflavin (Ac$_4$rF) in C^2HCl_3 and riboflavin 5'-monophosphate (FMN) in aqueous solution at 90.5 MHz and the calculated values for the anisotropy in the chemical shift ($\Delta\sigma$)*
The precision of the T_1^{CSA} values for Ac$_4$rF is ± 10% and that for FMN about ± 20%. For the accuracy of the $\Delta\sigma$ values, see text

Compound	^{13}C atom	T_1^{CSA}	$\Delta\sigma$
Ac$_4$rF in C^2HCl_3	C2	8.5	205
	C4	10.2	185
	C4a	6.3	239
	C10a	6.0	245
FMN in ^2H_2O	C2	6.6	161
	C4	4.8	188
	C4a	3.4	224
	C10a	2.7	250

the T_1 values at different magnetic field strengths the T_1^{CSA} can be calculated [32] (Table 2). It is evident from Tables 1 and 2 that both the CSA and the ^{13}C-^1H dipole-dipole relaxation mechanisms are important, especially at high magnetic field strength. In contrast, other relaxation mechanisms such as the ^{13}C-^{14}N dipole-dipole interactions contribute little to the overall relaxation rates.

A similar relaxation study was done on FMN in H_2O. Treatment of these data obtained at two field strengths (not shown) in analogy to those of Ac$_4$rF lead to the same conclusions as mentioned above for Ac$_4$rF, i.e. the CSA relaxation plays an important role in the overall relaxation of the carbon atoms studied. The overall T_1 values of FMN in ^2H_2O are summarized in Table 3. In D_2O the proton attached to N3 of FMN (cf. Fig. 1) exchanges easily with a deuteron of the solvent. The NOE values are therefore very small in this solvent. It should be noted that the accuracy of the results for FMN in ^2H_2O or H_2O is lower than that for Ac$_4$rF in $CHCl_3$ because FMN contains impurities consisting of monophosphate isomers and riboflavin and also forms aggregates [9]. The T_1^{CSA} values calculated for FMN are presented in Table 2.

Now the T_1^{CSA} values are known, the CSA parameters $\Delta\sigma$ can be evaluated. The $\Delta\sigma$ term is composed of the principal elements σ_{ii} ($i = x, y, z$) of the chemical shift tensor:

$$\Delta\sigma = \sigma_{zz} - {}^1/_2\,(\sigma_{xx} + \sigma_{yy}). \qquad (2)$$

Table 3. *The dependence on the magnetic field strength of the overall spin-lattice relaxation times (T_1), of some ^{13}C atoms of FMN in ^2H_2O*
The precision of the T_1 values is ± 10%

^{13}C atom	T_1 at	
	90.5 MHz	25.2 MHz
	s	
C2	3.5	6.9
C4	3.3	7.9
C4a	2.2	5.2
C10a	1.8	4.3

The general equation for T_1^{CSA} [22] also contains the term η, which expresses the deviation of the chemical shifts tensor from axial symmetry [33].

$$\eta = \frac{\sigma_{yy} - \sigma_{xx}}{\sigma_{zz} - \sigma_{av}} \qquad (3)$$

where σ_{av} is the average (liquid-state) chemical shift.

$$\sigma_{av} = {}^1/_3\,(\sigma_{xx} + \sigma_{yy} + \sigma_{zz}). \qquad (4)$$

The CSA parameter $\Delta\sigma$ of the investigated carbon atoms of the isoalloxazine moiety of Ac$_4$rF and FMN can now be calculated from the T_1^{CSA} values [28–30]. For an asymmetric flat aromatic ring system as present in flavin we can expect an anisotropic rotational diffusion tensor. The only information on the reorientational motion can be obtained from the T_1^{DD-H} values of C4 and C2, arising mainly from the interaction of these carbon atoms with the proton at N3 of flavin. In fact, however, this information represents only one term of the rotational diffusion tensor. In order to obtain an estimation of the $\Delta\sigma$ values, it is necessary to assume that the reorientational motion is isotropic, i.e. $J(\omega) = \tau_c$ in the motional narrowing limit. As we know the distance between the carbon atoms C2, C4 and the N3 proton (0.2 nm) from crystallographic studies [34], τ_c can easily be calculated. A τ_c of 74 ps is calculated for Ac$_4$rF in chloroform and a τ_c of 165 ps for FMN in H_2O at 26 °C. The difference between these values corresponds well with the difference in viscosity between the two solvents at 26 °C. To estimate the $\Delta\sigma$ values using Eqn (2) the additional assumption has to be made that the chemical shift tensor is axially symmetric, i.e. $\sigma_{xx} = \sigma_{yy}$ and therefore $\eta = 0$ (Eqn 3). The calculated $\Delta\sigma$ values for Ac$_4$rF and FMN are given in Table 2. The order of magnitude of the $\Delta\sigma$ values is about the same as that reported for indole [30] and for benzene [35]. In addition the $\Delta\sigma$ values for FMN and Ac$_4$rF are quite similar (Table 2). This result was expected since the electronic structure of the isoalloxazine moiety of FMN and Ac$_4$rF does not change drastically on going from water to chloroform. The largest difference observed is between C2 in Ac$_4$rF and in FMN. This difference probably reflects the greater polarization of the carbonyl group at position 2 in aqueous solution as compared to that in $CHCl_3$. This suggestion is supported by previously published work [8].

Solid-State NMR of Flavin

Solid-state NMR was applied to powdered riboflavin to test the validity of the two assumptions made above. Powder spectra enable determination of the principal elements of the chemical shift tensor [33, 36] and therefore provide a very

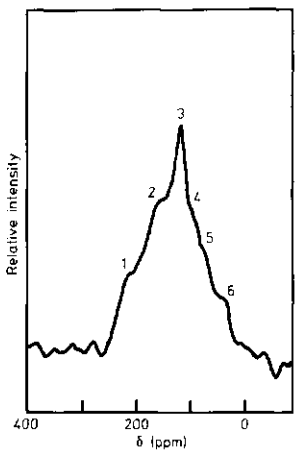


Fig. 3. *^{13}C solid-state NMR spectrum of powdered [4a-^{13}C, 5-^{15}N]riboflavin at 20 °C.* The repetition rate was 1 h. Total number of scans is 20. For the numbering of the peaks see text

straight-forward way to determine CSA parameters $\Delta\sigma$ and η using Eqns (2) and (3). This method can however only be applied for the chemical tensor of C4a of flavin. The reason for this is that C4 and C10a in flavin cannot be enriched independently and a doubly enriched flavin would yield a very complex powder spectrum. In addition, neighbouring quadrupolar nitrogen atoms (cf. Fig. 1) would make such powder spectra even more complex [37]. Synthesis of a flavin derivative enriched with ^{13}C at position 2 and ^{15}N at positions 1 and 3 would have been too expensive for the limited use of the compound. On the other hand, it is relatively easy in riboflavin to enrich C4a by ^{13}C and N5 by ^{15}N. The nearest hydrogen atom to the quaternary C4a atom in flavin is three bonds away. Consequently, cross-polarization from proton magnetization with or without 'magic-angle' spinning would probably be useless. For this reason, the experimental approach for this problem was the use of a single 90° pulse followed by a very short receiver dead time and acquisition (cf. Materials and Methods). In Fig. 3 a typical powder spectrum of [4a-^{13}C, 5-^{15}N]riboflavin is shown. Although the spectrum is rather complex a number of peaks and shoulders can be distinguished. We can expect very large T_1 values (perhaps even exceeding an hour) for non-protonated carbon atoms in powders. As riboflavin also contains protonated carbon atoms (characterized by much smaller T_1 values) we can expect intensity from some protonated carbon atoms, besides the intensity from the enriched C4a atom (Fig. 3). The differences in T_1 values mentioned above should however provide a method for the assignments. This was achieved experimentally by varying the repetition time between 1 h and 10 s. In this way it is possible to estimate roughly the T_1 values: the peaks at 20 ppm (peak 6), 60 ppm (peak 5) and 120 ppm (peak 2) have relatively short T_1 values. In liquid-state spectra of flavins [38] the C7 and C8 carbon atoms, the C atoms of the ribityl side chain and the C6 and C9 atoms are found respectively at these frequencies. The peaks 1, 3 and 4 in Fig. 3 exhibit a very long T_1 and this suggests that these peaks represent the principal values of the chemical shift tensor of the C4a atom. These tensor elements, i.e., σ_{xx}, σ_{yy} and σ_{zz}, are at 75 ppm, 104 ppm and 217 ppm. Calculation of the average chemical shift σ_{av} (Eqn 4) gives 132 ppm which is in good agreement with the value of 136 ppm observed in the liquid state [8, 38]. Using Eqns (2) and (3), $\Delta\sigma$ and η are calculated as 127 ppm and 0.34, respectively. This result differs from the values in Table 2 by a factor of almost 2. Although the assignments of the tensor elements should be regarded with caution, the results clearly show that the $\Delta\sigma$ values obtained in the previous section from liquid-state relaxation measurements are overestimated. The reason for this is that the above-mentioned assumptions are too rigorous. Thus we have only obtained a rough estimation of the CSA parameters for the C4a atom. Consequently the CSA values of Table 2 cannot be applied to a relaxation study of protein-bound FMN. Nevertheless, we will show that it is possible to obtain unambiguous information on the internal mobility of protein-bound FMN by other NMR data (cf. below).

Protein-Bound FMN

The rotational correlation time of the protein must be known to analyze the relaxation data on flavodoxin from *M. elsdenii*. The physical and chemical properties of *M. elsdenii* flavodoxin are very similar to those of the related flavodoxin from *Clostridium MP.*. Crystallographic studies on the latter protein revealed that it possesses an almost ideal globular structure [39]. These facts suggest that *M. elsdenii* flavodoxin also possesses a globular structure. This allows us, knowing that the translational diffusion coefficient $D_{20,w} = 11.4 \times 10^{-7}$ cm^2 s^{-1} [14], to calculate 5.9 ns for τ_c at 26 °C. On the other hand, a τ_c of 5 ns is calculated using the Stokes-Einstein relation and considering a hydration shell of the protein. Since the NMR measurements require a relatively high concentration of protein, we determined the macroscopic viscosity for a 3 mM solution of flavodoxin and found that the viscosity increases less than 10% at 26 °C as compared to that of a dilute solution. It was shown [9] by analytical ultracentrifugation experiments that even at these concentrations no aggregation occurs. The two independently determined τ_c values are thus in good agreement. In the following we will use the τ_c value of 5 ns, since, as will be shown below, the conclusions drawn from the relaxation data do not depend critically on the τ_c value used in the calculations. A representative ^{13}C T_1 measurement for selectively ^{13}C-enriched FMN bound to the apoflavodoxin from *M. elsdenii* is shown in Fig. 4. The ^{13}C resonances appear as doublets due to double labelling of FMN, i.e. C4 and C10a [8]. The spin-lattice relaxation times, measured at 62.8 MHz and 90.5 MHz, are collected in Table 4.

Theoretically [25], and also as deduced from model studies, we can expect the CSA and the dipole-dipole (DD) interactions to be possible relaxation sources. Let us first consider the theoretical dependence of T_1^{DD} and T_1^{CSA} on the isotropic τ_c value. The theoretical curves are shown in Fig. 5. Fig. 5B leads to the deduction that T_1^{CSA} values are smaller at high field strength than at low field strength. In addition, T_1^{CSA} gradually becomes less dependent on the field strength with increasing τ_c and for $\omega\tau_c \gg 1$, T_1^{CSA} is independent of the field strength. The opposite is true for T_1^{DD} (Fig. 5A). In Fig. 5A only the ^{13}C-^1H dipole-dipole relaxation is shown. The curves for ^{13}C-^{14}N dipole-dipole relaxation are similar to those of ^{13}C-^1H dipole-dipole relaxation [41] however. These theoretical data, in combination with overall field-dependent T_1 values of protein-bound FMN (Table 4), allow us to draw some important conclusions: (a) the observed increase in the overall T_1 values with increasing field strength

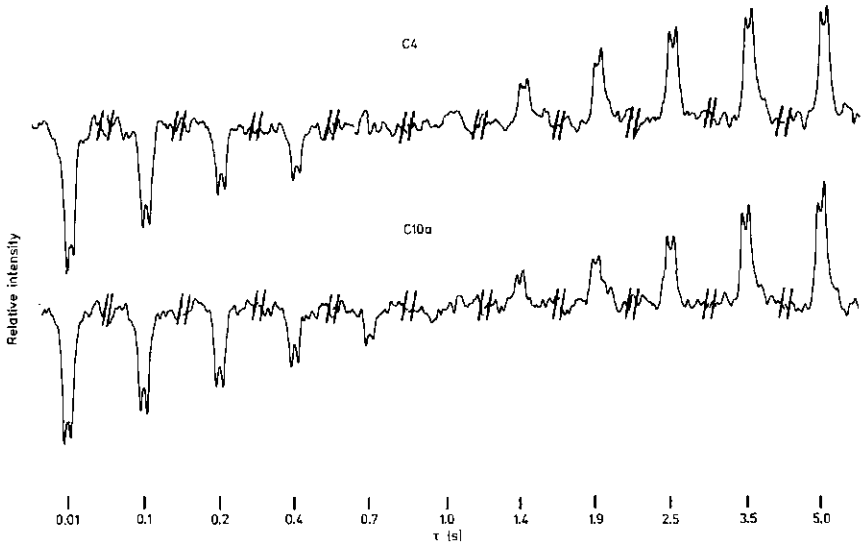


Fig. 4. *^{13}C T_1 and NOE measurement at 62.8 MHz.* Partially relaxed ^{13}C-NMR spectra of [4,10a-$^{13}C_2$] FMN bound to *M. elsdenii* flavodoxin measured at 62.8 MHz obtained by a 180°-τ-90° pulse sequence and Fourier transformation

Table 4. *The overall spin-lattice relaxation times (T_1) of certain atoms of selectively ^{13}C-enriched FMN bound to the apoflavodoxin from* M. elsdenii *measured at two different frequencies*

^{13}C atom	T_1 at	
	62.8 MHz	90.5 MHz
	s	
C2	1.5 ± 0.1	1.9 ± 0.15
C4	1.5 ± 0.05	2.1 ± 0.1
C4a	1.0 ± 0.1	1.4 ± 0.15
C10a	1.2 ± 0.05	1.8 ± 0.1

must be due to the dipole-dipole relaxation mechanism; (b) the observed increase in T_1 values at high field strength must be due to a significant contribution of slow motions of protein-bound FMN (i.e. $\omega\tau_c \gg 1$) to the spectral density function. Thus, a very mobile protein-bound FMN can already be excluded. In the following we will analyze the data of Table 4 in detail. Calculations show that for a τ_c of 5 ns and assuming no internal mobility T_1^{DD} increases by a factor of 1.88 on going from 62.8 MHz to 90.5 MHz. As can be checked easily the value of 1.88 is independent of the relative contributions of ^{13}C-^1H and ^{13}C-^{14}N to the T^{DD} value. If an internal mobility on the nanosecond or subnanosecond timescale exists, the factor of 1.88 will decrease (cf. Fig. 5A). The T_1^{CSA} value will always decrease on going from 62.8 MHz to 90.5 MHz (cf. Fig. 5B). Consequently, the magnitude of the increase in overall T_1 values for all four carbon atoms at high field strength allows the determination of the lower limit of the contribution of the dipole-dipole interactions to the overall T_1 relaxation. As an example, an increase in the overall T_1 from 1.5 s to 2.1 s is observed for the C4 atom (Table 4). Only dipolar interactions can explain this increase. Knowing that the ratio of T_1^{DD} (90.5 MHz)/T_1^{DD} (62.8 MHz) is smaller than or equal to 1.88, we simply calculate that T_1^{DD} makes up at least 60% of the overall T_1 relaxation at 62.8 MHz. In analogy a lower limit of the relative contribution of the dipolar interactions is determined as 45% for C2, 62% for C4a and 71% C10a. Both the ^{13}C-^1H and the ^{13}C-^{14}N dipole pairs contribute to T_1^{DD}. The situation is simple for C2 and C4, as the proton attached to N3 is very close (0.2 nm) and accordingly the ^{13}C-^{14}N dipolar pairs can be safely neglected. A more complex situation exists for C4a and C10a as the closest hydrogen atoms are probably at van der Waals distances. Owing to the small values of T_1^{DD} however, the contribution of the ^{13}C-^{14}N dipole interactions to T_1^{DD} is probably also minor for these carbon atoms. Summarizing, the overall T_1 values of C2 and C4 have a T_1^{DD-H} contribution of at least 45% and 60%, whereas for C4a and C10a the contribution of T_1^{DD-H} is not known exactly, but is probably at least 50%. This means that ^{13}C-^1H nuclear Overhauser effects should yield valuable information. This is illustrated for the C4 atom where T_1^{DD-H} makes up at least 60% of the overall T_1 value at 62.8 MHz. As the ^{13}C-^1H NOE only deals with the ^{13}C-^1H dipole interactions, the NOE will be (depending on the mobility) between 1 and at least 2.2 (60% of the maximal increase). Thus an NOE measurement should give the desired information on the internal mobility. Fig. 5C demonstrates that only a very small NOE will be detected at 62.8 MHz if no internal motion is present. A considerable contribution from fast internal motions ($\tau_{in} < 1$ ns) will enhance the NOE substantially however.

We have carried out NOE measurements at 62.8 MHz. No NOE could be detected for the carbon atoms investigated. For at τ_c of 5 ns we calculate an NOE of 1.19 at 62.8 MHz if the ^{13}C-^1H dipole-dipole interaction is the only relaxation source. Since the minimal contribution of the ^{13}C-^1H dipole-dipole interaction is 60% for C4, the NOE at 62.8 MHz for C4 is between 1.11 and 1.19 for a τ_c of 5 ns and no internal motion. Within the accuracy of the measure-

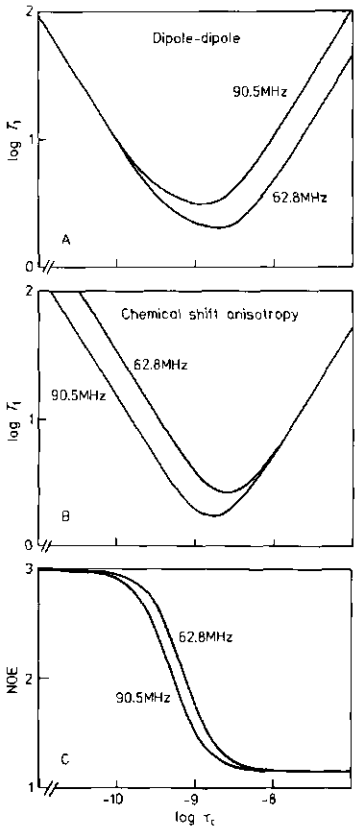


Fig. 5. *The theoretical dependence of (A) the dipole-dipole, (B) the chemical shift anisotropy relaxation, and (C) the nuclear Overhauser effect on the isotropic rotational correlation time (τ_c) is shown for the case that Eqn (1) holds for the spin density function (see text).* The dependence is calculated for two different field strengths as indicated

ments the experimentally observed NOE is smaller than 1.15. As no NOE is detected for the other investigated carbon atoms either, we can state that the bound isoalloxazine moiety contains no considerable internal motion in the nanosecond or subnanosecond timescale. We can also see from Fig. 5C that this conclusion does not depend critically on the accurate knowledge of the overall rotational correlation time of the flavodoxin, as the NOE in the presence of a substantial motional freedom in the nanosecond or subnanosecond region would be hardly dependent on the exact overall rotational correlation time. We can discuss in more detail the internal motions which are possible in the light of our results (cf. Eqn 1). Our results cannot exclude an internal motion characterized by a $\tau_w > 3$ ns. On the other hand, an internal motion characterized by a $\tau_w < 1$ ns is only possible when the half-angle θ_{max} is smaller than 20°. In this case the contribution of the internal motion to the spectral density function is too small to allow its detection by the current limitation of the instruments.

The conclusion that no internal motion contributes significantly to the overall relaxation time allows us to calculate T_1^{DD-H} from the field-dependent measurements and therefore also the distance between the ^{13}C and the nearest hydrogen atom(s). The distance to the nearest hydrogen atom is known for C2 and C4, i.e. the hydrogen atom attached to N3. The distance is 0.2 nm for both carbon atoms as deduced from crystallographic data of flavins [34]. Distances of 0.19 nm between C2 and its nearest hydrogen atom and 0.18 nm between C4 and its nearest hydrogen atom are calculated from our data. As hydrogen atoms in van der Waals' contact might contribute to a small extent to the T_1^{DD-H} relaxation the calculation might yield an underestimation of the distances. Either way, the calculated distances are in good agreement with the crystallographic data [34].

The conclusion that the isoalloxazine ring is immobilized by the apoprotein can be checked on the basis of the CSA parameters of the C4a atom, i.e. $\Delta\sigma$ (127 ppm) and η (0.34). Using these data, the overall T_1 values of the C4a atom of protein-bound FMN should be in agreement with a spectral density function in which only the overall reorientational motion of the protein is accounted for. A T_1^{CSA} of 2.81 s is then calculated for C4a at 62.8 MHz. As the overall T_1 is 1.0 s (Table 4), we calculate a T_1^{DD} of 1.55 s at 62.8 MHz. Using the theoretical dependence of T_1^{DD} and T_1^{CSA} on the magnetic field strength, T_1^{DD} and T_1^{CSA} are calculated as 2.91 s and 2.51 s at 90.5 MHz, respectively. From these values an overall T_1 of 1.35 s is calculated. This value agrees well with that determined experimentally (1.4 s, Table 4). Consequently, this supports the conclusion that the protein-bound isoalloxazine ring does not have a significant motional freedom which is characterized by a $\tau_w < 1$ ns.

Also T_2 values were determined from linewidth ($\Delta\nu_{1/2}$) measurements ($T_2 = 1/2\pi\Delta\nu_{1/2}$). The linewidth was 2 ± 0.7 Hz for all four carbon atoms, independent of the field strength. These values are in accordance with our conclusions given above, but the inherent inaccuracy does not permit additional conclusions.

From this study and results published previously on ^{31}P NMR on *M. elsdenii* flavodoxin [9], it must be concluded that the prosthetic group in flavodoxin from *M. elsdenii* is tightly associated with the apoprotein. This is an interesting observation with regard to nanosecond fluorescence data on the pyruvate dehydrogenase complex [42] for which it was found that the protein-bound flavin possesses a certain internal freedom on the nanosecond timescale. It is possible that these observations are linked to the specific biological functions of the proteins under consideration.

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Chapter 5

A REINVESTIGATION OF THE STRUCTURE OF OXIDIZED AND REDUCED FLAVIN. A ^{13}C and ^{15}N nuclear magnetic resonance study

Chrit T.W. Moonen, Jacques Vervoort and Franz Müller

SUMMARY

Several chemically substituted flavins are investigated in the oxidized and the reduced state by ^{13}C and ^{15}N NMR techniques. The dependence on the polarity of the solvent and on the concentration are studied. In combination with already published results a semi-empirical theory is developed to interpret the chemical shifts in terms of the solution structure of flavins. Where possible the results are compared with crystallographic data. The two sets of data appear to be in good agreement.

In contrast to common ideas the oxidized state is not fully coplanar, but the N(10) atom is situated out of plane to a certain degree. Polarizing the flavin by hydrogen bonds in a high dielectric medium moves the N(10) atom into the molecular plane and the flavin molecule becomes coplanar. In the coplanar molecule π electrons are delocalized from the N(10) atom mainly to O(2α) and O(4α).

The NMR results show that the solution structure of reduced flavin is mainly governed by sterical hindrance and hydrogen bonds. The findings are in contrast to commonly accepted ideas that reduced flavin is strongly bent. In an apolar solvent the reduced neutral isoalloxazine is only slightly bent. The formation of hydrogen bonds in a protic solvent of a high dielectric constant decreases the bend. The N(10) atom is now almost fully sp^2 hybridized and the N(5) atom has an endocyclic angle of 115^o-117^o, indicating its predominant sp^2 character. The results have several important implications for flavin catalysis. Among these, it is shown that the altered redox potential of the semiquinone-fully reduced redox couple of flavodoxin is probably not caused by the planarity of the reduced protein-bound $FMNH^-$.

INTRODUCTION

The flavins are especially remarkable among the various known natural redox coenzymes because of two outstanding features: i) they can function as one-electron as well as two-electron redox carriers, ii) they act with considerable

efficiency in a wide variety of enzymatic reactions. As a consequence, the flavins are known as very versatile redox coenzymes. This versatility suggests that nature has many possibilities to 'tune' the function of the flavin. This has led to the proposal that specific interactions between flavin and apoflavoprotein play a particular role in determining the pathway of flavin catalysis (Müller, 1972; Müller et al., 1970; Hemmerich and Massey, 1982). Other factors such as mobility of the flavin (Moonen and Müller, 1983), microenvironment of the flavin binding site as well as the planarity of the reduced flavin molecule (Tauscher et al., 1973; Simondsen and Tollin, 1980) may also be important.

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

various chemically modified flavins. The aim of this detailed study is to detect the various possibilities of modulating the physical and chemical properties of flavins and to provide a basis for the interpretation of ^{13}C and ^{15}N NMR results on flavoproteins.

MATERIALS AND METHODS

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

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

Measurements were performed on a Bruker CXP 300 NMR spectrometer operating at 30.4 MHz for ^{15}N and 75.6 MHz for ^{13}C NMR measurements. Broadband decoupling of 2 Watt was applied for ^{13}C NMR. No decoupling was applied for ^{15}N NMR. The temperature of all samples was kept constant at 26 ± 2°C. All spectra were recorded using 30° pulses and a repetition time of 0.8 - 1.3 s. Dioxan was used as an internal reference in aqueous solution and TMS in C^2HCl_3 solutions for ^{13}C NMR ($\delta_{dioxan} - \delta_{TMS} = 67.84$ ppm). Chemical shifts are expressed relative to TMS. Neat $[^{15}N]CH_3NO_2$ was used as an external reference in all solutions for ^{15}N NMR using a coaxial cylindrical capillary (Witanowski et al., 1981). Chemical shifts are reported as true shieldings (i.e. corrected for bulk volume susceptibilities) relative to liquid NH_3 ($\delta_{CH_3NO_2} - \delta_{NH_3} = 381.9$) (Witanowski et al., 1981; Levy and Lichter, 1979).

RESULTS AND DISCUSSION

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

The same rules can be applied for ^{15}N chemical shifts, although with some caution with regard to the marked differences between pyridine- and pyrrole type nitrogens (Witanowski et al., 1981, and references therein). The explanation of the ^{15}N chemical shift of a deprotonated nitrogen atom in heteroaromatic systems is theoretically more difficult to explain. The investigated systems always follow the same rules however, in that the deprotonated atom shifts con-

siderably downfield, although the electron density is increased (Pugmire and Grant, 1968, 1971; Quirt et al., 1974). These studies showed in addition that the atoms in α position to the deprotonation site show also a downfield shift, for all other atoms the π electron increase or decrease governs the chemical shift.

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

In the interpretation of our results we consider, taking into account the above-mentioned semi-empirical rules, the following parameters (for a discussion see Levy and Lichter, 1979; Witanowski et al., 1981): i) the degree of hybridization, ii) the π electron density, iii) the different behaviour of pyridine- and pyrrole-type nitrogen atoms and iiii) the influence of a negative charge on the deprotonation site and on the neighbouring atoms (only relevant for the interpretation of anionic reduced flavins).

Oxidized flavin

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

Polarization of the C(2) carbonyl function in aqueous solutions gives rise to a π electron decrease (hence a downfield shift) at C(2) and subsequently via mesomeric structures at C(10a), N(5), C(6), C(8) and C(9a). The downfield shift of C(7) and C(9) shown in Figure 2A and Table 1 cannot be explained solely by

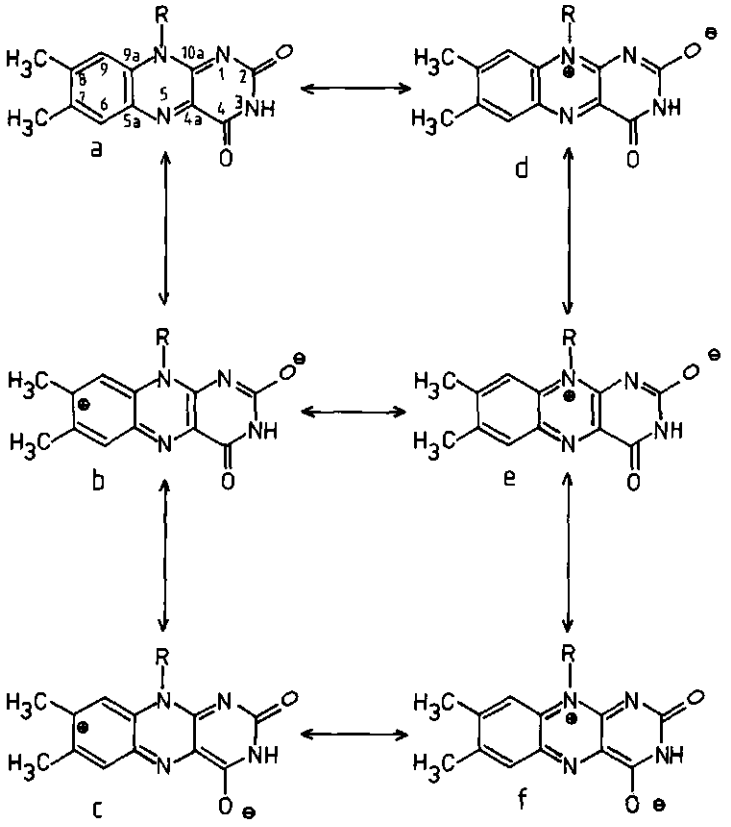


Figure 1: Possible mesomeric structures of oxidized flavin as deduced from NMR results.

structure b in Figure 1. Evidently structure b gives a too simplified picture of the polarized isoalloxazine ring. To describe the polarized structure of oxidized flavin more accurately it is necessary also to include the ^{15}N chemical shifts.

In heteroaromatic systems the nitrogen atoms can roughly be divided in pyrrole- or α-type and pyridine- or β-type nitrogen atoms (Witanowski et al., 1972). The chemical shifts of the two classes of nitrogen atoms depend differently on the polarity of the solvent. The origin of these effects lies in the orthogonality of the electron lone pair with respect to the aromatic system in pyridine-type nitrogens (N(1) and N(5) in flavin) and in the incorporation of the electron lone pair into the system in pyrrole-type nitrogens (N(3) and N(10)

Table I. The relevant ^{13}C chemical shifts of various oxidized flavins.

Compound	Solvent	^{13}C chemical shift (in ppm)[a]											
		C(2)	C(4)	C(4a)	C(5a)	C(6)	C(7)	C(8)	C(9)	C(9a)	C(10a)	C(10α)	C(8α)
[2,4,4a,10a-$^{13}C_4$]FMN[b]	$H_2O/^2H_2O$[c]	159.8	163.7	136.2	--	--	--	--	--	--	152.1	--	--
FMN[d]	$H_2O/^2H_2O$[c]	159.0	162.1	134.9	135.4	132.8	140.6	151.9	118.2	131.5	151.0	48.8	22.0
MeIMN	$H_2O/^2H_2O$[c]	159.2	162.6	136.9	136.8	131.7	140.5	140.2	118.5	132.8	151.3	49.2	--
TARF	C^2HCl_3	155.2	159.8	135.6	134.6	132.8	136.6	147.5	115.5	131.2	149.1	44.5	21.4
TARF	C^2HCL_3MeOH[e]	155.9	161.4	136.0	134.9	133.1	136.8	147.8	115.6	131.9	149.2	45.3	21.4
MeLfl	C^2HCl_3/MeOH[e]	156.7	160.6	135.5	135.0	132.6	137.5	148.8	115.7	132.0	149.2	32.2	21.6
MeLfl	DMF	155.9	160.5	137.1	135.0	132.8	136.7	147.7	117.0	132.0	150.2	32.1	20.8
Me_2IA	C^2HCl_3/MeOH[e]	156.5	160.3	136.7	136.2	132.6	137.9	138.2	115.4	131.7	149.3	32.3	--

a) Relative to TMS.

b) Chemical shifts extrapolated to infinite dilution.

c) Mixture contained 10% ^2H_2O (by vol.).

d) Concentration is 40 mM.

e) Mixture contained 10% MeOH (by vol.).

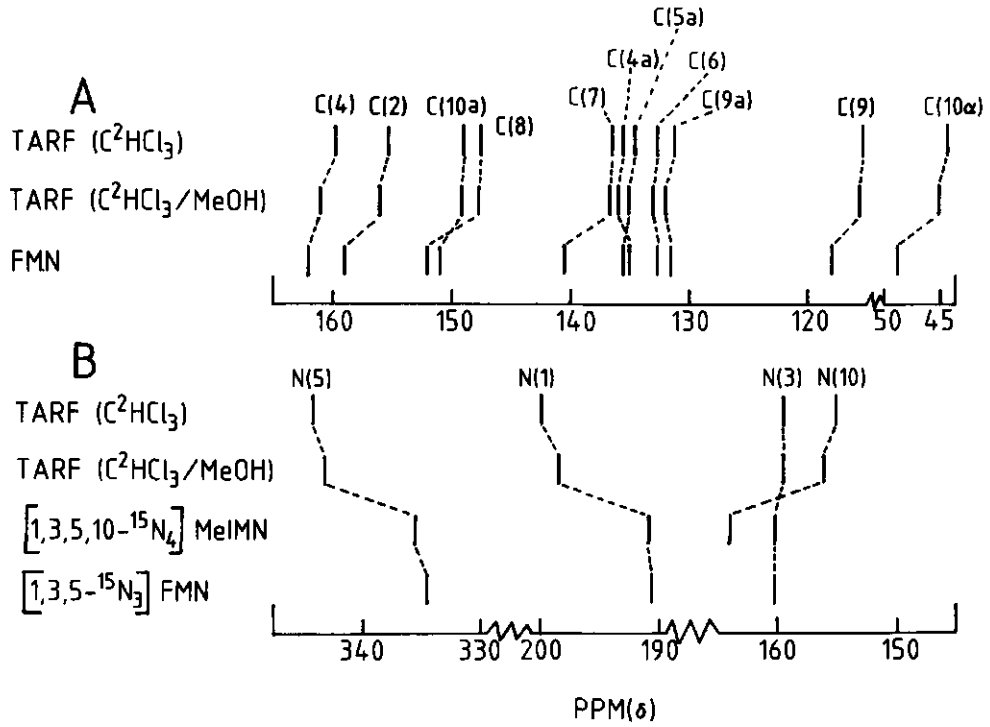


Figure 2: Correlation diagram of ^{13}C and ^{15}N NMR chemical shifts of oxidized flavins. The ^{15}N chemical shifts of TARF are from natural isotope abundance ^{15}N NMR spectra.

in flavin). The chemical shifts of pyridine-type nitrogens show a strong upfield shift on going from apolar to aqueous solution whereas those of the pyrrole-type nitrogens exhibit a smaller and opposite effect. As shown in Figure 2B and Table II the chemical shifts of the N(1), N(5) and N(3) atoms in flavin behave as expected on the basis of the above given classification. The large upfield shift of the resonances due to N(1) and N(5) observed in going from apolar solvents to aqueous solution indicates hydrogen bond formation between these atoms and water molecules, as already previously shown (Franken et al., 1983). The chemical shift of the N(3) atom shows the expected solvent dependent shift for a pyrrole-type nitrogen. However, the chemical shift of the N(10) atom is unexpected and a drastic downfield shift, amounting to 15 ppm, is observed. This observation strongly indicates that the N(10) atom is not as isolated as commonly assumed, but is strongly affected by polarization of the molecule.

Table II

^{15}N chemical shifts of oxidized flavins as dependent on solvent and concentration.

Compound	Concentration	Solvent	^{15}N Chemical shift (in ppm)[a]			
			N(1)	N(3)	N(5)	N(
[1,3,5-$^{15}N_3$]FMN	4 mM	^2H_2O[b]	190.8	160.5	334.7	-
FMN[c]	120 mM	^2H_2O[b]	192.9	161.1	334.4	16
[1,3,5,10-$^{15}N_4$]MeIMN	0.6 mM	^2H_2O[b]	190.5	160.4	335.5	16
[1,3,5,10-$^{15}N_4$]MeIMN	6 mM	^2H_2O[b]	191.1	160.4	335.6	16
TARF[c]	350 mM	C^2HCl_3	199.9	159.8	344.3	15
TARF[c]	350 mM	C^2HCl_3/MeOH[d]	198.8	159.8	343.4	15
[1,3,5,10-$^{15}N_4$]Me_2TARI[e]	7 mM	C^2HCl_3	201.1	160.7	346.7	15

a) Relative to NH_3 (cf. Material and Methods)
b) p^2H is 7.5
c) Natural isotope abundance ^{15}N NMR spectra
d) Mixture contained 10% MeOH (by vol.)
e) From Franken et al. (1983).

As the N(10) atom in flavin is a substituted pyrrole-type nitrogen, not able to form hydrogen bonds with the solvent, the large downfield shift must be ascribed to a hybridization effect, i.e. in aqueous solutions N(10) possesses more sp^2 character than in apolar solutions. This implies that N(10) must be out of plane to some degre in apolar solvents and more in plane in polar solvents. This configurational change of the N(10) atom is independent of the substituent at N(10) (Table I) and is therefore an intrinsic property of the isoalloxazine ring. It should be noted that the chemical shift due to the C(10α) atom parallels that of the N(10) atom (Figure 2). The flat structure in polar solutions can be rationalized by the mesomeric structure d-f in Figure 1. The creation of a partial positive charge on the N(10) atom can be expected to lead either to an upfield shift of the resonances due to N(1), N(3), C(4a), C(5a), C(7) and C(9) (increase of π electron density) or to a downfield shift of these resonances, owing to delocalization of the partial positive charge. Although these effects might influence the resonance position of N(1) and N(3) to some degree the observed upfield shifts are caused predominantly by hydrogen bond formation (cf. above). These two nitrogen atoms are therefore not further considered in the following. With respect to the carbon atoms, all of them are shifted downfiel

indicating that the π electron density at these sites is not increased when the flavin is polarized and that the observed downfield shifts are caused by delocalization of the partial positive charge of the N(10) atom. It should be noted that the small sensitivity of the C(4a) resonance to solvent polarity indicates that the two opposing effects are almost cancelling each other at C(4a). It must therefore be concluded from the ^{13}C NMR spectra that O(2α) and O(4α) are the main π electron acceptors upon polarization of flavin. The resulting mesomeric structures are stabilized by hydrogen bonds to the two oxygen atoms. The mesomeric structure d-f (Figure 1) are thus in agreement with the NMR results.

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

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

It has already been shown (Sarma et al., 1968; Kotowycz et al., 1969;

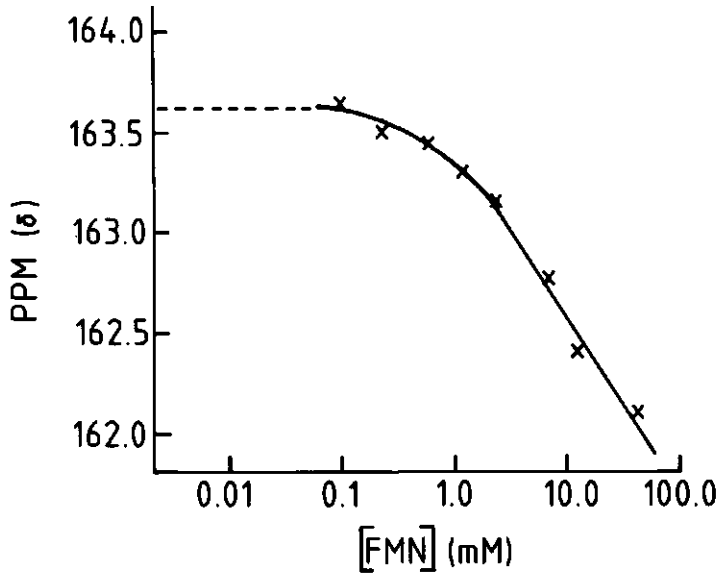


Figure 3: The concentration dependence of the ^{13}C chemical shift due to C(4) of [4-^{13}C]FMN in aqueous solution.

Kainosho and Kyogako, 1972; Moonen and Müller, 1982a; Franken et al., 1983) that FMN in aqueous solution forms aggregates. In Figure 3 the ^{13}C chemical shift of the C(4) atom of FMN is shown in dependence of the concentration. A rather strong concentration dependence of the ^{13}C chemical shift is observed. Similar effects are also observed for C(4a), C(2) and C(10a). The chemical shift values extrapolated to infinite dilution are presented in Table I. The observation that stacking is strongly inhibited at concentrations below 0.1 mM is in excellent agreement with light absorption studies (Müller et al., 1973). The ^{13}C NMR results also indicate that stacking of FMN inhibits its full polarization as evident from the extrapolated ^{13}C chemical shifts (Table I). We suggest that stacking leads to a microenvironment with a lower effective dielectric constant than that of the pure solvent, preventing full polarization of the molecule. It should also be noted that the C(4) resonance is most influenced by stacking. Under this condition the hydrogen bond to O(4α) is weaker than in dilute solution (Table I). The concentration dependence of the C(4) and C(4a) resonances strongly suggests that C(4a) becomes an important π electron acceptor in the absence of hydrogen bonding to O(4α) and at the same time renders O(4α) to a less favourable π electron acceptor.

This interpretation is in agreement with the fact that in monomeric FMN C(4a) shifts downfield indicating transfer of electron density to O(4α).

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

The particular behaviour of the N(10) atom in flavin is also supported by crystallographic data. Such data on oxidized flavin show that the N(10) atom is placed somewhat out of the molecular plane (Kierkegaard et al., 1971). In addition theoretical calculations on the oxidized flavin molecule (Dixon et al., 1979) have shown that only the 8.4 kJ/mol is required to bend the molecule slightly out of the molecular plane. Furthermore, the calculated π electron densities for the N(3) and the N(10) atoms are 1.731 and 1.680 electrons, respectively. Considering that both nitrogen atoms possess pyrrole-type character it would be expected that the N(3) atom should resonate at higher field than the N(10) atom. This is found for FMN in water, but not for TARF in chloroform. Since the calculations have been performed for the coplanar structure the result is not surprising. Consequently the incorrect prediction of the chemical shift for the N(3) atom of TARF in chloroform can be attributed to the different structure of flavin in apolar solvents.

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

1,5-Dihydro flavin

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

In principle a combination of ^{13}C and ^{15}N NMR techniques should also yield detailed information on the solution structure of reduced flavin, as has been demonstrated above for the oxidized molecule. In the following analysis we make use of published data and of a personal study of several model compounds. Owing to the higher complexity of the structure of reduced flavin independent ^{13}C (Van Schagen and Müller, 1980; Van Schagen and Müller, 1981) and ^{15}N NMR (Franken et al., 1983) studies on free and protein-bound reduced flavin did not allow development of a correlation between the chemical shifts and the structure of a particular flavin molecule. With the data now available such a correlation appears to exist, which is presented here. For practical reasons (sensitivity, availability of compounds) ^{13}C NMR results were preferably collected and supplemented by a few ^{15}N NMR results. The data are presented in Table III and IV. For convenience some selected data are presented diagrammatically in Figure 4. The structure of the less common reduced flavins are given in Figure 5.

It is evident from Table III that subsequent substitution of the various N atoms of flavin influences certain ^{13}C resonance lines dramatically. For conve-

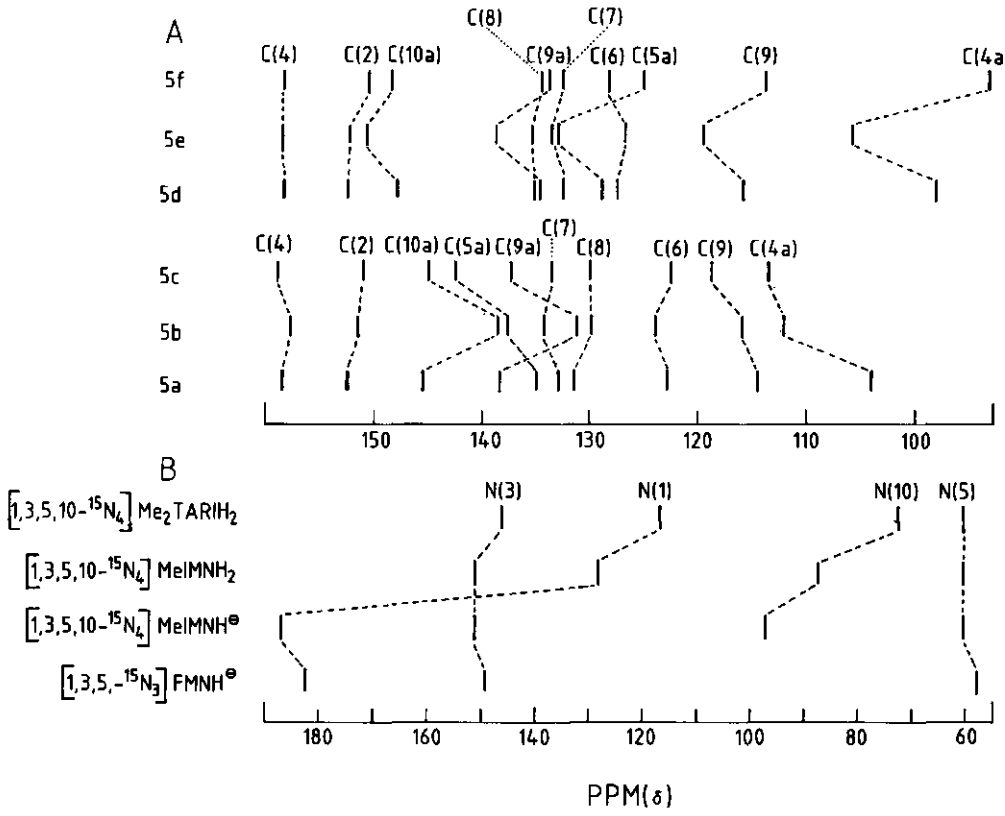
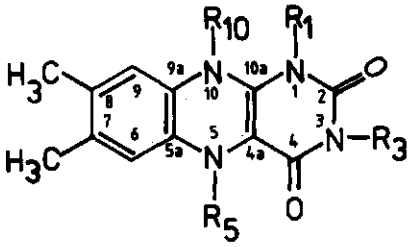


Figure 4: Correlation diagram of ^{13}C and ^{15}N NMR chemical shifts of reduced flavins.

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

In case of optimal folding of reduced flavin the benzene and the pyrimi-



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

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

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

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

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

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

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

Figure 5: The structure of less common reduced flavins, used in this study (*cf.* Table III, IV).

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

In the following an attempt will be undertaken to analyse the chemical shift changes in terms of the conformation of the reduced flavin. To do this we have

Table III

The relevant ^{13}C chemical shifts of 1,5-dihydroflavins as dependent on subsitution, concentration and solvent

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

a) For the structures, see Figure 5.

b) Relative to TMS.

Table IV

The ^{15}N chemical shifts of reduced flavins in aqueous and chloroform solution.

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

a) Relative to external NH_3.

to consider first the electronic structure of the N(10) and N(5) atoms which should yield valuable information for the analysis.

If the reduced flavin were planar then both N(5) and N(10) atoms would be pyrrole-type nitrogen atoms, i.e. both atoms are potential π electron donors. In the folded state, however, both nitrogen atoms are sp^3 hybridized and the electron lone pair can hardly participate in mesomeric structures. This does not necessarily mean that both N atoms have to attain the same configuration. It is possible that the degree of hybridization is different for both N atoms. The degree of hybridization of the N atoms for a few available flavin derivatives can be estimated from ^{15}N NMR data (Figure 4B, Table IV). The N(1) and the N(3) atoms resonate at lower field than the N(5) and the N(10) atoms. The chemical shifts indicate that N(1) and N(3) are predominantly pyrrole-type, i.e. sp^2 type, whereas N(5) and N(10) are in the region of aniline-type N atoms i.e. somewhere between sp^2 and sp^3 type. It has already been argued (Axenrod et al., 1971) that ^{15}N chemical shifts in aniline-type compounds reflect the participation of the nitrogen electron lone pair in the π electron system. ^{13}C chemical shifts can also be used to monitor the degree of participation of the electron lone pair of the N(5) and N(10) atoms in the aromatic subsystems. Increasing the planarity at the N(10) atom, for example, results in π electron donation via mesomeric structures especially to C(4a) and C(5a) (cf. Figure 6) leading to an upfield shift of the latter atoms. On the other hand, increasing the planarity at the N(5) atom results

in π electron donation to C(10a) and C(9a). The carbon pairs C(4a) and C(5a), and C(9a) and C(10a) actually show parallel shifts, supporting our notion that the configuration of N(10) and N(5) is mainly reflected by the chemical shift of the mentioned carbon pairs. Cpd 5c contains methyl groups at positions 1, 3, 5 and 10 leading undoubtedly to a strong steric overlap between the methyl groups at N(10) and N(1), and a strong overlap between the N(5) methyl group and the carbonyl group at C(4). The downfield shifts of all four quaternary carbon atoms in cpd 5c as compared to those of cpd 5b clearly indicate the inhibited π electron donating characters of N(5) and N(10), suggesting a strong bending of the flavin molecule. Comparing cpd 5a with 5c it is seen that C(9a) and C(10a) show about the same chemical shifts, whereas C(5a) and C(4a) in cpd 5a are shifted to high field by 6.4 and 9.5 ppm, respectively. This indicates that the π electron donating character of the N(10) atom is increased in cpd 5a and that the configuration of N(5) remains about the same in both compounds. Cpd 5b exhibits opposite effects as cpd 5c. The π electron donating character of N(5) is strongly enhanced, whereas that of N(10) is decreased. These interpretations are in agreement with the expected steric effects in these flavins.

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

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

The endocyclic angle φ is calculated using the following equation:

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

where C_i is C(4a) or C(5a) for the calculation of the endocyclic angle of N(10), and C(9a) or C(10a) for the calculation of the endocyclic angle of N(5), $\delta(C_{i,sp^3})$ and $\delta(C_{i,sp^2})$ are the corresponding limit chemical shifts of carbon atom C_i for a sp^3 and sp^2 hybridized nitrogen atom, respectively. 109.5^o is the endocyclic angle for a sp^3 hybridized nitrogen atom and 10.5^o is the difference in angles between a sp^2 and sp^3 hybridized nitrogen atom. The limiting values for $\delta(C_{i,sp^3})$ and $\delta(C_{i,sp^2})$ must be known to perform the calculations. Assuming that N(10) in cpd 5f approximates total sp^2 character the endocyclic angle of N(10) in the various compounds is calculated using the limiting values of $\delta(C_{4a,sp^2})$ = 93.3 ppm and $\delta(C_{5a,sp^2})$ = 124.9 ppm (Table III, cpd 5f). For an approximately sp^3 hybridized N(10) atom the chemical shifts due to C(4a) and C(5a) of cpd 5c (Figure 4A) are assumed to represent the needed limiting values, i.e. $\delta(C_{4a,sp^3})$ = 113.3 and $\delta(C_{5a,sp^3})$ = 141.6 ppm, respectively. The endocyclic angle of the N(10) atom of various flavin derivatives, as determined from experimental ^{13}C chemical shift values, are presented in Table V, together with published crystallographic data. Table V demonstrates that the two sets of data agree surprisingly well, indicating that our method of determining the endocyclic angle of the N(10) atom in reduced flavin can be used reasonably safe. The minor differences between the two sets of data are probably caused by the fact that we have completely neglected possible effects on the chemical shifts of the atoms in question by substitution of nearby atoms. Apparently such effects are only of minor importance.

The determination of the endocyclic angle of the N(5) atom is much more difficult because no safe indications are available demonstrating that a particular 1,5-dihydroflavin possesses a sp^2 hybridized N(5) atom. We therefore, used the following modified procedure. It is known from crystallographic studies (Norrestam and Von Glehn, 1972) that the N(5) atom in cpd 5b possesses an endocyclic angle of 116.4^o, i.e. the atom is about 65% sp^2 and 35% sp^3 hybridized.

Table V Comparison of the endocyclic angles of the N(10) and N(5) atoms in reduced flavins as determined by crystallographic and NMR methods. For details, see text.

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

a) Taken from Norrestam and von Glehn (1972).

b) Values used in the calculations, see text.

c) Taken from Norrestam et al. (1969).

d) Taken from Werner and Rönnquist (1970).

e) Taken from Leijonmarck and Werner (1971).

f) These values must be considered with great caution, for an explanation, see text.

The degree of hybridization is in agreement with the $^1J(^{15}N-^1H)$ coupling constant of 87.5 Hz for a similar compound (Franken et al., 1983). Thus the chemical shift values of the C(9a) and C(10a) atoms of cpd 5b (Table III) are used as reference chemical shifts for an endocyclic angle of 116.4° (Eq. 1). In addition it was assumed that a change from sp^3 to sp^2 hybridization of N(5) causes similar chemical shift changes of C(9a) and C(10a), as a hybridization change of N(10) on the chemical shift of C(4a) and C(5a), as discussed above. Although somewhat arbitrary, this assumption is supported by the fact that the introduction of peri-overcrowding effects between a N(5) substituent and the C(4) carbonyl function causes chemical shift changes at the C(9a) and C(10a) centers comparable with analogous effects on the chemical shift changes at the C(4a) and the C(5a) centers by N(1) and N(10) substituents. In addition the value of 109.5° in Eq. 1 was replaced by 116.4° for the calculations.

The calculated values are compared with crystallographic data in Table V.

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

In Table V the calculated endocyclic angles for $TARFH_2$ are also given. The results indicate that the N(5) atom is considerably sp^2 hybridized. This result is in excellent agreement with the $^1J(^{15}N(5)-^1H)$ coupling constant of $TARFH_2$ (Franken et al., 1983). The N(10) atom, on the other hand, possesses less sp^2 character than the N(5) atom. The results indicate that $TARFH_2$ possesses a fairly planar conformation, more bent at the N(10) than the N(5) center.

The calculated endocyclic angles for $FMNH_2$ in aqueous solution are also presented in Table V. The calculated endocyclic angle for the N(5) atom, especially that calculated from the chemical shift of $^{13}C(10a)$, is smaller than that for $TARFH_2$. Based on published light absorption data (Dudley et al., 1964) it was expected that the endocyclic angle of the N(5) atom in $FMNH_2$ would be at least as large as that in $TARFH_2$. The reason for this apparent discrepancy will be explained in the following on the basis of the ^{13}C and ^{15}N chemical shifts of $FMNH_2$ (Table III and IV). As compared to Me_2TARIH_2 in $CHCl_3$ the ^{15}N chemical shifts of $MeIMNH_2$ in aqueous solution appear, with the exception of that of the

N(5) atom, at lower fields (Figure 4B, Table IV). The large downfield shift of the resonance line of N(10) indicates a drastic change to enhanced sp^2 character. The increased sp^2 character of N(10) is also reflected in the upfield shift of the resonances due to C(4a) and C(5a). This upfield shift is however less than would be expected on the basis of the downfield shift of the N(10) resonance. The N(5)H and N(3)H groups in reduced flavin form hydrogen bonds with the solvent in aqueous solution leading to downfield shifts of the corresponding ^{15}N chemical shifts. This is clearly observed on the chemical shift of N(3). The small effect observed on the N(5) resonance suggests that the expected downfield shift is almost cancelled by an increase of the sp^3 character of N(5). This interpretation is supported by the downfield shift of the resonances due to C(9a) and C(10a), but the downfield shift observed for C(10a) is much larger than expected by the change of hybridization of the N(5) atom. Changing the solvent from $CHCl_3$ to aqueous solution also leads to an unexpectedly large downfield shift of the resonance line of N(1). This downfield shift of the resonance of the pyrrole-type N(1) atom is too large to originate solely from hydrogen bond formation. The large downfield shift suggests a considerable increase in sp^2 character of the N(1) atom. The similar (in magnitude) and parallel shifts of the resonances due to the N(1) and N(10) atoms indicate that both atoms can in some way act cooperatively. In contrast to the oxidized flavin the chemical shifts due to C(2) and C(4) are only slightly affected when going from $CHCl_3$ to aqueous solution. This indicates that the change in solvent hardly increases the partial positive charge of the C(2) and the C(4) centers. The explanation is that the π electron density of the O(2α) and O(4α) centers is increased by a cooperative electron donation from N(10) and N(1), and even from N(3). As a consequence O(2α) and O(4α) can no longer polarize C(2) and C(4) to such an extent as in oxidized flavin. It should be noted that the ^{13}C chemical shifts are only slightly dependent on concentration, although aggregation does occur as judged from the increase of linewidths upon increasing the concentration.

In our opinion the electronic structure of $FMNH_2$ can, based on the ^{13}C and ^{15}N NMR data, best be described by the structures shown in Figure 6. The

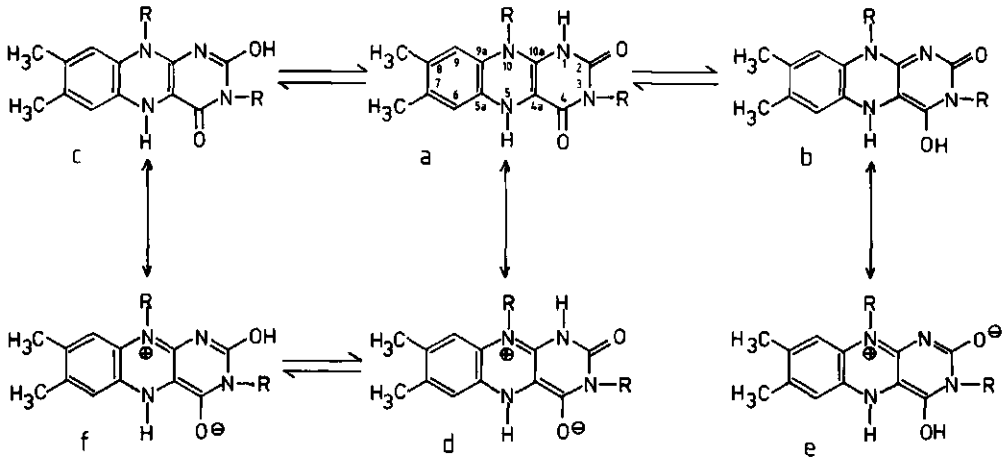


Figure 6: Possible mesomeric structures of reduced flavin as deduced from NMR results.

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

It becomes evident from the discussion above that our semi-empirical approach to calculate the endocyclic angles of reduced flavin in aqueous solution only yields lower limits. In our calculations we assumed that the change in hybridization of the N(5) and N(10) atoms will lead to a π electron density increase or decrease at the centers C(9a) and C(10a), and C(4a) and C(5a), respectively. In apolar solutions where mesomeric structures, as presented in Figure 6, are not favoured, the semi-empirical approach yields reasonably good results. In highly

polar solvents, where mesomeric structures play a role, the ^{15}N chemical shifts due to the N(5) and N(10) atoms are more reliable parameters to estimate the endocyclic angles. At any rate it is estimated from the combination of ^{13}C and ^{15}N NMR results that in $FMNH_2$ the endocyclic angle for N(10) is about 116-118^{o} and that for N(5) is slightly less.

The N(1)H group of reduced flavin deprotonates with a pK_a of 6.6 (Dudley et al., 1964; Van Schagen and Müller, 1981; Franken et al., 1983). The negative charge at N(1) leads to a strong downfield shift of the ^{15}N chemical shift of N(1) and the ^{13}C chemical shifts of the neighbouring carbon atoms C(2) and C(10a). These shifts are similar to those observed in other heteroaromatic systems (Pugmire and Grant, 1968,1971; Ewers et al., 1974; Tokuhiro and Frankel, 1969). The $^{13}C(2)$ resonance is shifted less downfield than the $^{13}C(10a)$ resonance, owing to the increased π electron density at O(2α). It was expected that the negative charge at N(1) would increase the π electron density at N(10), leading to an upfield shift of the resonance of N(10). In contrast a downfield shift of 10 ppm is observed, indicating an increase of sp^2 character of N(10). The increased sp^2 character of N(10) is also reflected in the small upfield shift of the resonances due to C(4a), C(5a) and C(7). Deprotonation of reduced flavin influences the chemical shift of the N(5) resonance only slightly, the small upfield shift indicates a slight decrease of the predominant sp^2 character of the N(5) atom. These results indicate that the N(10) atom in ionized reduced flavin possesses almost full sp^2 character, while that of the N(5) atoms is somewhat less.

The analysis presented above shows that the conformation of reduced flavin is mainly governed by the polarity of the solvent and by steric hindrance introduced by N-substitution. In polar solvents, where mesomeric structures are stabilized, unsubstituted reduced flavin attains an almost planar structure. It has been suggested that the pyrazine subnucleus of reduced flavin possesses anti-aromatic character causing a strong bending of the molecule (Tauscher et al., 1973). The mesomeric structures presented in Figure 6 show that the molecule can 'escape' this anti-aromaticity and attain a fairly coplanar structure. The previous interpretation of the light absorption spectra of reduced flavin (Dudley et al., 1964) should be reconsidered in view of our NMR results, i.e. the observed

molar absorption coefficient at 450 nm for a particular flavin is most probably solely related to the degree of sp^2 hybridization of the N(5) atom of reduced flavin. Our results are also in agreement with theoretical calculations by Dixon et al. (1979), who showed that the reduced flavin molecule is only slightly bent and that only a relatively small activation energy is needed for the molecule to acquire a planar conformation. These results were recently supported by photoelectron spectroscopy (Eweg et al., 1980b).

Our results have several biological implications. It has been shown by crystallographic studies (Ludwig et al., 1976) that $FMNH^-$ is almost coplanar when bound to Clostridium MP flavodoxin. In M.elsdenii flavodoxin which is closely related to Clostridium MP flavodoxin, the sp^2 character of the N(5) atom of the reduced prosthetic group is confirmed by the coupling constant (Franken et al., 1983). Simondsen and Tollin (1980) suggested that the planar structure of $FMNH^-$ in these proteins is mainly responsible for the altered redox potential of the couple semiquinone/fully reduced, as compared to free $FMNH^-$. Our results, however, indicate that free $FMNH^-$ already possesses an almost planar conformation. It is therefore unlikely that the redox potential in flavodoxin is governed mainly by the conformation of protein-bound $FMNH^-$. Probably other factors such as negative charges in the vicinity of the bound flavin play a much more important role.

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

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Chapter 6

A CARBON-13 NUCLEAR MAGNETIC RESONANCE STUDY ON THE DYNAMICS OF THE CONFORMATION OF REDUCED FLAVIN

Chrit T.W. Moonen, Jacques Vervoort and Franz Müller

SUMMARY

Several flavin model compounds in the reduced state have been investigated by ^{13}C NMR techniques. The NMR spectra were recorded in dependence of temperature, in the range of 30^{o} to $-100^{o}C$. The results show that the interpretation of similar results by Tauscher et al. (1973) are incorrect. Nitrogen inversion, as previously suggested by these authors, is not observed, but ring inversion is. The ring inversion is strongly dependent on solvent polarity and hybridization of the N(5) atom. Symmetry arguments in the dynamic processes of the inversion reactions are discussed.

INTRODUCTION

Reduced (1,5-dihydro)flavin plays an important role in biological reactions catalyzed by flavoproteins. It is known from crystallographic studies (Kierkegaard et al., 1971) on flavin model compounds that reduced flavin possesses a bent conformation, often referred to as "butterfly" conformation. It has been proposed that the activation barrier for the transition from the bent to the planar conformation provides a means of regulating the redox potential of protein-bound flavin (Simondsen and Tollin, 1980; Tauscher et al., 1973; Van Schagen and Müller, 1981). In this concept the apoflavoprotein forces the bound flavin to attain a certain conformation. This concept seems to be supported by theoretical calculations indicating that an activation barrier of 145 kJ/mol exists between the bent and the planar conformation (Palmer and Platenkamp, 1979). Tauscher et al. (1973) investigated the nitrogen inversion in reduced flavin by ^{1}H NMR. An activation barrier of about 42 kJ/mol for the inversion of the N(5) atom was calculated from these results. This seems to be in contradiction with our recent ^{13}C and ^{15}N NMR data (Moonen et al., 1984). These results demonstrate that the conformation of reduced flavin is mainly governed by intramolecular overcrowding effects (substitution at N(1) and N(5)) and the polarity of the solvent. The ease in which the endocyclic angle of the N(5) and the N(10) atom can be modulated independently is especially striking. This suggests that the activation barrier for reduced flavin

to attain a planar conformation must be low.

Preliminary ^{13}C NMR results (van Schagen and Müller, 1980) strongly indicated that this technique is particularly useful for investigation of the dynamics of the conformational changes of reduced flavin. Since the ^{13}C chemical shifts of flavin are mainly governed by the π electron density at the carbon atoms under study (Van Schagen and Müller, 1980), ^{13}C NMR should yield a much better insight into the dynamics of the conformational change than ^{1}H NMR where only a limited number of nuclei in flavin are available. Furthermore the resolution of ^{13}C spectra at high field strength is much better than the splitting of a single proton resonance line to an AB pattern, as used by Tauscher et al. (1973). Our results demonstrate that the temperature dependent ^{13}C NMR spectra of reduced flavin yield detailed information on the conformation of reduced flavin and allow calculation of a more accurate value for the activation barrier. This study also shows that nitrogen inversion is not observed and that the spectral changes are related to the "butterfly" motion of the molecule.
This is in contrast to published results (Tauscher et al., 1973).

MATERIALS AND METHODS

The compounds used in this study were prepared according to published procedures: 1,3,7,8,10-pentamethyl-5-acetyl-1,5-dihydro-isoalloxazine, 1,3,7,8,10-pentamethyl-5-benzyl-1,5-dihydro-isoalloxazine, 1,3,5,7,8,10-hexamethyl-1,5-dihydro-isoalloxazine and 1,3,7,8-tetramethyl-5-acetyl-5,10-dihydroalloxazine, (Dudley and Hemmerich, 1967); 3,7,8-trimethyl-1,10-ethano-5-acetyl-1,5-dihydro-isoalloxazine (Müller and Massey, 1969), the parent compound was prepared from riboflavin (Grande et al., 1977); 3-methyl-5-acetyl-1,5-dihydro-tetraacetylriboflavin (van Schagen and Müller, 1980); 3-methyl-1,5-dihydro-tetraacetylriboflavin was prepared directly in the NMR tube by a two-phase reduction (van Schagen and Müller, 1980). Subsequently the aqueous phase was removed by means of a syringe under anaerobic conditions.

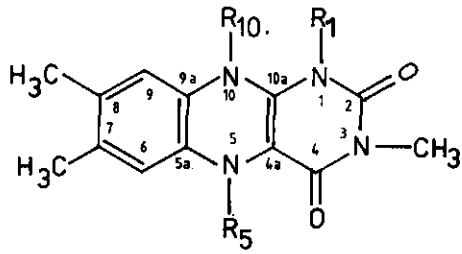
Wilmad 10 mm precision tubes were used. ^{13}C NMR measurements were performed at 75.5 MHz on a Bruker 300 CXP spectrometer. Broadband decoupling (3 Watt) was

applied. A repetition time of 1 s was used. Pulse width was 30^{0}. 5000 transients were usually accumulated. Conversion rates were determined from the coalescence points according to the method of Binsch (1975). Spectra exhibiting unequal populations of the various forms were analyzed using the method of Shanan-Atidi and Bar-Eli (1970). Activation barriers were determined using the Eyring equation as described by Binsch (1975).

RESULTS AND DISCUSSION

As has been discussed previously by Tauscher et al. (1973) the following dynamic processes have to be considered by the analysis of temperature-dependent conformations of reduced flavin: 1) the rotation of the substituent at N(5) around the N(5) - N(5α) bond; 2) the inversion of the N(5) and the N(10)atoms; and 3) the "butterfly" motion of the reduced flavin molecule, i.e. the flipping around the N(5)-N(10) axis. It is often difficult to discriminate experimentally between nitrogen inversion and ring inversion ("butterfly" motion) in heterocycles (Lambert et al., 1971). Some certainty with regard to this problem can be obtained using various model compounds, as will be shown below.

The structure of the compounds investigated in this study are shown in Figure 1. The dependence on the temperature of the ^{13}C NMR-spectra of cpds. 1 and 2 is shown in Figures 2 and 3, respectively. At low temperature both compounds give rise to splitting of all or almost all resonances, although the population stoichiometry of the splitting is different. The activation barrier calculated from ^{13}C NMR spectra for several compounds is summarized in Table 1. The question now arises which dynamic process causes the observed splitting of the resonances. Solely freezing the rotation of the acetyl group would probably not cause splitting of the resonances due to the carbon atoms of flavin. At most splitting of the resonances due to the acetyl side chain would be expected. The splitting patterns shown in Figures 2 and 3, therefore, reflect a dynamic process, which influences the electronic structure of the whole isoalloxazine ring. This effect could be caused by inversion of one of the nitrogen atoms (N(5) or



1: $R_1R_{10}=CH_2-CH_2, R_5=COCH_3$
2: $R_1=R_{10}=CH_3, R_5=COCH_3$
3: $R_1=CH_3, R_{10}=H, R_5=COCH_3$
4: $R_1=H, R_{10}=$tetraactylribityl$, R_5=COCH_3$
5: $R_1=R_5=H, R_{10}=$tetraactylribityl
6: $R_1=R_{10}=CH_3, R_5=C_7H_7$
7: $R_1=R_5=R_{10}=CH_3$

Figure 1: The structure of the compounds investigated.

N(10)) or by the so-called butterfly motion or by a combination of effects, both resulting in a change of the nitrogen substituents from axial to equatorial position and vice versa. An inversion of the N(10) atom can be excluded since the gross effects observed for cpds. 1 and 2 are very similar, with almost equal activation barriers. As has been shown by Moonen et al. (1984), cpd. 1 contains an sp^2 hydridized N(10) atom, whereas the N(10) atom in cpd. 2 exhibits considerable sp^3 character. Nitrogen inversion is strongly dependent on the endocyclic angle and consequently on the hybridization of the nitrogen atom. (Lehn and Wagner, 1970; Lambert et al., 1971).

Although the hybridization of the N(10) atom differs in cpds. 1 and 2, very similar effects are observed for both compounds. We therefore conclude that the splitting patterns are not caused by N(10) inversion. This leaves us with the dynamic process of the N(5) inversion and/or the "butterfly" motion. Whichever process predominates, a partial rotation of the acetyl group is needed, as demonstrated in Figure 4. The acetyl function has to align along the N(5)-N(10) axis before ring inversion or N(5) inversion can take place. Due to considerable overlap between the carbonyl function at C(4) and the acetyl group at N(5),

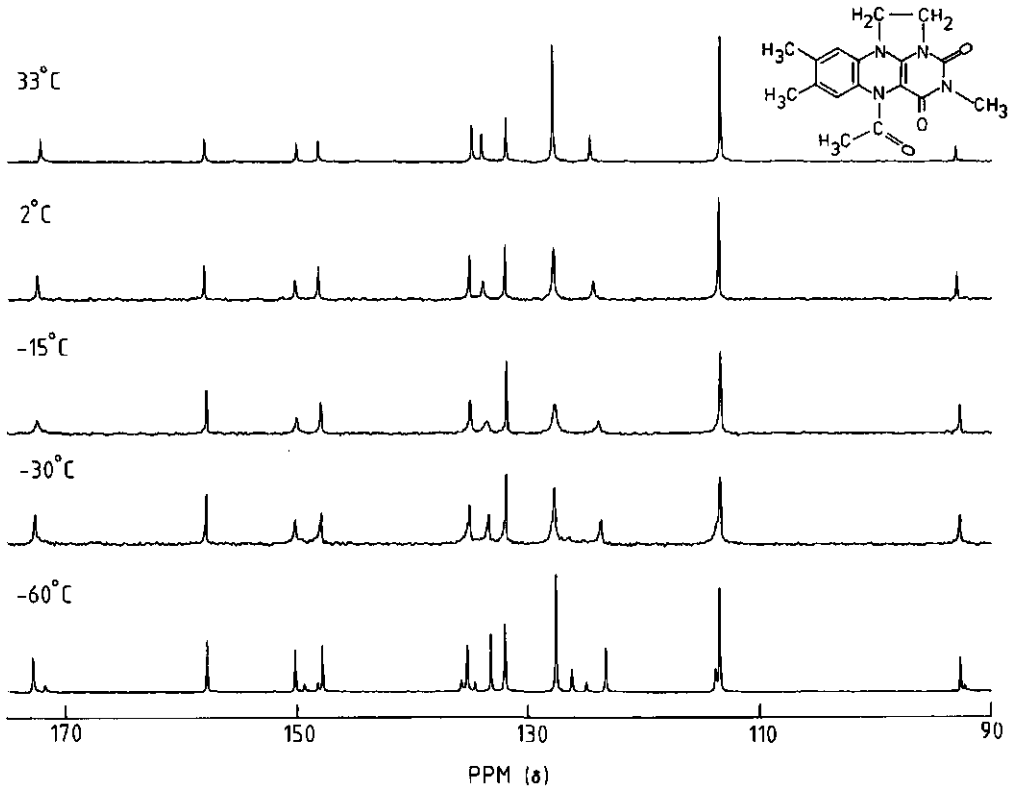


Figure 2: Temperature dependence of the natural abundance ^{13}C NMR spectrum of 3,7,8-trimethyl-1,10-ethano-5-acetyl-1,5-dihydroisoalloxazine (1) in chloroform.

this leads to an energetically rather unfavorable situation. This also means that the activation barrier of the two possible processes is strongly influenced by the activation barrier of the acetyl rotation. The activation barrier of the acetyl rotation can be roughly estimated from published data on amino and amide rotation (for a review, see Binsch (1975)). A rotation barrier of 39.5 kJ/mol was determined for example, for 1,1,1,-trimethyl-2,2-dichloroaminomethane, containing an sp^3 hybridized nitrogen atom (Bushweller et al., 1973). In amides, possessing a partial double bond, the barrier was found to be about 60 kJ/mol if only a small steric hindrance is present in the molecule, and the barriers

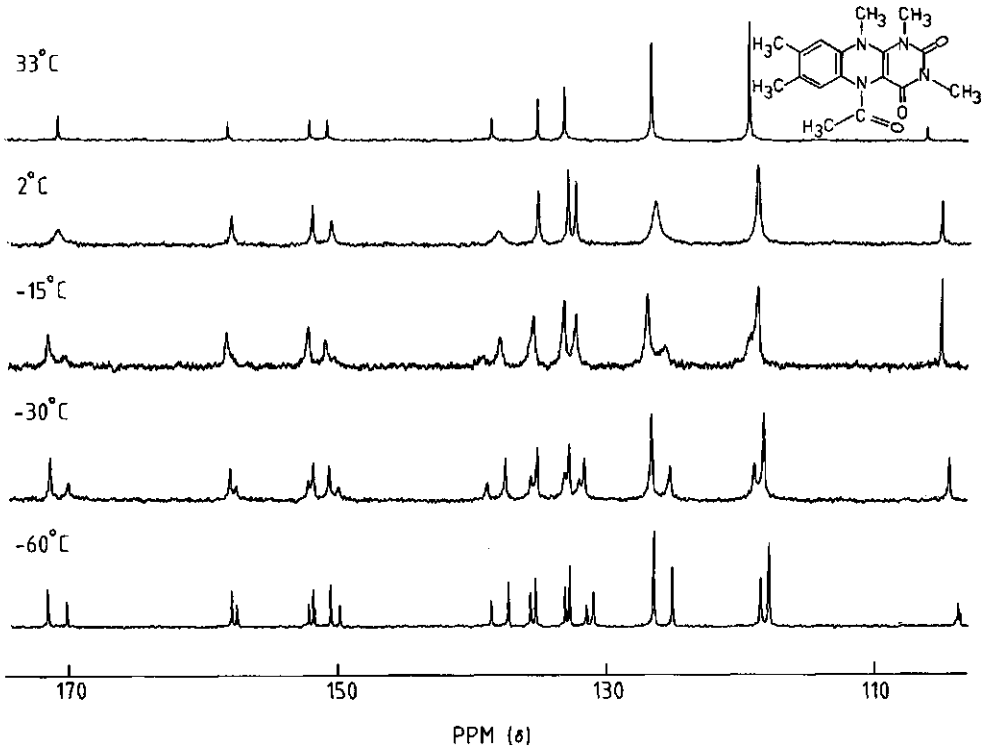


Figure 3: Temperature dependence of the natural abundance ^{13}C NMR spectrum of 1,3,7,8,10-pentamethyl-5-acetyl-1,5-dihydroisoalloxazine (2) in chloroform.

become much higher if steric hindrance is increased (Binsch, 1975).

The activation barrier for the acetyl rotation in flavin is estimated from these data to be at least 60 kJ/mol. Furthermore, ^{13}C NMR spectra of cpd. 7 in chloroform does not show a splitting or even a broadening of the ^{13}C lines at $-60^{o}C$ which clearly demonstrates that the actual activation barrier for the ring inversion or N(5) inversion must be rather low. (data not shown). Tauscher et al.

Table 1 Activation barriers in chloroform calculated from the temperature dependent ^{13}C NMR spectra using the method of Shanan-Otidi and Bar-Eli (1970)

Compound	Activation barrier ΔG^* (kJ/mol) at the coalescence temperature	Coalescence temperature (^oK)
1	55.2	258
2	55.6	273
3	57.6	273
4	61.9	306

(1973) suggested that ring inversion in flavin is energetically the most favored process. This suggestion is in agreement with published data obtained on different compounds. For instance, Lehn and Wagner (1970) and Lambert et al. (1971) showed that the activation barrier for nitrogen inversion in cyclic compounds ranges from 40 kJ/mol to much higher values for sp^3 hybridized nitrogen atoms not containing a covalently bound hydrogen atom. On the other hand, ring inversion in cyclohexane shows an activation barrier of 42 kJ/mol (Anet and Anet, 1975). Introduction of a double bond into this system however, markedly decreases the activation barrier (Anet and Anet, 1975). Bernard and St. Jacques (1973) determined the barrier in cyclohexene to be 20-25 kJ/mol. The drastically decreased barrier was ascribed to the fact that cyclohexene cannot adopt the low energy chair conformation. This means that the more planar the conformation of a molecule becomes the lower the barrier will be, as has also been demonstrated for piperidine and derivatives thereof, containing a sp^2 center (Gerig, 1968; Jensen and Beck, 1968; Lambert and Oliver, 1968). The barriers determined for cyclohexane, cyclohexene and the piperidine derivatives are of about the same magnitude. This also strongly indicates that the replacement of a carbon atom in cyclohexane by a nitrogen atom does not influence the activation barrier much. This suggests that the properties of the pyrazine subnucleus of reduced

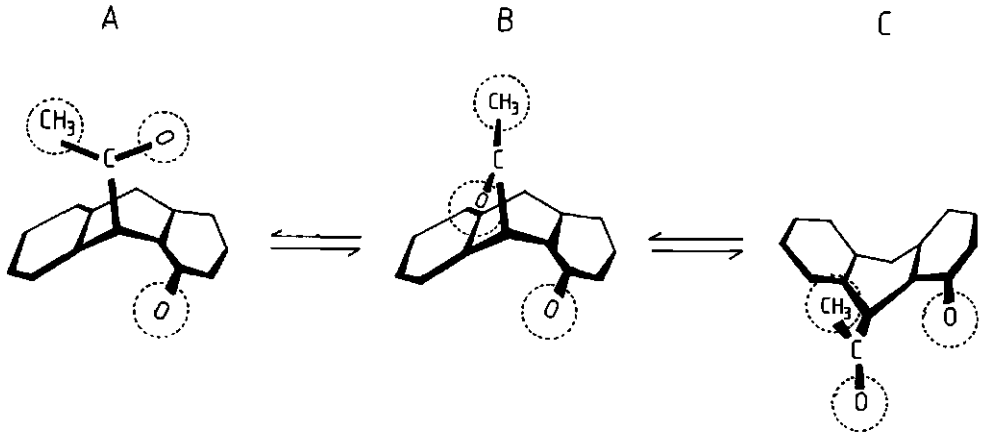


Figure 4: Schematic representation of the "butterfly" motion and the rotation of the N(5) acetyl side chain. The configuration of the acetyl side chain in A is favoured as the steric overcrowding effect with the carbonyl group at position 4 is minimized (also the para-overcrowding effect with the N(10) substituent is minimized). To allow the "butterfly" motion (B → C) it is necessary that the acetyl side chain rotates (A → B) to minimize the peri-overcrowding effect with the C(4) carbonyl group during the planar transition state. (see also text).

flavin are compatible with those of simple cyclohexane and cyclohexene derivatives. Thus, the actual activation barrier for ring inversion must indeed be far below 60 kJ/mol and the apparent high activation barriers reported in this study are caused by the high barrier for the acetyl rotation.

From this it can also be concluded that ring inversion in reduced flavin is considerably more favored than inversion of the acetylated N(5) atom. This implies that we are observing the effects of the ring inversion in Figures 2 and 3, although the calculated activation barriers from the splitting patterns represent its activation energy increased by the barrier of the unfavorable acetyl rotation.

The splitting patterns of all N(5) acetylated flavins investigated are rather similar and the activation barriers hardly differ (Table 1). This indicates that the activation barrier for the ring inversion is hardly dependent on the N(10) substituent (see Table 1). As the N(10) atom in flavin is always more sp^2 hybridized and steric effects are less for the N(10) than for the N(5) atom, it is

not surprising that the activation energy for the overall dynamic process is still mainly determined by the acetyl rotation at the N(5) atom. In MeTARFH$_2$, on the other hand, no splitting at all could be observed down to -40°C. In this compound the N(5) atom contains a hydrogen atom and therefore the butterfly motion is greatly facilitated, in accordance with our results.

This interpretation is in full agreement with earlier observations (Moonen et al., 1984) showing that the degree of hybridization of the N(5) and the N(10) atoms in reduced flavin can easily be influenced by substitution. Dixon et al. (1979) calculated an activation barrier of 16 kJ/mol for the "butterfly" motion of unsubstituted 1,5-dihydroflavin. In our opinion this value is, also considering the published results on cyclohexane, cyclohexene and piperidine derivatives, close to the actual activation barrier. This is also in agreement with our results on TARFH$_2$. In contrast, the theoretical value of 145 kJ/mol for the ring inversion of reduced flavin, calculated by Palmer and Platenkamp(1979), must be incorrect.

The interpretation of our results is in contradiction to work published by Tauscher et al. (1973). These authors investigated ^{1}H NMR spectra of N(5) alkylated flavin derivatives in dependence of temperature and ascribed the observed effects to the inversion of the N(5) atom. They excluded a rotation of the N(5) side chain around the N(5)-C(5α)- or C(5α)-C(5β)-axis. This conclusion was based on the fact that 1,3,7,8-tetramethyl-10-benzyl-5-acetyl-1,5-dihydro-isoalloxazine did not show a splitting of the methyl group of the N(5) acetyl group, not even at -107°C. It is, however, by no means certain that the two possible rotamers can be resolved using ^{1}H NMR. Even if the two rotamers could be resolved, their detection could well be obscured by a large population difference. Furthermore, the maximum activation energy for the acetyl rotation, derived by Tauscher et al. (1973) from the absence of a splitting in the ^{1}H NMR spectrum of 1,3,7,8-tetramethyl-10-benzyl-5-acetyl-1,5-dihydroisoalloxazine is far below what would be expected for such a rotation (cf. Binsch, 1975), taking into consideration

a) the partial double bond character in the amide-like N(5)-C(5α) bond,

b) the peri-overcrowding with the carbonyl function at C(4) and

c) the para-overcrowding with the large N(10) benzyl substituent.

Moreover, it can be concluded directly from the published spectra of Tauscher et al. (1973) that the interpretation of their data is incorrect. If it were true that rotation of the N(5) side chain in N(5) benzylated flavin is fast on the NMR timescale down to -100^{o}C, then the methylene protons of the benzyl side chain should remain magnetically equivalent because of this fast rotation. Consequently freezing the inversion of the N(5) atom would lead to a splitting of the singlet due to the methylene group into a doublet of probably unequal population. The actual observed pattern of a quartet (two doublets of equal population) by Tauscher et al. (1973) must be due to the fact that the rotation of the benzyl group is frozen rendering the methylene protons unequivalent, resulting in a multiplet of an AA'BB' pattern. The published value for the barrier 42.4 kJ/mol Tauscher et al., 1973) is also in the range expected for the side chain rotation (Binsch, 1975). Moreover, we could not detect any splitting or even a broadening in the ^{13}C spectra of cpd. 6 in acetone down to -95^{o}C.

The symmetry aspects of the dynamics of nitrogen inversion and ring inversion will be discussed briefly because these aspects are important for a thorough interpretation of the data (Lambert et al., 1971). Thus if the ring inversion ("butterfly") in reduced flavin were followed immediately by nitrogen inversion of the N(5) and the N(10) atoms the mirror image of the initial conformation would be formed, resulting in identical NMR spectra. Consequently the ring inversion in reduced flavin can only be observed in flavin derivatives in which the inversion barrier is higher than the ring inversion for one of the nitrogen atoms at least. In cpds. 1 to 4 for instance the ring inversion could only be studied because of the N(5) acetyl group. In the absence of the acetyl group the N(5) inversion follows the ring inversion (due to fast proton tunnelling) yielding the mirror image of the initial state in agreement with the experimental observation that NMR spectra of N(5) unsubstituted flavins show no dependence on temperature. The ring inversion of free 1,5-dihydroflavin, involved in biological reactions, can therefore only be studied by introduction of sterical hindrance at the N(5) atom since the N(10) atom will probably have a low inversion barrier, owing to its partial sp^{2} character. It follows from this that it will be

rather difficult to design experiments where the two coupled dynamic processes can be studied independently by ^{13}C and ^{1}H NMR. With respect to the study of the N(5) inversion by Tauscher et al. (1973) it must be mentioned that these authors did not consider symmetry arguments in the interpretation of their experimental results. Thus, if effects of N(5) inversion have been observed they have been caused either by a direct N(5) inversion or by ring inversion followed by N(10) inversion resulting in the enantiomeric form, which cannot be distinguished from the initial form by conventional NMR methods. The energetically favoured pathway will of course be dominant. This means that the N(5) inversion will not be observable if the other pathway is energetically favoured as is the case in cpd. 6, investigated by Tauscher et al. (1973).

The existence of mirror-images of flavin has some implications for studies of flavin and flavin analogues binding by apoflavoproteins. Since, as shown previously (Moonen et al. , 1984), in oxidized flavin the N(10) atom is somewhat out of the molecular plane, an enantiomer also exists in this state. If the apoflavoprotein binds the flavin molecule stereospecifically, as can be expected, then half the amount of an added stoichiometric concentration of flavin with respect to the apoflavoprotein will be bound faster by the apoprotein than the other half, because some time will be required for the conversion into the other enantiomeric form. However, as the conversion will occur with a high rate (low activation barrier) this aspect will probably only be of importance in kinetic studies using techniques of high time resolution.

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Chapter 7

On The Intermolecular Electron Transfer Between Different Redox States of Flavodoxin From *Megasphaera Elsdenii*: A 500 MHz ^{1}H NMR Study

Chrit T.W. Moonen and Franz Müller

SUMMARY

The electron transfer reactions between molecules of flavodoxin from M.elsdenii in different redox states have been investigated by proton nuclear magnetic resonance techniques at 500 MHz. The electron transfer between molecules in the oxidized and semiquinone state is shown to be at least 350 times slower than that between molecules in the semiquinone and hydroquinone state. The latter reaction was studied at different ionic strength and temperature. The rate of electron transfer increases with increasing ionic strength, as expected for a reaction between molecules of identical charges. The electron transfer reaction is only slightly dependent on temperature suggesting an outer sphere reaction mechanism. The results indicate that the activation energy for the electron transfer reaction between the semiquinone and hydroquinone state is negligible in contrast to that between the oxidized and semiquinone state. It is suggested that this feature renders M.elsdenii flavodoxin to an exclusive one electron donor/acceptor in the cell, thereby shuttling between the semiquinone and the hydroquinone state. Mechanistic implications of the findings are briefly discussed.

INTRODUCTION

Flavodoxins are a class of small flavoproteins (M_r = 15 000 - 23 000) which contain riboflavin 5'-phosphate as prosthetic group and function as electron carriers in biological reactions [1]. Upon reduction these flavoproteins form a stable semiquinone under anaerobic conditions. The flavosemiquinone can be reduced further to the two-electron reduced (1,5-dihydroflavin or hydroquinone) state (Fig. 1). In in vivo reactions flavodoxin from Megasphaera elsdenii

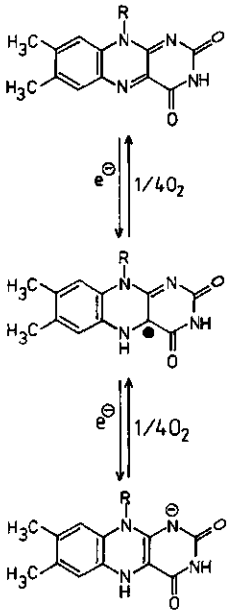

Fig. 1. The structures of the prosthetic group in the three redox states.

shuttles between the semiquinone and the hydroquinone state. The redox potential of this transition is strongly altered in flavodoxins as compared with that of the free prosthetic group. In M.elsdenii flavodoxin the transition between the quinone (oxidized) and the semiquinone state (E_2) is characterized by a redox potential of -115mV and that between the semiquinone and the hydroquinone state (E_1) by a redox potential of -375 mV at pH 7.0 [1].

The three-dimensional structure of the flavodoxin from Clostridium MP is known in all three redox states [2-4]. The crystallographic data show that FMN is shielded from solvent water except for the two methyl groups at position 7 and 8 of the isoalloxazine moiety of FMN. No gross conformational change has been observed between the different redox states, but the three-dimensional data indicate that a small conformational change takes place in the transition from the oxidized to the semiquinone state [3].

The chemical and physical properties of the two flavodoxins from M.elsdenii and Clostridium MP are similar indicating a close structural relationship between these flavodoxins [1]. This was recently further supported by a nuclear magnetic resonance (NMR) study [5] showing that the active centers in the two oxidized flavodoxins are similar. From NMR studies it was also learned that the phosphate group and the isoalloxazine moiety of FMN are strongly immobilized [6,7]. It was proposed that the immobilization of the protein-bound flavin may be related to the biological function of the flavodoxins [7].

It has been proposed from the fact that part of the benzene subnucleus of FMN in flavodoxin is exposed to the bulk solvent that the methyl group in position 8 of FMN, or less specifically, the 7,8-dimethylbenzene subnucleus of FMN plays an important role in the electron transfer reaction [8]. Apparent support for this proposal was deduced from kinetic studies using free and protein-bound modified reduced flavins and cytochrome c as an oxidant. However, the kinetics of the reduction of cytochrome c by the semiquinone of riboflavin and 7,8-dichlororiboflavin do not support the proposal, since the rate constants for both reactions are identical [9]. This study, on the other hand, nicely demonstrated that charges play an important role in biological electron transfer reactions.

In this paper we report on the rate of electron transfer between flavodoxin molecules of different redox states in equilibrium situations. Although these reactions are not of physiological importance, we have a relatively simple model system to investigate the rate of the electron transfer. A major advantage of

the use of the nuclear magnetic resonance techniques in this system is the possibility to follow the flux of electron transfer during equilibrium. The results allow us to draw some important conclusions with respect to the mechanism of electron transfer catalyzed by flavodoxins.

MATERIALS AND METHODS

Flavodoxin from Megasphaera elsdenii was isolated and purified according to published procedures [10]. Reduction of solutions of flavodoxin was achieved by the addition of a dithionite solution [11] or by light irradiation for 30 minutes of flavodoxin solution containing 1.6 mM EDTA and 8 μM deazaflavin [12]. Anaerobic solutions were prepared by carefully flushing the flavodoxin solution in the NMR tube with argon for 30 minutes. Stepwise reoxidation was done by injection of small volumes of air into the NMR tube followed by gentle mixing. During reduction and reoxidation the intermediate semiquinone state is quantitatively formed before full reduction or oxidation is accomplished [1].

Samples of flavodoxin solutions were prepared by exhaustive dialysis against Tris-HCl buffer, pH 8.3. Then the samples were lyophilized and redissolved in ^2H_2O. This procedure was repeated twice and was sufficient to exchange the easily exchangeable protons by deuterons. The desired concentration of flavodoxin and buffer were obtained by the addition of the appropriate volume of ^2H_2O to the final, lyophilized preparation. The ionic strength of the solutions was varied between 20 to 1000 mM by addition of KCl. The pH values given are those measured at the beginning of each experiment. It should be noted that the pH values of solutions reduced by excess dithionite and subsequently reoxidized by addition of small amounts of oxygen are somewhat lower than the starting pH value due to the reaction between O_2 and the excess dithionite.

^1H NMR spectra were recorded on a Bruker WM 500 spectrometer operating at 500 MHz. A spectral width of 6000 Hz and a repetition time of 1 s were used. 8K data points were used unless otherwise stated. Quadrature detection and quadrature phase cycling were used. The accuracy of the temperatures stated

is ± 1 °C. Chemical shifts are reported with respect to trimethylsilylpropionate (TSP). Wilmad 5 mm NMR tubes were used.

Spin-spin relaxation times were calculated from line width measurements. Line shape analysis under exchange conditions was performed according to McLaughlin and Leigh [13] in the extreme conditions of very fast or very slow exchange, and according to McConnel [14] in intermediate situations (cf. Results). In the latter case the analytical solution for the two site exchange problem was used [14,15].

RESULTS

Flavodoxin from M.elsdenii resembles that from Clostridium MP in many chemical and physical properties [1]. Crystallographic studies of the latter protein have shown that the conformation of the protein is the same in the hydroquinone and the semiquinone states. With regard to the FMN binding site, however, there are some minor conformational differences between the quinone state, on the one hand, and the semiquinone and the hydroquinone states on the other [2-4]. James et al. [16] proposed, using the ^{1}H NMR technique, that the overall conformation of M.elsdenii flavodoxin is almost independent of the redox states (Fig. 1). Van Schagen and Müller [17] confirmed this conclusion by a ^{1}H NMR study at higher field strength. These conclusions are further supported by a ^{31}P NMR study on M.elsdenii flavodoxin [6].

The high field part of the 500 MHz ^{1}H NMR spectra of M.elsdenii flavodoxin in the three redox states is shown in Fig. 2. The resolution of the spectra is enhanced by a Lorentzian to Gaussian transformation. In spite of the relatively high molecular weight of the protein (M_r = 15 000) the resolution is remarkable and even coupling patterns are clearly seen. This region of the ^{1}H NMR spectrum of M.elsdenii flavodoxin consists of methyl resonances strongly shifted from the random coil positions by ring current effects. These methyl resonances can therefore be regarded as intrinsic conformational probes of the protein. The resonance position of most of the lines is not affected by reduction or reoxidation, except for the resonances I, II and III (Fig. 2). In addition, the line width of

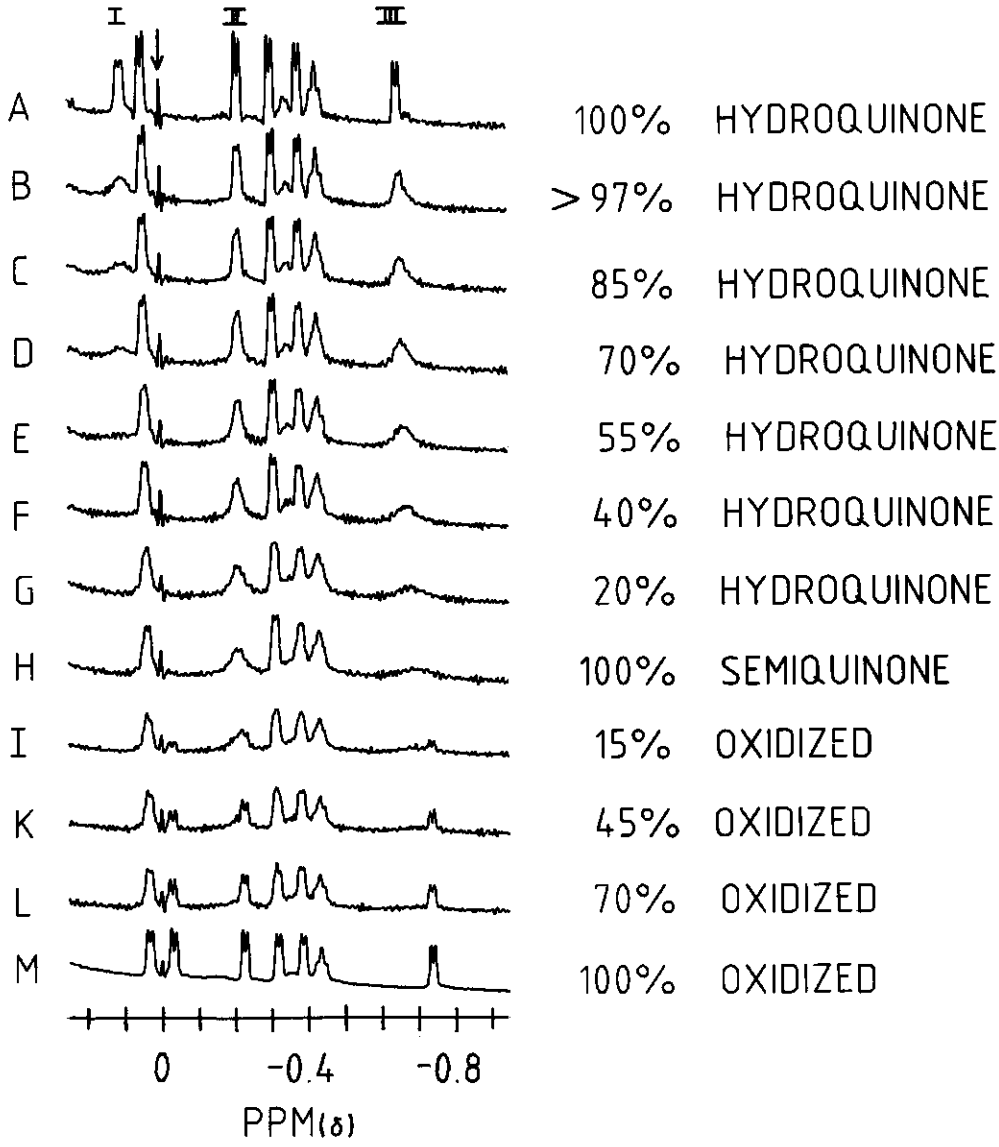

Fig. 2. The high field part of 500 MHz ^{1}H NMR spectra of M.elsdenii flavodoxin as dependent on the state of reduction, as indicated. The concentration of the protein was 3 mM. Spectra were taken at 33°C. The protein was dissolved in 20 mM Tris-HCl buffer, pH 8.3. The arrow indicates the resonance position of the internal standard. The roman numbers indicate the resonance lines used to calculate the rate of electron transfer. For further details, see Materials and Methods, and Results.

the resonances I and III is strongly dependent on the concentration of the semiquinone present at a certain degree of reduction or reoxidation of the flavodoxin. The line width of peak II is similarly but less strongly affected by the presence of semiquinone. The three peaks have already been assigned [17] and represent the β-methyl group of Ala-56 (peak I) and the two δ-methyl groups of Leu-62 (peak II and III). Peak I appears at -0.04 ppm in the oxidized and at 0.11 ppm in the hydroquinone state. Peak III resonates at -0.74 ppm in the quinone and at -0.65 ppm in the two electron reduced state, and peak II at -0.23 ppm in the oxidized and reduced state.

Flavodoxins form a relatively stable radical under anaerobic conditions [1]. In the semiquinone state the free electron is delocalized in the isoalloxazine part of the prosthetic group, but most of the spin density is confined to the central ring (pyrazine subnucleus) of the flavin molecule [18]. The effect of this paramagnetic center on the resonances in the neighbourhood of the prosthetic group is seen in spectrum H of Fig. 2. The peaks I and III are broadened almost beyond detection, whereas the other peaks are relatively unaffected as compared with those in the diamagnetic redox states (Fig. 2, spectra A and M).

On going from the hydroquinone to the semiquinone state peak I and III broaden, even in the presence of a relatively small concentration of semiquinone (Fig. 2, spectrum B). As can be deduced from Fig. 2, (spectra A to H) the broadening is clearly dependent on the relative concentration of hydroquinone and semiquinone. In contrast, on passing from the oxidized to the semiquinone state (Fig. 2, spectra M to H) no broadening of the peaks I and III is observed. Only a decrease in peak height can be detected, the linewidth remains unaltered. The following straight forward conclusions can be drawn from these observations: 1) when the flavodoxin molecules are partly in the hydroquinone and partly in the semiquinone state, the redox state of each flavodoxin molecule changes rapidly with respect to the NMR time scale (Fig.2, spectra A to G); ii) when the flavodoxin molecules are partly in the semiquinone and partly in the quinone state, such a rapid change of the redox state of each flavodoxin molecule does not occur. Similar conclusions can be drawn from subspectra of all parts of the ^{1}H NMR spectrum of M.elsdenii flavo-

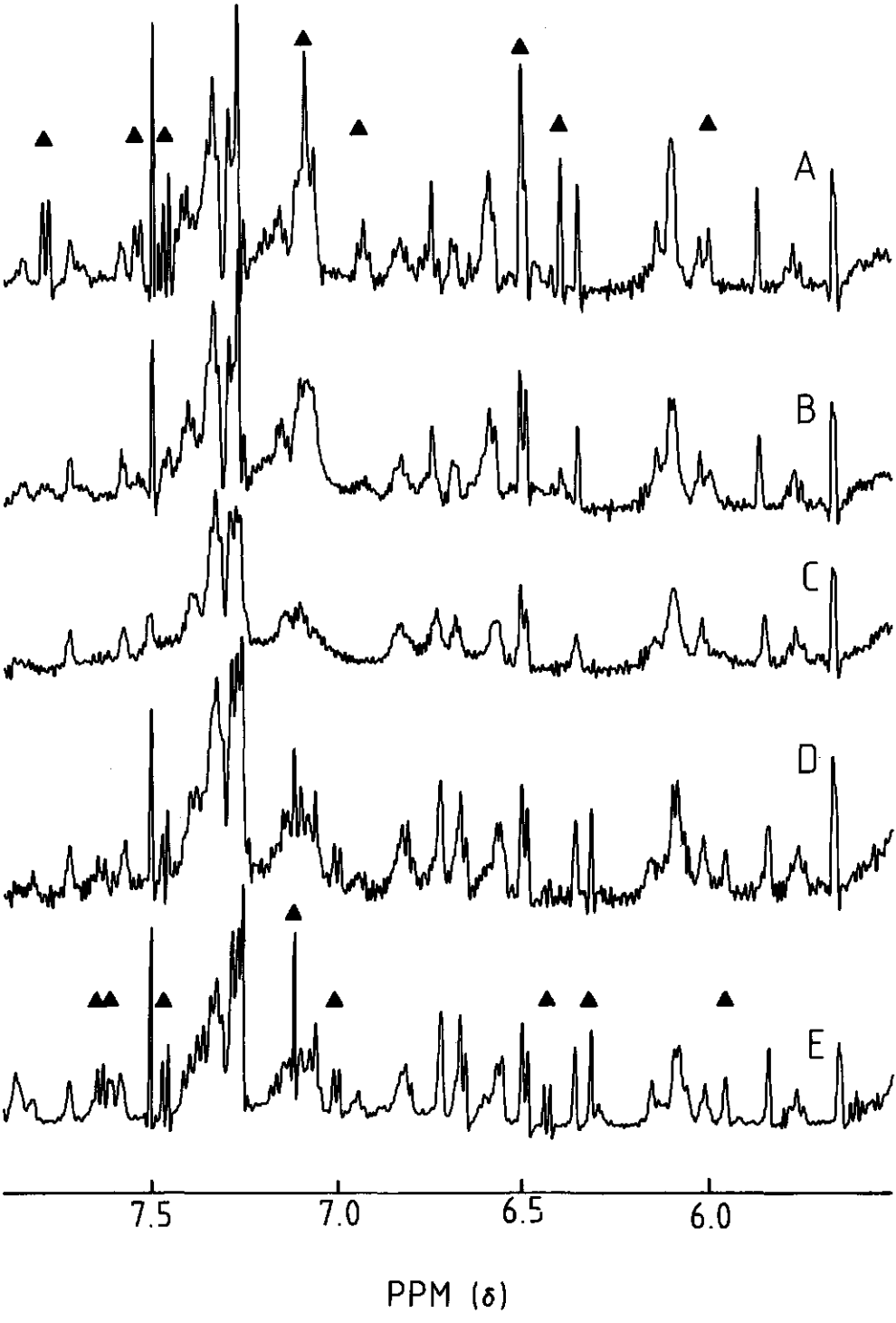

Fig. 3 The aromatic region of 500 MHz ^{1}H NMR spectra of M.elsdenii flavodoxin as dependent on the state of reduction. For further details, see Fig. 1. Resonance lines indicated with ▲ are broadened in the semiquinone state. A: 100% hydroquinone, b: 90% hydroquinone, C: 100% semiquinone, D: 70% oxidized, E: 100% oxidized.

doxin in the three redox states. This is illustrated in Fig. 3 for the aromatic part of the ^{1}H NMR spectrum. It has been shown by ^{1}H NMR techniques [5,17] that several aromatic amino acid residues are in the neighbourhood of the prosthetic group. The resonances of these amino acid residues behave in the same way as the methyl resonances at high field. Since the peaks at high field are much better resolved and separated from other proton resonances the calculation of the rate constants for the electron exchange reactions has been done with the experimental data obtained at high field.

The marked differences between the rate of electron exchange between the different redox states has been noticed previously in a ^{31}P NMR study [6]. Owing to the line width in the ^{31}P NMR spectra of M.elsdenii flavodoxin and the limited sensitivity of the spectrometer used, only approximate rates of the electron exchange reactions could be determined. On the other hand James et al. [16] arrived at the conclusion that the rates of electron exchange are similar for both redox shuttles. This conclusion cannot be correct, as already indicated above. The discrepancy must be ascribed to the fact that our ^{1}H NMR spectra of M.elsdenii flavodoxin are far better resolved than those of James et al. [16] who had to base their calculations on resonance lines composed of several methyl groups.

The spectra were recorded with flavodoxin solutions reduced by various concentrations of dithionite. In principle one has to consider the possibility that dithionite or its dissociation product SO_2^- [11] could function as a mediator in the electron exchange reactions. To check this possibility the flavodoxin was reduced by light irradiation in the presence of EDTA and a catalytic amount of deazaflavin [12]. It was found that the electron exchange rates are independent on the method of reduction. We therefore can conclude that the measured rate of electron exchange is due to the direct electron transfer between flavodoxin molecules in the different redox states. Thus, the line broadening observed in Figs. 2 and 3

represents the following reactions:

$$Fld_{sq}^{(1)} + Fld_{hq}^{(2)} \underset{k_1}{\overset{k_1}{\rightleftharpoons}} Fld_{hq}^{(1)} + Fld_{sq}^{(2)} \qquad (1a)$$

$$Fld_{sq}^{(1)} + Fld_{q}^{(2)} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Fld_{q}^{(1)} + Fld_{sq}^{(2)} \qquad (1b)$$

where Fld denotes M.elsdenii flavodoxin and the subscripts q, sq and hq are the quinone, semiquinone and hydroquinone states, respectively. Second order rate constants are denoted as k. The products of these reactions are the same as the reactants, i.e. the reactions are symmetric and therefore $k_1 = k_{-1}$, and $k_2 = k_{-2}$. With the NMR technique it is relatively easy to determine the flux of electrons during the equilibrium state because one of the reactants is paramagnetic and this paramagnetism is lost after the electron transfer.

According to the reactions 1a and 1b we are dealing with a simple two-site exchange system (hydroquinone and semiquinone or semiquinone and quinone). For this system the total number of electron transfers (in Ms^{-1}) can be expressed as:

$$n_t = k_{ex}\,[A][B] \qquad (2)$$

where [A] and [B] are the concentrations of flavodoxin in the redox states A and B, respectively, and k_{ex} is equal to k_1 or k_2 (cf. Eqn. 1a and 1b). The site lifetime τ_A in the redox state A, for instance, is now expressed as the number of molecules in the redox state A divided by the number of electron transfers per second:

$$\tau_A = \frac{[A]}{n_t} \qquad (3)$$

The site lifetimes in the different redox states are related to the lifetime τ [13]:

$$\frac{1}{\tau} = \frac{1}{\tau_A} + \frac{1}{\tau_B} \qquad \text{and} \qquad (4a)$$

$$\tau = \tau_A X_B = \tau_B X_A \qquad (4b)$$

where X_A and X_B are the mole fractions of flavodoxin, in the redox states A and B. The combination of Eqns. (2), (3) and (4) leads to the expression:

$$k_{ex} = \tau^{-1}([A] + [B])^{-1} = \tau^{-1}[Fld]^{-1} \qquad (5)$$

where [Fld] is the total concentration of flavodoxin in solution. The lifetime τ was determined from the broadening of the resonances I, II and III (Fig. 2). This was achieved by a fitting procedure of a theoretical lineshape to the experimental lineshape, using the analytical solution for the two-site exchange problem [14]. In the limits of slow or fast exchange the equations of McLaughlin and Leigh [13] were used.

The spin-spin (T_2) relaxation and the chemical shift of the analyzed atom or group in the two redox states must be known to simulate the lineshape [14]. T_2 was calculated from the line width. The chemical shift was determined directly from the ^1H NMR spectra. The parameters for the resonances I, II and III are summarized in Table 1. The spin-spin relaxation and the chemical shift of resonance I in the semiquinone state were determined indirectly from spectra A, B and C (Fig. 2) by extrapolation to 100% semiquinone, using the equations of McLaughlin and Leigh [13] for the limit of fast exchange.

Table 1. Chemical shift and T_2 values of the resonances I, II and III (cf. Fig. 2) of M.elsdenii flavodoxin in the three redox states

Resonance	Redox state					
	hydroquinone		semiquinone		quinone	
	δ(ppm)	T_2(ms)	δ(ppm)	T_2(ms)	δ(ppm)	T_2(ms)
I Ala-56, β-CH_3	0.11	26.5±0.2	0.05	0.6±0.2	- 0.04	26.5±0.2
II Leu-62, δ-CH_3	-0.23	26.5±0.2	-0.21	10.6±0.4	-0.23	26.5±0.2
III Leu-62, δ-CH_3	-0.65	26.5±0.2	-0.70	4.0±0.8	-0.74	26.5±0.2

Under the condition of Fig. 2 the electron transfer between the semiquinone and the hydroquinone states occurs in the limit of fast exchange and consequently only the lower limit of the second-order rate constant can be obtained. According to Eqn. 5 the electron exchange rate varies proportionally with the

flavodoxin concentration. Experiments at lower flavodoxin concentrations were therefore also conducted in order to arrive at the intermediate exchange region for which the fitting procedure allows an accurate determination of the lifetime and the rate constant. The fitting procedure was done for the resonances I and III for all experiments, yielding similar results. In situations where a relatively fast electron exchange (i.e. $\tau < 6$ ms) occurs, results were calculated from resonance I only, because its very small T_2 value (Table 1) in the semiquinone state allows the determination of the lifetime. The slower T_2 relaxation of the group due to peak III in the semiquinone state (Table 1) does not allow an accurate calculation for such short lifetimes. The exchange rates, determined under various conditions, are summarized in Table 2.

Table 2. Rate of electron exchange between the hydroquinone and semiquinone state of M.elsdenii flavodoxin under various conditions. Flavodoxin was dissolved in 20 mM Tris-HCl (pH 8.3). Lifetime τ was determined from a comparison between experimental and computer simulated spectra (cf. Material and Methods). Rate constants k_{ex} were determined using Eqn. 5. Accuracy of the rate constants is ± 20%.

Flavodoxin conc. (mM)	Ionic strength[a] (mM)	Temperature °C	Lifetime τ (ms)	Electron exchange $M^{-1}s^{-1} \times 10^5$
0.45	~ 5[a]	33	17.5	1.3
0.45	~ 5[a]	33	8.5	2.6
0.45	200	33	4.0	5.6
0.2	1000	33	<2.5	>20.0
0.25	200	8	12	3.3
0.25	200	20	9.5	4.2
0.25	200	33	6.5	6.2

[a] Ionic strength of the solutions is somewhat higher than indicated. This is partly due to the addition of dithionite (final concentration ± 1 mM) and partly due to the lyophilization procedure.

It should be noted that neither a temperature change from 10°C to 33°C nor a change in ionic strength up to 1 M KCl influences the structure of this flavodoxin, as we could not detect any change in the ^1H NMR spectra under these conditions. The rate of electron transfer between the semiquinone and the hydroquinone state is only slightly dependent on the temperature (Table 2.). In contrast the reaction is strongly dependent on the ionic strength of the solution. The exchange rate increases with increasing concentration of ionic strength.

Only an upper limit of the rate constant ($<5.7 \times 10^3 M^{-1} s^{-1}$) could be determined. for the electron transfer between the quinone and the semiquinone state. Since this upper limit was calculated under optimal experimental conditions (i.e. 8 mM flavodoxin, 1M KCl at 33°C) we had no choice to perform meaningful experiments under different experimental conditions.

The distance to the paramagnetic center can be calculated in the semiquinone state from the T_2 values of the groups due to the resonances I, II and III according to the Solomon-Bloembergen equation [19,20]:

$$\frac{1}{T_2} = \frac{1}{20} \frac{\gamma_H^2 g^2 \beta^2}{r^6} \left(4\tau_R + \frac{3\tau_R}{1+(\omega_H \tau_R)^2} + \frac{13\tau_R}{1+(\omega_S \tau_R)^2}\right) \qquad (6)$$

where γ_H is the gyromagnetic ratio of hydrogen, g the electron g-value, β the Bohr magneton, r the distance between the hydrogen atom and the free electron, ω_S and ω_H respectively the electron and hydrogen Larmor precession frequencies, τ_R the correlation time, and S the spin quantum number. The correlation time consists of contributions from the rotational correlation time τ_c and the electron relaxation time τ_s:

$$\frac{1}{\tau_R} = \frac{1}{\tau_c} + \frac{1}{\tau_s} \qquad (7)$$

The rotational correlation time of the protein is about 5 ns [7]. τ_s is about 100 ns [21] and can thus be neglected in the calculations. Only the dipolar part of the Solomon-Bloembergen equation is considered in Eqn. 6 because no electron

density of the free electron is delocalized onto the groups under consideration. The following values are calculated for the distance between the paramagnetic center and Ala-56 (peak I), 0.73 nm; Leu-62 (peak II), 1.02 nm; Leu-62 (peak III), 1.28 nm. The following distances, with respect to the N(5) atom of the isoalloxazine ring, were determined from crystallographic data of the related flavodoxin from Clostridiu MP [2]: 0.49, 1.12 and 1.28 nm, respectively. Our calculated values agree better with the crystallographic data as the distance increases. The reason for this effect probably lies in the fast rotation of the methyl groups, for which Eqn. 6 does not account. The effect of fast rotating methyl groups has been clearly demonstrated by Kalk and Berendsen [22]; the closer the methyl groups to the paramagnetic center, the more the angle of the rotating vector increases. Consequently, the influence of the fast rotation of the methyl groups to the relaxation rates in the semiquinone state increases when the distance to the paramagnetic center decreases. Furthermore, it cannot be excluded that the conformation of the active center of M.elsdenii flavodoxin differs somewhat from that of Clostridium MP flavodoxin.

DISCUSSION

The maximum electron exchange rate for the electron transfer between the semiquinone and the hydroquinone state is observed at a high salt concentration (Tab 2). Under the same conditions (I=1M, 33^{0}C) the upper limit for the electron transfer between the quinone and the semiquinone state was found to be at least 350 times slower ($k_{ex} < 5.7 \times 10^{3} M^{-1} s^{-1}$) than the electron exchange between the semiquinone and the hydroquinone state. As we have measured the flux of electrons during equilibrium and identical conditions, we conclude that this difference must be due to a difference in activation energy, i.e. the activation energy for the electron transfer between the semiquinone and the hydroquinone state is markedly lower than that for the electron transfer between the semiquinone and the quinone state. A lower limit of the difference of the activation energy for the two elec-

tron exchange reactions can be calculated from the difference of the two rate constants. This yields ΔE_a > 15 kJ/mol. The activation barrier between the semiquinone and the hydroquinone state can be calculated from the temperature dependence of the rate constant (Table 2). An increase of the reaction rate by a factor of 1.9 ± 0.4 is observed on going from 8°C to 33°C. From these results an activation energy of 18.5 kJ/mol can be calculated. However, an increase in temperature leads to a decrease in the viscosity of water and consequently to an increase in the diffusion constant by a factor of 1.85. This strongly indicates that the activation energy for the electron transfer between the semiquinone and the hydroquinone state is rather small, if not negligible. This means that the reaction is diffusion controlled and strongly suggests that we are dealing with an outer sphere reaction mechanism [23]. From the apparent absence of an activation energy it must be concluded that the rate of electron transfer is determined by the number of times that the active sites are in contact with each other.

Flavodoxin from M.elsdenii has a net charge of -17 at pH 7 as calculated from the primary structure [24,25]. It is known from three-dimensional data of the related flavodoxin from Clostridium MP that most of the net charge is concentrated near the FMN binding site [2-4]. In addition the C-terminal of a helix dipole moment points towards the prosthetic group. These facts suggest that a strong repulsion between flavodoxin molecules exists. This suggestion is supported by data of Moonen and Müller [6] showing that no apparent association occurs between flavodoxin molecules, even at a concentration of 3 mM. The strong ionic strength dependence of the exchange rate is in accordance with the latter results (Table 2) and with results of Simondsen et al. [26], who showed that the electron transfer rate between the positively charged cytochrome c and flavodoxin decreased with increasing ionic strength. At 1M KCl the repulsive forces of the charges are approximately totally diminished and under this condition only a lower limit of the exchange rate between flavodoxin molecules could be determined.

The absence of an activation barrier for the electron transfer reaction be-

tween the semiquinone and the hydroquinone state allows us to compare directly the experimentally determined exchange rate (k_{ex}) with the calculated collision frequency (k_c) in order to determine the efficiency of the reaction. Mathematically this can be expressed with the following equation:

$$k_{ex} = 0.5\, k_c \times f(D_{rot}, O_{eff}, r, p) \qquad (8)$$

where f is a function of the effective surface (O_{eff}), the distance needed for an effective transfer and the geometrical constraints for the reaction (factor p). Van Leeuwen [27] has pointed out that rotation of the reacting molecules during the encounter time increases the efficiency of the reaction (D_{rot} in Eqn. 8). This term is probably of minor importance in our case since the dissociation rate of the complex must be rather high. A lower limit for the dissociation rate, respectively an upper limit for the lifetime of the complex can be estimated neglecting the effect of charges. Assuming that equilibrium conditions exist when a certain volume contains the theoretically maximal amount of flavodoxin which is calculated to be 67 mM. Under these conditions the flavodoxin molecules are forced to interact with each other. It is then calculated [27] that the lifetime of the complex is < 1.7 ns.This value must be considerably smaller owing to the existing repulsion forces (see above). Since the rotational correlation time τ_c of the protein is 5 ns [6] it can be concluded that D_{rot} (Eqn. 8) can indeed be neglected. This conclusion is further supported by the facts that no association between flavodoxin molecules was observed [6], that the dipole moment of the molecules is situated such that repulsive forces develop on approach of the active sites (Van Leeuwen, personal communication) and that the negative charges in the flavodoxin molecule are concentrated around the active site. These latter repulsive forces are not diminished at a high salt concentration since by a close approach of the flavodoxin molecules counterions are forced out of the intersecting area. The factor 0.5 in Eqn. 8 takes into consideration that even under ideal conditions the electron might either be transferred or stay on the

same molecule. If we assume that there are no geometrical constraints for the reaction and that a very close contact is needed (i.e. $r \sim o$) we can determine the effective area of the reaction using Eqn. 9:

$$k_{ex} = 0.5\ k_c \left[\frac{O_{eff}}{O_{tot}}\right]^2 \qquad (9)$$

where O_{eff} and O_{tot} are the effective and the total surface of the protein, respectively. The power 2 originates from the fact that both active centers have to meet each other for an effective electron transfer. The collision frequency k_c can be calculated from Eqn. 10 [27] under the condition that charges can be neglected. The latter condition is sufficiently fulfilled at 1 M KCl.

$$k_c = 4\pi N_A R D x 10^3 \qquad (10)$$

In Eqn. 10 N_A is the number of Avogadro, R the sum of the radii of the two colliding flavodoxin molecules ($r_1 = r_2 = 1.8$ nm [2]), D the sum of the diffusion constants of the two flavodoxins ($D_w^{20} = 11.4 \times 10^{-7}\ cm^2 s^{-1}$ [10]) and 10^3 a scaling factor. A collision frequency of $8.7 \times 10^9 M^{-1} s^{-1}$ is calculated at 33°C from Eqn. 10. Using this value and the lower limit of k_{ex} ($2.0 \times 10^6 M^{-1} s^{-1}$) and a total surface of 4.07×10^3 Å^2 we arrive, under the condition of Eqn. 9 at an effective surface of 87 Å^2, i.e. 2.1% of the total surface. The calculated effective surface would increase if geometrical constraints for the reaction existed. If electron transfer, on the other hand, would also be possible at larger distances, the effective surface would decrease (cf. Eqn.8). The calculated value should therefore be regarded with caution. Nevertheless, we can conclude that a relative large effective surface is needed or the electron transfer is possible without a very close approach of the molecules. It should be noted that both possibilities argue against the view that the fairly exposed methyl groups at C(7) and C(8) of the prosthetic group have to be in direct contact with the electron acceptor or donor. This notion is supported by electron transfer experiments using 7-methyl-10-ribityl-isoalloxazine-5'-phosphate as a prosthetic group. M.elsdenii

flavodoxin reconstituted with this flavin yielded identical electron transfer rates (data not shown) to those obtained with the native protein. This result seems to be in contradiction with the conclusion of Simondsen and Tollin [9] that the dimethylbenzene subnucleus is directly involved in the electron transfer reaction. This conclusion was mainly based on kinetic experiments using 8-chloro substituted flavins. However, such substitutions might introduce an activation barrier in the redox transitions. Another possibility is that the chloro substituted flavins have less orbital overlap with the acceptor or donor. Moreover, the vibrational modes of flavin are strongly altered upon this chlorine substitution [28]. But sufficient orbital overlap is a condition in an outer sphere mechanism following Franck-Condon principles [23].

The rate constants of the electron transfer reactions for M.elsdenii flavodoxin, determined under equilibrium conditions, are in excellent agreement with kinetic studies by Mayhew and Massey [29] , using stopped flow techniques. Owing to the high reactivity of the semiquinone of M.elsdenii flavodoxin towards dithionite the rate of the reduction of the semiquinone form could not be determined, but it was estimated that the semiquinone is about 450 times more reactive than oxidized flavodoxin. The rate constant for the comproportionation reaction, i.e. the reaction between flavodoxin molecules in the oxidized and reduced state to form semiquinone, was determined to be 2.3×10^3 $M^{-1}s^{-1}$ (I=0.16 M, 25^oC). Considering the different reaction conditions this value is in excellent agreement with our maximum value of $5.7 \times 10^3 M^{-1}s^{-1}$ (I=1.0 M, 33^oC) for the electron exchange reaction between molecules in the oxidized and the semiquinone state. This means that the comproportionation reaction is solely governed by the rate of the one-electron reduction of oxidized flavodoxin, i.e. the activation energy of the transition from the oxidized to the semiquinone state.

The conformation and the conformational stability of flavodoxin in the three redox states [2-7] can also be rationalized by an outer sphere reaction mechanism. In an outer sphere reaction mechanism the electron is transferred from one

molecule to the other, while no nuclear rearrangement occurs according to the Franck-Condon principles. Thus the first step will be an electron transfer from the HOMO of the reduced state to the LUMO of the semiquinone state. The next (much slower) step is the electronic relaxation, accompanied by nuclear rearrangement to the newly formed hydroquinone and semiquinone state. It is well known that during the first step (the electron transfer) the polarity of both reactants are considerably changed. As a consequence polar residues and especially solvent water have to rearrange and, therefore, a high activation energy can be introduced. This is the case for the transition quinone-semiquinone, where a conformational change was observed in the FMN binding site, using crystallographic methods [3]. In the semiquinone and hydroquinone states however, the conformation is identical. Moreover, the very tight package of the active center of flavodoxin [5-7] shields the flavin ring from solvent water. Thus nuclear rearrangement is considerably hampered. This rationalizes the tight and identical structures of the semiquinone and the hydroquinone states, whereas the introduction of a considerable nuclear rearrangement during the quinone/semiquinone transition inhibits the involvement of the oxidized state in the electron transfer. This mechanism probably renders flavodoxin from M.elsdenii to an exclusive one-electron transferring protein. Furthermore, our results place some doubt on the suggestion that specific complex formation is needed between donor and acceptor. We have given evidence in this paper, that considerable transfer rates can be observed without any specific complex formation. Specific complex formation might of course enhance the transfer rate but it seems not to be a prerequisite for the electron transfer reaction.

It should be noted that electron carriers as flavodoxins and ferredoxins are often interchangeable, although structures and chromophores are quite different. Mayhew and Ludwig [1] explained the interchangeability as an apparent lack of recognition in the electron transfer reactions. This explanation fits well with our suggestion, that tight and specific complex formation is not a

necessary condition for the electron transfer. We suggest, that this is especially valid when a negligible activation barrier exists for the involved redox transitions. If a high barrier exists, than specific complex formation can be necessary in order to overcome the barrier.

From our data it is likely that the difference in activation energy is the control mechanism in the living cell for the preference for the shuttle between the hydroquinone and the semiquinone state above that between the semiquinone and the quinone state. In this respect it is interesting to note that M.elsdenii flavodoxin is an inducible protein which replaces ferredoxin in the absence of iron [1]. Ferredoxin transfers one electron with a midpoint potential of -375 mV [30]. The redox potential of the semiquinone/hydroquinone couple of flavodoxin is -375 mV whereas the midpoint redox potential of the quinone/semiquinone couple is -115 mV [1,30]. From the comparison of these redox potentials and the interchangeability of the two proteins it has already been deduced [1] that the function of M.elsdenii flavodoxin is likely to shuttle between the hydroquinone and the semiquinone state. In this study we have found strong support for this suggestion. It is questionable, however, if this feature is common to all flavodoxins as some flavodoxins are constitutive. Thus no clear interchangeability with a one-electron transferring protein exists for the other class of flavodoxins. Thus it cannot be excluded that these flavodoxins can also act as two electron donors or acceptors. This aspect is currently under study in our laboratory.

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Chapter 8

A ^{13}C and ^{15}N Nuclear Magnetic Resonance Study on The Interaction between Riboflavin and Riboflavin-Binding Apoprotein

Chrit T.W. Moonen, Willy A.M. van den Berg, Marleen Boerjan and Franz Müller

ABSTRACT

Riboflavin-binding apoprotein from egg yolk and egg white have been reconstituted with ^{15}N and ^{13}C enriched riboflavin derivatives. These protein preparations were investigated in the oxidized and two-electron reduced state by ^{15}N and ^{13}C NMR techniques. The chemical shift values of the protein-bound flavin are compared to those of free flavin in water or chloroform. The results are interpreted in terms of interactions between the apoprotein and its prosthetic group and the conformation of bound flavin.

In the oxidized and in the reduced state the chemical shifts and line broadening effects of the bound riboflavin are extremely similar for the two different proteins indicating similar binding sites for riboflavin. The solvent accessibility to the bound riboflavin is similar in the reduced and the oxidized complexes, i.e. the N(3), O(2α) and N(1) atoms are exposed, whereas the O(4α) atom is shielded from solvent water.

A hydrogen bond to the N(5) atom exists in the oxidized state. This hydrogen bond is weaker than the corresponding one between free FMN and water. At best only a weak hydrogen bond exists to the O(4α) atom. Binding of oxidized riboflavin forces the N(10) atom of flavin into the molecular plane. The isoalloxazine ring is strongly polarized.

No hydrogen bond to the O(4α) atom is formed in the reduced neutral state of the complex. Although the N(1) atom is accessible to solvent water, it possesses more sp^3 character than in free $FMNH_2$. The data also suggest a rapid motion of the N(1) atom in and out of the molecular plane. The reason for these effects is probably the absence of a hydrogen bond to the O(4α) atom which makes π electron delocalization from the N(1) atom unfavourable. The N(10) atom exhibits about the same configuration as in the oxidized state. In contrast the N(5) atom shows increased sp^3 hybridization.

The pK value of the deprotonation of the N(1)H group is measured as 7.45, a shift of about 0.8 pH unit to more alkaline pH values as compared to that of free $FMNH_2$. This pK_a shift might be caused by the absence of a hydrogen bond to the O(4α) atom. In the anionic reduced state the N(1) atom shows even more sp^3 character than in the neutral complex, which is explained by the absence of a

hydrogen bond to the O(4α) atom. The ^{15}N resonance line due to the N(1) atom is severely broadened, probably due to its motion in and out of the molecular plane. The N(5) atom shows almost the same ^{15}N chemical shift as in the neutral complex indicating an increased sp^3 character. This is confirmed by the coupling constant of 78 ± 5 Hz with the covalently bound hydrogen atom. No coupling was detected in the neutral complex due to rapid hydrogen exchange with solvent water. These results show that the N(5) atom is shielded from solvent at high pH values. The N(10) atom exhibits considerable sp^2 character in the anionic complex.

The results are discussed in the light of the possible function of the protein.

INTRODUCTION

Riboflavin-binding protein (RBP) is found in the yolks and whites of all avian eggs (Osuga and Feeney, 1968). The function of the protein is still not fully understood, although it is known, that it mediates the transport of riboflavin from the maternal system to the developing oocytes, where riboflavin is essential for embryonic growth and development (Winter et al., 1967; Ostrowski et al., 1968). It has also been suggested that RBP in egg whites functions as an inhibitor of bacterial growth (Board and Fuller, 1974).

As flavodoxins (Mayhew and Ludwig, 1975) RBP is often used as a model system for flavin-protein interactions. The easy availability of large quantities of these proteins makes them especially suitable for a study of the flavin-apoflavoprotein interactions. The importance of these interactions in flavin biochemistry is obvious since it has been suggested that specific interactions in flavin-apoflavoprotein complexes are responsible for the "tuning" of the flavoprotein for its specific catalysis (Müller, 1972; Eweg et al., 1982).

Several physicochemical studies have been reported on RBP and its interaction with riboflavin. It has been shown that RBP from egg yolk (RBPY) resembles RBP from egg white (RBPW) with respect to the thermodynamics of riboflavin binding (Matsui et al., 1982a, 1982b). The two proteins have roughly the same

molecular weight (Ostrowski and Krawczyk, 1963; Blankenhorn, 1978; Froehlich et al., 1980), the same amino acid composition and flavin-protein association constants (Farrell et al., 1969; Ostrowski and Krawczyk, 1963; Steczko and Ostrowski, 1975), and are immunologically indistinguishable (Farrell et al., 1970). They differ however with respect to the content of sialic acid (Jacubczak et al., 1968; Kawabata and Kanamori, 1968). In addition Matsui et al. (1982a and 1982b) have shown that the volume of the cavity for the binding site of riboflavin differs for the two proteins. This has however no influence on the kinetics of the association between flavin and apoprotein, as shown by Nishina (1977).

The use of riboflavin analogues provided new insights into the relative importance of the different atoms of the isoalloxazine ring for the binding by RBPY and RBPW (Matsui et al., 1982a and 1982b; Choi and McCormick, 1980; Becvar and Palmer, 1982; Nishikimi and Kyogoku, 1973). These studies resulted in the conclusion that the dimethylbenzene ring of flavin is buried in the interior of the protein, i.e. bound differently than in flavodoxins (Mayhew and Ludwig, 1975). Thermodynamic studies on RBPW support this conclusion (Matsui et al., 1982a). In addition it was shown by chemical modification studies that a tryptophan and a tyrosine residue are involved in the binding of riboflavin by RBPW (Blankenhorn, 1978) and probably also by RBPY (Steczko and Ostrowski, 1975). Kumosinski et al. (1982) suggested for RBPW that an aromatic-rich cleft opens at pH values $3.7<pH<7.0$ and concommitantly releases the riboflavin.

The ^{13}C and ^{15}N NMR techniques are powerful methods for detecting subtle differences in hydrogen bonding and conformation of the flavin in both the oxidized and the reduced state (Moonen et al., 1984) and for elucidation of dynamic processes (Moonen and Müller, 1983). These methods have successfully been applied, using specifically ^{13}C and ^{15}N enriched flavins, to flavodoxins from Megasphaera elsdenii and Azotobacter vinelandii (Van Schagen and Müller, 1981; Franken et al., 1983), where specific hydrogen bonding was detected. Some ^{13}C NMR experiments on oxidized RBPW were carried out by Yagi et al. (1976). No

theory for the interpretation of the data was developed at that time. We now report on a detailed study on the complex between RBPY and riboflavin, and RBPW and riboflavin using specifically labelled ^{13}C and ^{15}N riboflavin derivatives. Several specific interactions are detected in both the oxidized and the reduced systems. In addition novel information is revealed concerning the hydrogen exchange of hydrogen-carrying nitrogen atoms, which show an unexpectedly strong dependence on pH. Dynamic information about the riboflavin binding site is deduced from these results.

MATERIALS AND METHODS

Apo-RBPY was isolated and purified according to Murthy et al. (1979) with the exception that an Amicon concentrator was used instead of aquacide treatment. Apo-RBPW was isolated and purified according to Farrell et al. (1969). Specific ^{13}C enrichment of riboflavin was performed according to Van Schagen and Müller (1981). Specific ^{15}N enrichment was performed as described by Müller et al. (1983).

The relative concentrations of riboflavin-binding apoprotein and riboflavin was 1:1 in all samples. These were prepared as follows. Reconstitution of riboflavin-binding apoprotein with riboflavin was carried out with 0.1 mM apoprotein and an excess of riboflavin in 50 mM potassium phosphate (pH 7.0). Excess of riboflavin was removed by exhaustive dialysis against the same buffer. In the final dialysis the buffer concentration was decreased to 1 mM. The protein-riboflavin complex was then concentrated by lyophilization.

Wilmad 10 mm precision NMR tubes were used. The samples contained 1-3 mM RBP, 100 mM potassium phosphate buffer of varying pH (see Results and Discussion). Depending on the kind of experiment, the samples contained 10% or 100% $^{2}H_2O$. The sample volume was 1.6 ml. The pH measurements were carried out before and after the NMR measurements and are reported without correction for the deuteron isotope effect. Reduction was conducted by addition of the desired amount of a dithionite solution to the anaerobic solution of RBP. Anaerobiosis

was achieved by carefully flushing the solutions in the NMR tube with argon for about 20 min. The NMR tube was sealed with a serum cap.

All measurements were performed on a Bruker CXP 300 spectrometer operating at 30.4 MHz for ^{15}N and 75.6 MHz for ^{13}C NMR experiments. Broadband decoupling of only 0.5 Watt was applied for ^{13}C measurements to prevent heating up of the sample. There was no need for a higher decoupling power for ^{13}C NMR as we were mainly interested in quaternary carbon atoms. No decoupling was used for ^{15}N NMR measurements in order to be able to observe the exchange rate of the NH protons. Dioxane (3 µl) was used as an internal reference for ^{13}C NMR. Chemical shift values are reported relative to TMS ($\delta_{dioxan}-\delta_{TMS}$ = 67.84 ppm). Neat [^{15}N]CH_3NO_2 was used as an external reference for ^{15}N NMR using a coaxial cylindrical capillary as recommended by Witanowski et al. (1981). Chemical shift values are reported relative to liquid NH_3 at 25°C as recommended by Levy and Lichter (1979) ($\delta_{CH_3NO_2}-\delta_{NH_3}$ = 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 as described by Witanowski et al. (1981). The accuracy of the reported values is ±0.1 ppm for ^{13}C and ±0.3 ppm for ^{15}N chemical shift values, unless otherwise stated. The temperature of all samples was kept constant at 26±2°C. All spectra were recorded using 30° pulses and a repetition time of 0.5-1 s.

RESULTS AND DISCUSSION

Studies on the oxidized state

The dissociation constant of RBPW and RBPY with riboflavin is about 50 nM (Choi and McCormick, 1980; Zak et al., 1972). For the samples used in this study it is calculated that only about 0.1% riboflavin is free in solution. Consequently the reported data are not distorted by free riboflavin. Spectrum A of Figure 1 shows the low field part of the ^{13}C NMR spectrum of RBPY complexed with riboflavin. The possible interference with protein resonances for the determination of the chemical shift values of bound riboflavin was elimi-

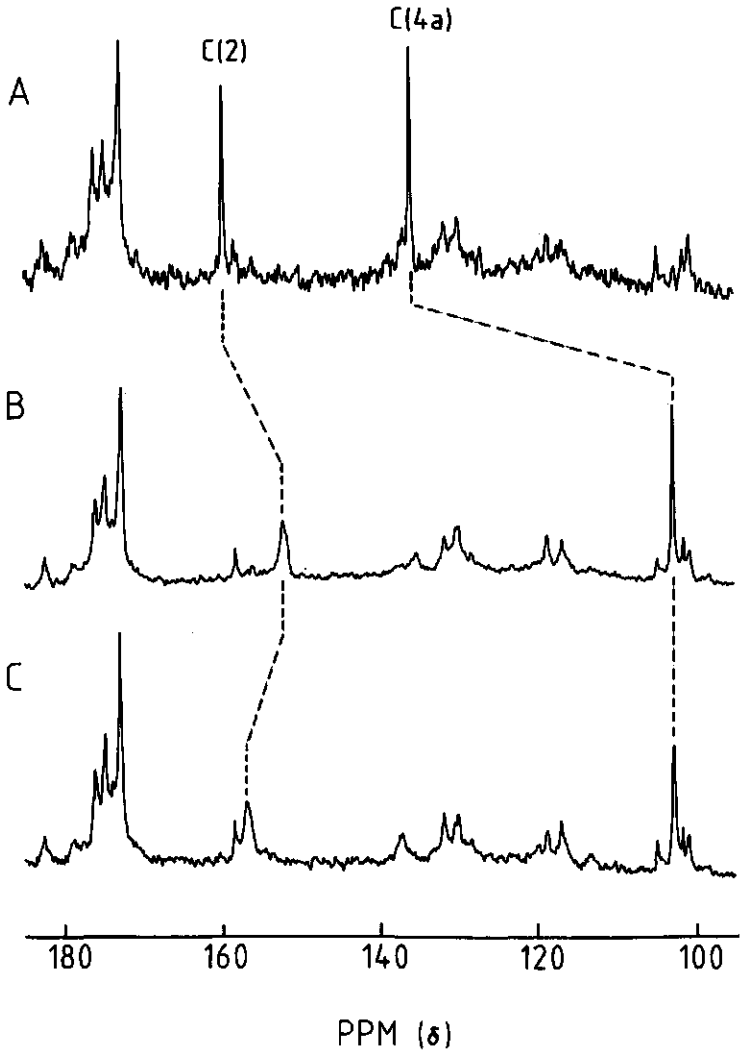


Figure 1: ^{13}C NMR spectra of riboflavin-binding apoprotein from egg yolk reconstituted with [2,4a-$^{13}C_2$]riboflavin.
Spectrum A: oxidized protein, in 100 mM potassium phosphate, pH 8.5.
Spectrum B and C: two-electron reduced protein in 100 mM potassium phosphate, pH 6.0 and in 100 mM sodium borate, pH 9.0, respectively.

nated by the use of difference spectra (data not shown). The resonances due to natural abundance ^{13}C of the protein in the spectra can partly be assigned according to Allerhand (1979) and Gratwohl and Wüthrich (1974). All peptide carbonyl and carboxyl resonances appear between 170 and 180 ppm. The Arg C ζ atoms are

found at 158.2 ppm. Aromatic carbon atoms of Trp, Tyr, Phe and His resonate between 110 and 140 ppm. An interesting region lies between 95 and 105 ppm where normally no carbon atoms of amino acids resonate. However several natural abundance resonances of RBPY are found in this region. As already pointed out in the introduction, RBPY contains sialic acid. The C-1 atoms of some carbohydrate components of sialic acid appear in the region between 95 and 105 ppm. The resonance position should in principle allow a rough determination of the different stereoisomers (Bock and Thogerson, 1982). In addition, several lines due to other carbohydrate residues were observed in the spectral region between 60 en 80 ppm (data not shown). It should be mentioned that these regions differ between RBPY and RBPW, as would be expected from the difference in sialic acid content. However, these spectral regions are not further considered here as this lies beyond the scope of this study.

The flavin ^{13}C chemical shifts for the complexes of RBPY and RBPW are summarized in Table I. Spectrum A of Figure 2 shows a ^{15}N NMR spectrum of ^{15}N enriched riboflavin bound to RBPW. Only small natural abundance resonances near 120 ppm are observed which are due to peptide nitrogens (Levy and Lichter, 1979). The ^{15}N chemical shifts are also summarized in Table I. The structure of the flavin labeled at all four nitrogen atoms with ^{15}N is given in Figure 3.

In the accompanying paper (Moonen et al., 1984) we have shown that the ^{13}C and ^{15}N chemical shifts are very sensitive to hydrogen bonding, conformation of the flavin and the polarity of the solvent used. The extreme situations of strong hydrogen bonds and high polarity around the isoalloxazine ring (e.g. FMN in water) as against no hydrogen bonds and low polarity (e.g. TARF in chloroform), are also listed in Table I in order to facilitate the analysis.

A rough comparison between the data of riboflavin bound to RBPY and RBPW shows (Table I) that the chemical shifts for the two proteins hardly differ. The differences are all within the experimental accuracy of the data. This leads to the conclusion that the binding site for the oxidized isoalloxazine ring must be very similar in the two proteins. Consequently we can discuss the interactions for both complexes together.

Table I. Carbon-13 and nitrogen-15 chemical shifts of oxidized riboflavin bound to riboflavin-binding apoprotein from egg yolk (RBPY) and from egg white (RBPW). Linewidth of ^{13}C resonances is 10±4 Hz and that of ^{15}N resonances 15±5 Hz, unless otherwise stated. For comparison also ^{13}C and ^{15}N chemical shifts of FMN in water and of TARF in C^2HCl_3 are included.

Compound	Solvent	Chemical shifts in ppm			
		Carbon Atoms			
		C(2)	C(4)	C(4a)	C(10a)
RBPW[a)]	pH 8.5	159.7	162.1	135.9	152.3
RBPY[a)]	pH 8.5	159.6	162.0	135.9	152.4
FMN[b,c)]	pH 8.0	159.8	163.7	136.2	152.1
TARF	C^2HCl_3	155.2	159.8	135.5	149.1
		Nitrogen Atoms			
		N(1)	N(3)	N(5)	N(10)
RBPW[a,d)]	pH 9.0	191.5	159.2	338.2	165.4
RBPW[d,e)]	pH 6.4	191.5	158.5[f)]	337.7[g)]	165.1
RBPY[a,d)]	pH 6.2	191.6	159.4[f)]	338.2[g)]	165.3
MeIMN[b,c,d)]	pH 8.0	190.5	160.4	335.5	164.6
MeTARI[d)]	C^2HCl_3	201.1	160.7	346.7	150.4

a) Solvent is 90% H_2O, 10% ^2H_2O.

b) FMN or MeIMN were used for the comparison because of their higher solubility in H_2O than riboflavin analogues

c) Independent of pH in the range $5<pH<9$

d) See Figure 3 for structure of the flavin

e) Solvent is 100% ^2H_2O

f) The resonance signal of $^{15}N(3)$ is broadened at a pH value of about 6, linewidth ~40 Hz. Accuracy of the chemical shift given is ±1 ppm (See text).

g) Linewidth is about 30 Hz.

Contrary to ^{1}H NMR where ring current effects of neighbouring aromatic amino acids influence chemical shifts such effects are of relative negligible importance in ^{13}C and ^{15}N NMR. Therefore, the data in Table I can be analyzed directly in terms of hydrogen bonding, specific interactions and conformation of the flavin in the complexes following the semi-empirical rules of the accompanying paper (Moonen et al., 1984).

In the oxidized state the $^{15}N(3)$ atom in riboflavin bound to the two proteins is the only nitrogen atom carrying a covalently bound hydrogen atom. A $^{1}J_{^{15}N(3)-^{1}H}$ of 92 Hz is expected (Franken et al., 1983). No splitting of the ^{15}N signal due to the N(3) atom of flavin is observed on use of H_2O as a solvent. This indicates that the exchange rate of the proton is high (lifetime $\tau << \frac{1}{^{1}J}$, i.e. τ <<10 ms). This result strongly suggests that the N(3) atom is exposed to solvent and also explains why RBP can be easily purified using riboflavin coupled via the N(3) position to a matrix (Blankenhorn et al., 1975). Choi and McCormick (1980) arrived at the same conclusion by studying the interaction between RBP and flavin analogues.

The resonance position of C(2) is almost the same as that of FMN in water. This suggests that O(2α) is also exposed to solvent water, besides N(3), again in accordance with the binding studies of flavin analogues. The resonance position of C(4) clearly shows that the hydrogen bond to O(4α) is considerably weaker than in free FMN. The upfield shift of N(3) is probably a consequence of the weak hydrogen bond to O(4α), as by approximation the N(3)H-C(4)-O(4α) group in oxidized flavin can be regarded as a "peptide bond". It is known that the nitrogen resonance of peptides is mainly governed by the hydrogen bond to the carbonyl function (Witanowski et al., 1981). The resonance position of N(1) indicates that a strong hydrogen bond is formed to the N(1) atom of bound riboflavin. This hydrogen bond is comparable to that of FMN in water. Thus, with regard to hydrogen bonding and solvent exposure, the data indicate that N(3), O(2α) and N(1) are exposed to solvent, whereas N(5) exhibits a somewhat weaker hydrogen bond as compared with free FMN.

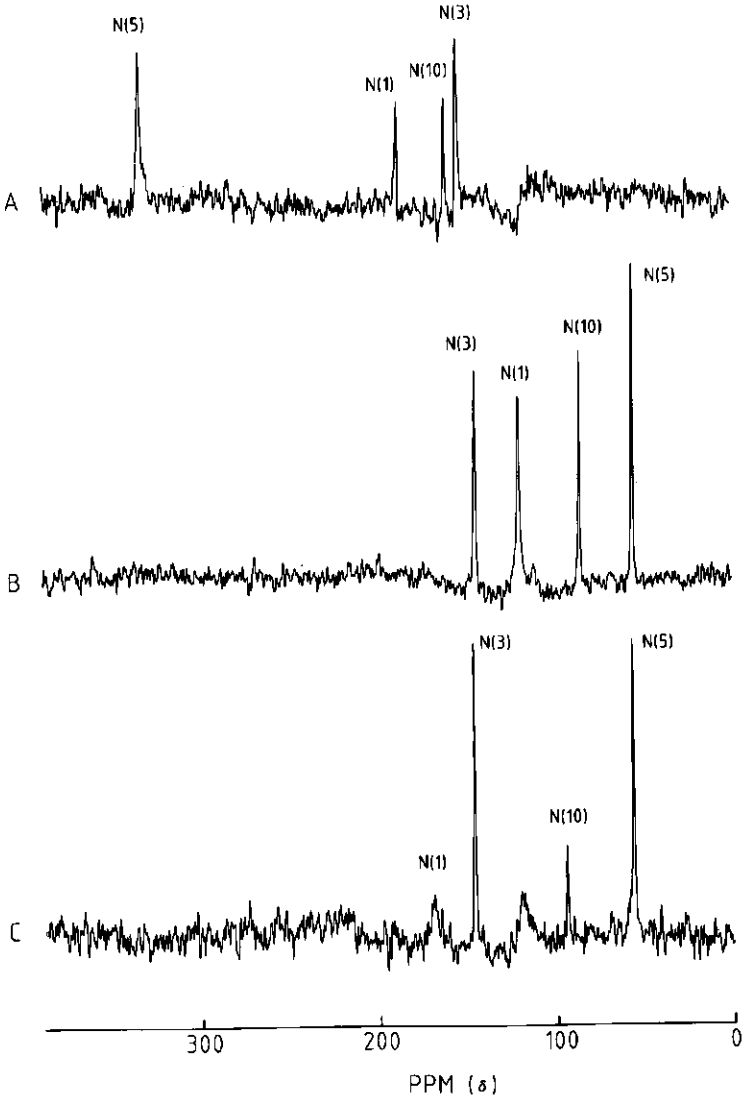
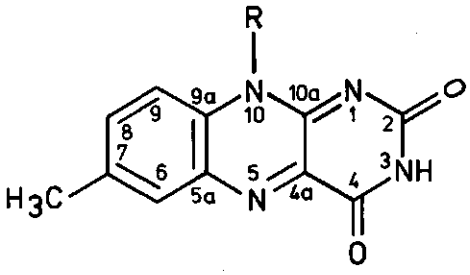


Figure 2: ^{15}N NMR spectra of riboflavin-binding apoprotein from egg white reconstituted with [1,3,5,10-$^{15}N_4$]7-methyl-10-ribityl-isoalloxazine. Spectrum A: oxidized protein in 100 mM potassium phosphate, pH 6.4. Spectrum B and C: two-electron reduced protein in 100 mM potassium phosphate, pH 6.3 and 100 mM sodium borate, pH 9.0, respectively.

It has been observed that the pK_a value of the deprotonation of N(3)H of protein-bound riboflavin is increased (Miura et al., 1983) as compared to that of free riboflavin. It is suggested that the weak hydrogen bond to O(4α) of the

protein-bound riboflavin is responsible for the increased pK_a value.

The chemical shifts due to N(10) and C(10a) can be used as monitors with regard to the polarization of the isoalloxazine ring and the configuration of the N(10) atom. The N(10) resonance position is shifted even further downfield than in free FMN. The same holds for C(10a), although to a lesser extent. This clearly demonstrates that N(10) is forced into the molecular plane and the isoalloxazine ring is strongly polarized. These results suggest that riboflavin interacts strongly with the apoprotein and that the neighborhood of the N(10) atom and the N(10) side chain of riboflavin are strongly involved in the interaction. This interpretation is in accord with binding studies of flavin analogues (Choi and McCormick, 1980).



a: $R=CH_2-(CHOH)_3-CH_2OPO_3H_2$

b: $R=CH_2-(CHOCOCH_3)_3-CH_2OCOCH_3$

Figure 3: Structure of flavin derivatives enriched with ^{15}N at positions 1,3,5, and 10. R = ribityl, tetraacetylribityl and ribityl-5'-monophosphate.

The ^{15}N NMR data at lower pH values show that polarization or hydrogen bonding is hardly altered. The resonance line of N(3) is broadened however (linewidth ~20 Hz) at pH 6.2 compared to that at higher pH values. This broadening is absent if ^2H_2O is used as the solvent. This strongly indicates that we are dealing with a hydrogen exchange reaction, which is slower at low than at high pH values, in agreement with base catalyzed hydrogen exchange.

Studies on the two-electron reduced state

The dissociaton constant of RBPY with reduced riboflavin is about 1 μM (Scheller et al., 1979). Consequently, our samples investigated contain about 2% riboflavin free in solution. The dissociation constant of RBPW with riboflavin is about 1000 times higher in the reduced than in the oxidized state (Blankenhorn, 1978), i.e. 8% of free riboflavin is calculated to be present in our samples. Our chemical shift data will therefore mainly reflect the complex between RBP and riboflavin. Spectra B and C of Figure 1 show ^{13}C NMR spectra for RBPY at different pH values. Spectra B and C of Figure 2 show ^{15}N NMR spectra of RBPW. The chemical shift values are summarized in Table II. The data for $FMNH_2$ and $FMNH^-$ in water, and $TARFH_2$ in chloroform (cf. also accompanying paper, Moonen et al., 1984) are included for comparison.

A comparison of the data for RBPY and RBPW shows that in the reduced state the differences in the chemical shift values are also within the experimental error. From this we conclude, in agreement with the results on the oxidized state, that the binding site for reduced riboflavin is similar in RBPY and RBPW. Again our analysis concerns consequently both proteins.

In reduced free FMN the deprotonation of N(1)H occurs with a pK_a of about 6.6 (Dudley et al., 1964). Using ^{13}C (Van Schagen and Müller, 1981) and ^{15}N NMR (Franken et al., 1983) it has been shown that the chemical shifts due to $^{15}N(1)$, $^{13}C(2)$ and $^{13}C(10a)$ reflect this deprotonation clearly. Figure 4 shows the dependence of the chemical shift on the pH for RBPW complexed with [2-^{13}C]-riboflavin. The curve and the extreme chemical shift at low and high pH values are identical with those of reduced FMN in the same buffer, but the pK_a value is shifted from ∿6.6 to 7.45±0.05. The pK_a value for RBPY was determined as 7.35±0.05. This supports our conclusion drawn above that the binding sites in the two proteins must be very similar. An explanation for the increased pK_a values for the complexes as compared with reduced free FMN will be offered below.

The chemical shift of C(2) in the protein-bound, neutral 1,5-dihydroriboflavin is identical to that of $FMNH_2$, suggesting that O(2α) interacts with

Table II. Carbon-13 and Nitrogen-15 chemical shifts of reduced riboflavin bound to RBPY or RBPW. For comparison the values for $FMNH_2$ in water and $TARFH_2$ in chloroform are also included. Linewidth of ^{13}C resonances is 10±4 Hz and that of ^{15}N resonances 13±5 Hz, unless otherwise stated.

Compound	Solvent	Chemical shifts in ppm			
		Carbon Atoms			
		C(2)	C(4)	C(4a)	C(10a)
RBPW[a)]	pH 6.0	151.1	156.5	102.8	143.9[h)]
RBPY[a)]	pH 6.0	151.1	156.6	102.8	144.2[h)]
$FMNH_2$[b)]	pH 5.0	151.1	158.3	102.8	144.0
$TARFH_2$	C^2HCl_3	150.6	157.0	105.1	137.1
RBPW[a)]	pH 9.0	158.1	156.8	102.8[h)]	155.0
RBPY[a)]	pH 9.0	158.0	157.0	102.4[h)]	155.0
$FMNH^-$[b)]	pH 9.0	158.2	157.7	101.4	155.5
		Nitrogen Atoms			
		N(1)	N(3)	N(5)	N(10)
RBPW[d,e)]	pH 5.5	123.5[j)]	148.3	59.1	89.9
RBPW[a,d)]	pH 6.3	128.5[k)]	148.2[l)]	59.8[l)]	89.9
RBPY[a,d)]	pH 6.3	129.8[k)]	148.9[l)]	59.9[l)]	90.3
$MeIMNH_2$[b,d)]	pH 5.0	128.1	150.7	60.6	87.3
$MeTARIH_2$	C^2HCl_3	116.7	145.8	60.7	72.2
RBPW[a,d)]	pH 9.0	176.0[m)]	150.3	60.1[n)]	97.7
$MeIMNH^-$[b,d)]	pH 9.0	186.9	150.7	60.6	97.2

For superscripts a-e, see Table I.

h) Linewidth ∿70 Hz, precision of chemical shift is ±0.6 ppm.

j) Linewidth ∿40 Hz, precision of chemical shift is ±0.4 ppm.

k) Linewidth ∿80 Hz, precision of chemical shift is ±1.0 ppm.

l) Linewdith ∿50 Hz, precision of chemical shift is ±0.8 ppm.

m) Linewdith ∿200 Hz, precision of chemical shift is ±2.0 ppm.

n) Doublet with a coupling constant of 78±5 Hz.

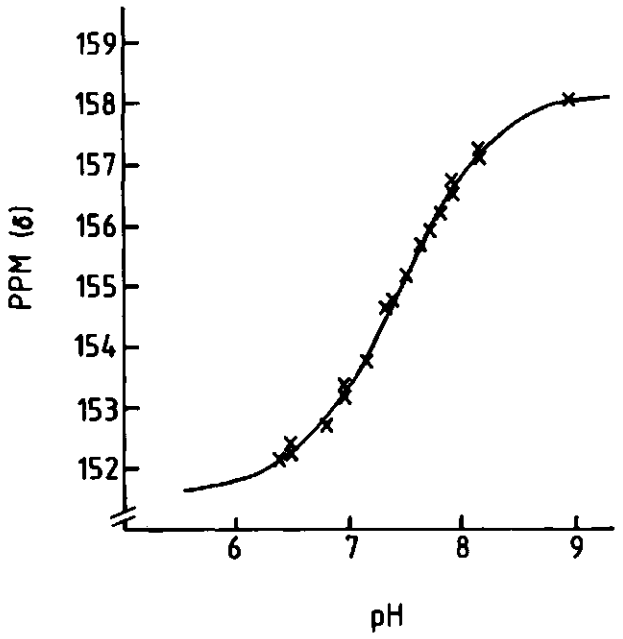


Figure 4: The pH-dependence of the ^{13}C chemical shift of [2-^{13}C]1,5-dihydroriboflavin bound to riboflavin-binding apoprotein from egg white. The solid curve has been calculated using a pK_a value of 7.45.

solvent water. No coupling could be observed with H_2O as a solvent for the ^{15}N resonance of the N(3)H group. This indicates the exposure of this site to the solvent. The chemical shift of N(3) however, resembles more that of $TARFH_2$ than that of $FMNH_2$. As in the oxidized molecule a hydrogen bond to O(4α) is absent, clearly demonstrated by the upfield position of C(4). The situation is more complex with regard to the N(1) atom. The fact that a good titration curve for the deprotonation of N(1)H could be obtained demonstrates that the protonation/deprotonation reaction is faster than the chemical shift separation of C(2) in the two states (Binsch, 1975), which is about 500 Hz. Moreover, no sharp resonance line for N(1) could be observed in H_2O indicating hydrogen exchange with the solvent being faster than that expected on the one-bond NH coupling constant. These data clearly indicate exposure of N(1) to solvent water. On the other hand the chemical shift correlates better with that of $TARFH_2$ suggesting a weak ex-

posure to solvent water. This apparent discrepancy is explained as follows. The accompanying paper (Moonen et al., 1984) shows that N(1) in $TARFH_2$ is not of 100% sp^2 character and that the sp^2 character of the N(1) and the N(10) atom is increased on polarization of the flavin. Although the N(10) atom thus possesses considerable sp^2 character in the complex, as illustrated by its chemical shift, the N(1) atom shows more sp^3 character than in $FMNH_2$. Steric hindrance by the apo-RBP could explain this observation, but this explanation is not in agreement with the easy accessibility to solvent water. It is therefore suggested that the absence of a hydrogen bond to O(4α) causes the observed effect. As O(4α) is an unfavourable π electron acceptor in the absence of a hydrogen bond, the delocalization of the π electrons of N(10) and N(1) is less favoured. The N(10) atom is likely forced into the molecular plane by the apoprotein, which makes sp^2 hybridization of the N(1) atom unlikely. This could explain why the resonance lines due to N(1) and also C(10a) are broadened considerably at about pH 6.0 (Table II). This broadening and the other data suggest, that the N(1) atom in RBP moves in and out of the molecular plane. The C(10a) atom is probably also influenced by this motion. At pH 5.5 the linewidth of the N(1) resonance line decreases to about 50 Hz (Table II), indicating that the frequency of this motion is increased. The latter result is compatible with the observed swelling of the protein at low pH values (Kumosinski et al., 1982). The ^{19}F NMR data of Miura et al. (1983) can be similarly explained. The N(5) atom is also fairly exposed to solvent as no coupling constant could be detected in H_2O.

In the reduced state the degree of bending around the N(5)-N(10) axis (i.e. the "butterfly" conformation) is expressed directly by the N(5) and N(10) resonances and indirectly by the C(10a) and C(4a) resonances which are under the influence of the π electron donating character of the N(5) and N(10) atoms (Moonen et al., 1984). The N(10) atom resonates at about 2 ppm downfield from that of $FMNH_2$, whereas the N(5) atom is shifted upfield. These results indicate strongly that the bending is more pronounced at the N(5) than the N(10) position, as compared to the conformation of free $FMNH_2$. The C(10a) atom resonates at a position comparable with that in $FMNH_2$, which supports this conclusion. The

absence of a hydrogen bond to O(4α) disfavours π electron delocalizaton from N(10) and N(1) to the O(4α) position. An increase in the π electron density is therefore expected at C(4a). Since the N(1) atom is less sp^2 hybridized than the N(10) atom however, the cooperative π electron delocalization of the two nitrogen atoms is decreased as compared to that in $FMNH_2$. The overall apparent effect is that the resonance due to C(4a) seems to be unaffected.

The conclusions remain roughly the same with regard to the anionic 1,5-dihydroriboflavin bound to the protein as given for the neutral reduced form. The chemical shift of C(2) and the absence of a coupling constant for N(3)H again indicate the exposure of these centers to solvent water. The resonance position of C(4) indicates the absence of a hydrogen bond. The upfield shift of the resonance due to N(1), as compared with $FMNH^-$, indicates that the sp^2 character of this atom is further decreased as compared to that at the lower pH values. The considerable linewidth even suggests that the N(1) atom rapidly changes its configuration (i.e. configurational averaging). The broadened resonance of C(4a) might be caused by the configuration averaging of N(1). The results further indicate that the configuration of the N(5) and the N(10) atoms are roughly the same as in the neutral protein-bound flavin molecule. The small downfield shift of the resonance due to C(4a) as compared with $FMNH^-$ supports this interpretation and indicates that π electron delocalization from the N(1) atom is indeed decreased. It can be concluded from these results that most features of the bound neutral reduced riboflavin are maintained in the negatively charged system. There are two exceptions to this conclusion; i) the sp^3 hybridization of the N(1) atom is increased; ii) the N(5)H group shows a coupling constant of 78±5 Hz. The magnitude of this coupling constant clearly shows that the N(5) atom possesses increased sp^3 character, already concluded from its resonance position. The fact that the coupling constant could only be observed at high pH values indicates that the protein undergoes a conformational change when the pH of the solution is increased. This change in conformation shields N(5)H to solvent water at high pH. The result is compatible with a swelling of the protein at low pH and a shrinking at high pH, as proposed recently by Kumosinski et al. (1982).

We return now to the point of the higher pK_a value for the deprotonation of N(1) as compared with reduced free riboflavin. The apparent destabilization of the negative charge on N(1) might be due to a negative charge in the vicinity of N(1) in the complex. Another more likely possibility is the absence of a hydrogen bond to O(4α). As we have seen in the accompanying paper (Moonen et al., 1984), the structure of the flavin (especially N(10)) changes upon deprotonation in such a way that the π electron delocalization of N(1) is also favoured. As has been indicated the C(4a) and O(4α) are important centers for this delocalization. The delocalization to O(4α) is not favoured in the complex, which probably influences the degree of hybridization of N(1) and thus renders the deprotonation unfavourable.

The apparently decreased sp^2 character of N(1) in the reduced state might explain the weaker binding of reduced as compared with oxidized riboflavin. However, at the moment we cannot quantify this effect.

This study has demonstrated that detailed information can be obtained from flavoproteins using ^{13}C and ^{15}N NMR techniques and isotopically enriched flavocoenzymes. The information thus obtained is more specific than binding studies with flavin derivatives (Choi and McCormick, 1980). Some results can be compared directly with published results such as the solvent accessibility, which is in excellent agreement with the binding study of flavin derivatives. Other features such as a strong interaction of O(4α) and N(10) of riboflavin with the apoprotein have been suggested from binding studies and are now confirmed by our results. The data on dynamics and strength of hydrogen bonding are novel however and describe the characteristics of binding in a very detailed way.

Finally, in the light of our results we wish to briefly discuss the question of the function of the protein. It is known (McDonnell et al., 1951) that the egg is under anaerobic conditions during the first stage of development. It might well be that riboflavin is in the reduced state in this stage. The relative exposure of RBP-bound riboflavin could suggest that reduced flavin serves as an electron donor for the reduction of the disulfide bonds of ovomucine, a phenomenon which is long known (McDonnell et al., 1951) but still

remains unexplained. Our hypothesis further implies that the mentioned redox reaction requires the consumption of one proton if the reduced riboflavin is in the anionic state. This mechanism could also explain the observation that the pH value of the albumen of a fresh egg is 7.6 and increases to 8.5 within the first day of development (Carter, 1968). The initial pH value of a fresh egg is thus above the pK_a value of the reduced complex, i.e. reduced riboflavin is predominantly in the anionic state. Thus, our NMR results and the hypothesis derived from them, seem to offer an explanation for the observed pH changes in the developing embryo. The egg yolk is highly structured and pH gradients are important for the differentiation of the cells. It is therefore not unlikely that the redox reactions mentioned are related to the phenomena of pH gradients.

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Chapter 9

The different classes of flavoproteins: Miscellaneous NMR results and mechanistic implications

9.1. Introduction

In chapter 1 a classification of the different flavoproteins is given. In this chapter NMR results of a particular flavoprotein belonging to one of these classes of flavoproteins are discussed, i.e. the flavodoxins, the dehydrogenases and the hydroxylases. Flavodoxins, especially that from Megasphaera elsdenii has been discussed already in previous chapters and will be considered further below, where attention is paid to the redox potential (9.2). Lipoamide dehydrogenase from Azotobacter vinelandii has been chosen as a representative of the dehydrogenases (9.3) and parahydroxybenzoate hydroxylase from Pseudomonas fluorescens as a representative of the hydroxylases (9.4.).

9.2. Modulation of the redox potential in flavodoxin.

In the foregoing chapters various results on M.elsdenii flavodoxin have been discussed, i.e. the mobility of the prosthetic group FMN, the structure of protein-bound FMN and the protein in the different redox states and the activation barriers in the redox transitons. In this paragraph attention is paid to the modulation of the redox potential of FMN by the apoprotein. In Table 1 the redox potentials of FMN and some flavodoxins are summarized. Although the redox potentials among the flavodoxins show only minor differences, a drastic change is observed with regard to free FMN, amounting to about a 300 mV decrease for the E_1 and +150 mV for the E_2 value. Smith et al. (1977) pointed out that the E_2 value is difficult to rationalize as the protein undergoes distinct conformational changes by the transition from the oxidized to the semiquinone state.

Table 1. Redox potentials of some flavodoxins and free FMN. E_1 represents the redox potential of the transition between the semiquinone and the hydroquinone state, E_2 represents the redox potential of the transition between the oxidized and the semiquinone state. For the redox potentials of the flavodoxins, see Mayhew and Ludwig (1975) and references therein. For the redox potentials of FMN, see Anderson (1983).

		Redox potentials	
Compound	pH	E_1(mV)	E_2(mV)
M.elsdenii flavodoxin	8	-375, -413[a)]	-175
Clostridium MP flavodoxin	8	-408	-152
D.vulgaris flavodoxin	7.8	-440	-150
FMN		-124	-314

a) Calculated value for I = 100 mM, see text.

However, the situation is much simpler for the biologically relevant redox transition between the semiquinone and the hydroquinone state, as crystallographic data on Clostridium MP flavodoxin (Ludwig et al., 1977) and NMR data on M.elsdenii flavodoxin (Moonen and Müller, 1982, 1984) have shown that the protein structure must be extremely similar in these two states. Moreover, crystallographic data (Smith et al., 1977, Ludwig et al., 1976) and NMR data (Van Schagen and Müller, 1981; Franken et al., 1983) have shown that the isoalloxazine ring is almost coplanar in both redox states. An identical structure with respect to the flavin is also observed in Desulfovibrio vulgaris flavodoxin (Watenpaugh et al., 1976). This has led Simondsen and Tollin (1980) and Tauscher et al. (1973) to propose that the low E_1 value in flavodoxins is a result of the coplanar structure of flavin which is forced onto the flavin by the apoprotein. At that time it was thought that a

planar conformation of reduced flavin is energetically not favored. However, it has been shown recently (Moonen et al., 1984a) that polarized free $FMNH^-$ is hardly bent, i.e. the N(10) is almost fully sp^2 hybridized, whereas N(5) is approximately 70% sp^2 and 30% sp^3 hybridized. Moreover, the activation energy of the ring inversion (or "butterfly" motion) must be very low as deduced from dynamic ^{13}C NMR results (Moonen et al., 1984b), theoretical calculations (Dixon et al., 1979) and ^{13}C and ^{15}N NMR data of model compounds (for references see Moonen et al., 1984a and 1984b). Therefore the observed small bending angle in flavodoxin cannot cause the drastically decreased redox potential. The obvious alternative origin until now not considered, is the influence of the negative charge at N(1) in the hydroquinone state, which has been demonstrated to exist by NMR results (Van Schagen and Müller, 1980; Franken et al., 1983). It should be mentioned that the neutral semiquinone state is present in flavodoxins. It should,however, be possible to quantify the effect of the negative charge on N(1) on the redox transition E_1, as (from NMR and crystallographic results) charges are located in the vicinity of the flavin .
For example, it has been shown that the phosphate group of FMN in M.elsdenii flavodoxin is in the dianionic form regardless of the redox state (Moonen and Müller, 1982). From this study, the distance to the N(1) atom is also known i.e. about 0.8 nm. Moreover, this flavodoxin contains a large net negative charge (-17), contributing also to repulsion of the charge at N(1) of flavin. A third effect is the monopole-dipole interaction between the latter charge and the dipole of the protein. The relevant equations for the quantification of the charge interactions were developed recently by Van Leeuwen (1983). In the following the different contributions to the E_1 value will be discussed.

1. The monopole-monopole interaction of the negative charge at N(1) of flavin and the dianionic phosphate group: The distance between the charges is 0.8 nm (Moonen and Müller, 1982). No countercharges are near the N(1) atom and the phosphate group of flavin, or in the region between the charges as evidenced by

crystallographic results on the related Clostridium MP flavodoxin (Ludwig et al., 1976). Both the phosphate group and the isoalloxazine ring are tightly bound and buried in the protein (Moonen and Müller, 1982). The charges are thus separated by a rather low dielectric constant. A reasonable estimation for the dielectric constant is 20. This results in a repulsion energy of 7.2 kT or 180 mV.

2. The monopole-monopole interaction of the negative charge of the N(1) atom of flavin and the overall net negative charge of the flavodoxin.
   M.elsdenii flavodoxin possesses a net negative charge of -15 (excluded the phosphate group). Upon comparison to the related flavodoxin from Clostridium MP several charged residues appear to be invariant in the primary sequence (Mayhew and Ludwig, 1975). Crystallographic data show that all charges (except that of the phosphate moiety) are exposed to solvent. The charge interaction is thus sensitive to the ionic strength of the solvent. At a very low ionic strength (I = 0) a charge repulsion of 160 mV is calculated, for I = 100 mM the repulsion is 57 mV.
3. The monopole-dipole interaction of the negative charge at N(1) and the overall dipole moment of the protein:
   Recently, the dipole moment of Clostridium MP flavodoxin has been calculated (Van Leeuwen, unpublished results). The dipole moment is 450 Debye with the negative pole pointing towards the isoalloxazine moiety. From these data a repulsion energy of 52 mV is calculated.

From the calculations presented above it follows that only interaction 2 is dependent on the ionic strength. Thus, using I = 100 mM the total repulsion is 289 mV and using I = 0 mM the total repulsion is 392 mV. This result strongly suggests that the negative charge at N(1) in the hydroquinone state causes the dramatic decrease in the redox potential owing to strong repulsion. The close resemblance of the actual and calculated values of the redox potentials shows that the almost planar structure of the fully reduced isoalloxazine ring in flavodoxin

is not the cause of the decrease of the redox potential.

The analyses given above clearly show that redox potential modulation can be easily achieved by charge interactions. As will be shown in subsequent paragraphs the negative charge at N(1) of flavin is found in all flavoproteins investigated so far in our laboratory (i.e. luciferase from Vibrio harveyi, flavodoxin from M.elsdenii and D.vulgaris, lipoamide dehydrogenase from A.vinelandii and parahydroxybenzoate hydroxylase from P.fluorescens). Thus charge interactions are in all these cases potential mechanisms for redox potential modulation. It should be noted that charged substrates might play also a significant role in this modulation.

9.3. Lipoamide dehydrogenase

9.3.1. Lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) catalyzes the reversible reaction

$$NAD^+ + \text{dihydrolipoamide} \rightleftharpoons \text{lipoamide} + NADH + H^+.$$

The enzyme contains two redox centers, disulfide and FAD. Consequently, the enzyme can be fully reduced by four electrons, which species is thought to be catalytically inactive. The reaction mechanism, proposed by Massey and Veeger (1961) shows the essential role of the two-electron reduced enzyme (EH_2), which has later been confirmed by many investigators (for a review, see Williams (1976)). The first step in the reaction is the oxidation of dihydrolipoamide by the disulfide thereby forming the EH_2 species. In this form of the enzyme the electrons are shared by the disulfide group and FAD. This species is characterized by a 'charge-transfer' band at 530 nm. Although several proposals have been made for the electronic structure of EH_2, (Palmer and Massey, 1968; Searls et al., 1961; Massey et al., 1960), recently Wilkinson and Williams (1979) clearly showed for the E.coli enzyme that EH_2 is in fact a mixture of distinct species. The second step in the reaction is the reduction of NAD^+ by EH_2 yielding the oxidized enzyme and NADH. Several kinetic data are in agreement with the model, but others remain difficult to explain (for a review see Williams, 1976). Among these especially

the requirement for NAD^+ in the NADH oxidation by the enzyme and the possible significance of a second binding site for NAD^+ are mentioned. Matthews et al., (1979) suggested that NAD^+ functions not only as an acceptor but also as an effector which influences flavin-disulfide interaction in EH_2. In this study specifically ^{13}C labeled FAD is used to investigate the interactions between the apoflavoprotein of Azotobacter vinelandii and FAD in the oxidized, the EH_2 and the 4 electron reduced state (EH_4). Moreover, the electron distribution in EH_2 is investigated resulting in additional mechanistic information.

9.3.2 Materials and Methods

Lipoamide dehydrogenase (Lipdh) from Azotobacter vinelandii was isolated and purified from the α ketoglutarate dehydrogenase complex as described by Bosma (1984). Preparation of the apoprotein was performed as follows. Purified enzyme (140 mg) was loaded on a phenylsepharose column (Pharmacia) using a 50 mM potassium phosphate buffer which contained 30% ammonium-sulphate, 1 M KBr and 1 mM EDTA (final pH 6.5). Subsequently, FAD was removed from the protein by washing the column with 10 ml of 50 mM potassium phosphate buffer, containing 30% ammoniumsulphate, 1 M KBr and 1 mM EDTA (final pH 4.0). Subsequently the column was washed with 15 ml of the same mixture omitting KBr. Recombination with ^{13}C labeled FAD was performed on the column immediately after the preparation of apoprotein using 2 ml of a solution containing 5 mM FAD, 50 mM potassium phosphate, 30% ammonium sulphate, 1 mM EDTA (final pH 6.5). The column was then washed with the same mixture, from which FAD was omitted, for 4 h at room temperature and subsequently for 16 h at 4^oC. The reconstituted protein was eluted from the column with a mixture of 10 mM potassium phosphate containing 1 mM EDTA and 50% ethyleneglycol (final pH 6.9). The first 3 ml of the eluate contained about 75% of the total protein reconstituted. The remainder eluted in a rather diffuse band of about 50 ml. The reconstituted protein was collected and subsequently dialyzed and concentrated using an ultra-

filtration procedure (Amicon). The recovery of reconstituted protein was higher than 80% of the starting material. The specific activity and absorption spectrum of the reconstituted protein were identical with those of the starting material. This procedure appeared to be superior to the procedure described by Van den Broek (1971).

^{13}C NMR measurements were performed with a Bruker CXP 300 spectrometer operating at 75.5 MHz. 10 min Wilmad precision tubes were used. Samples contained 1.6 ml solution consisting of 1.2 mM protein and 50 mM potassium phosphate buffer (pH 7.0). 10% D_2O was added for the stabilization of the magnetic field and 3 µl dioxan was used as the internal reference. 30^o pulses and a repetition time of 0.6-0.8 s were applied. Quadrature detection and quadrature phase cycling were used. Temperature was kept constant at 26^oC. Anaerobiosis was achieved by carefully flushing the sample with argon for 20 min. Reduction was performed with dithionite (Mayhew, 1978). Oxygen-free samples were sealed with a serum cap. ^{13}C enrichment of the isoalloxazine ring was performed as described by Van Schagen and Müller (1980). FAD was synthesized and purified as described by Cramer and Neunhoeffer (1962).

9.3.3. Results

9.3.3.1. The oxidized state.

In Fig. 1 the ^{13}C spectrum is shown of oxidized lipoamide dehydrogenase, enriched with ^{13}C (>90%) at the position C(4) and C(10a) of FAD. The resonance due to C(4) is overlapping with a natural abundance resonance at 158.2 ppm, probably representing the C^{ζ} atom of arginine residues. Where necessary, difference spectra between natural abundance spectra and ^{13}C spectra of samples containing ^{13}C enriched flavin were performed to identify the resonances due to the prosthetic group. The chemical shift due to C(2), C(4), C(4a) and C(10a) atoms in oxidized and 4 electron reduced lipoamide dehydrogenase are summarized in Table 2. In a previous ^{13}C NMR study on the interaction between riboflavin

and riboflavin binding protein it was shown that the combination of ^{13}C and ^{15}N NMR data allow a detailed description of the structure of the flavin and specific hydrogen bonds between FAD and the apoprotein (Moonen et al., 1984). Therefore

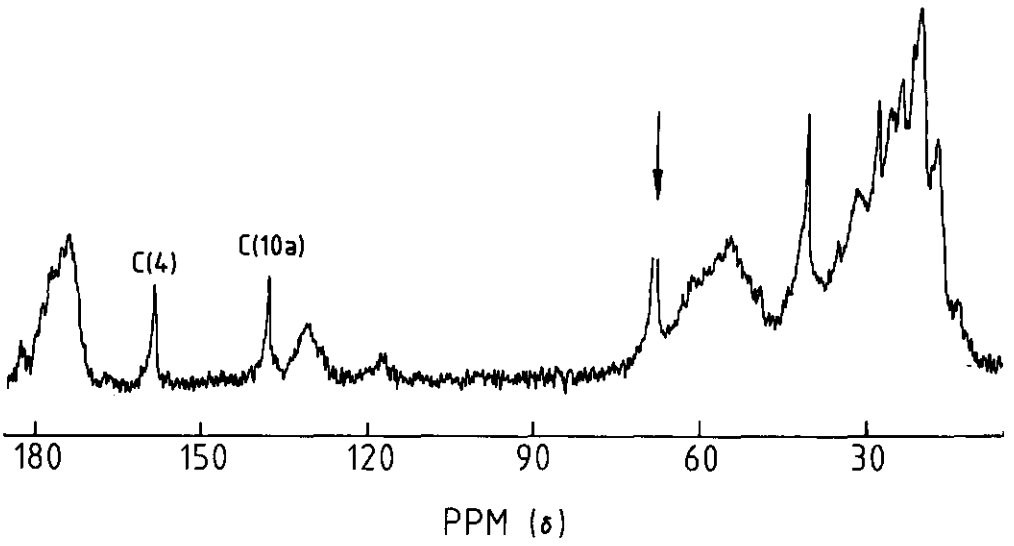


Figure 1. ^{13}C NMR spectrum of oxidized lipoamide dehydrogenase reconstituted with specifically ^{13}C enriched (>90%) FAD at position C(4) and C(10a). The position of these atoms is indicated in the spectrum. Chemical shifts are reported relative to TMS. As the internal reference dioxan was used (indicated by the arrow at 67.8 ppm). Data acquisition lasted approximately 12h. Sample contained 1.2 mM enzyme (on the basis of M_r = 56 000) in 50 mM potassium phosphate, pH 7.0).

one must be cautious in this study because only ^{13}C data are available. In Table 2 for comparison the chemical shifts of the isoalloxazine ring in water (i.e. monomeric FMN) and in chloroform (i.e. tetraacetylriboflavin) are added. In oxidized lipoamide dehydrogenase both resonances due to the C(2) and the C(4) of flavin are downfield shifted as compared with TARF in chloroform, and are almost identical with those of FMN. This clearly demonstrates that both carbonyl groups are polarized indicating hydrogen bond formation, although slightly

weaker than for FMN in water. The downfield shifted resonance due to C(4a) indicates that N(10) is a weak electron donor and that it is probable somewhat out of the molecular plane. For free isoalloxazine this occurs also in a medium with a relatively low dielectric constant (Moonen et al., 1984). The downfield shifted resonance of C(10a) can be caused by two effects i) by mesomeric structures via the polarization of O(4α) and O(2α) ii) by the absence of a hydrogen bond to the pyridine-like N(1) atom. Without ^{15}N NMR data it is not possible to determine the actual background of this shift. However, summarizing the general picture of the oxidized FAD bound to lipoamide dehydrogenase, it is concluded that both carbonyl groups are fairly polarized, but the polarization seems not to extend to the N(10) nucleus, probably due to a microenvironment of a relatively low dielectric constant. It has already been concluded from absorption data that the isoalloxazine ring is in a hydrophobic environment (Williams, 1976). This is in rough agreement with our data, but it should be mentioned that despite the hydrophobic milieu the carbonyl groups are polarized.

9.3.3.2. The 4-electron reduced state.

The chemical shifts of C(2) and C(10a) of the 4-electron reduced lipoamide dehydrogenase (EH_4) clearly indicate that the N(1) atom is ionic in 50 mM potassium phosphate buffer, pH 7.0. From the chemical shifts of C(2) and C(4) it can be concluded that both carbonyl groups are strongly polarized. In addition, the resonance line of C(4a) is strongly upfield shifted as compared with $FMNH^-$, showing that the N(10) atom is a strong π electron donor and thus must have almost full sp^2 character. The resonances of C(10a) is somewhat upfield shifted as compared to $FMNH^-$. This suggests that the degree of sp^2 hybridization of the N(5) atom is slightly increased compared with $FMNH^-$. Thus, the combined ^{13}C NMR data of $FADH^-$ in EH_4 show that both carbonyl functions are polarized and that the isoalloxazine ring is probably almost planar. The latter conclusion suggests that the reduced bound isoalloxazine ring is in a hydrophilic milieu in contrast to

Table 2. Chemical shift of ^{13}C labelled atoms of FAD bound to lipoamide dehydrogenase (50 mM, potassium phosphate, pH 7.0). For comparison the chemical shifts of FMN in water and TARF in chloroform are added. a) lipdh is lipoamide dehydrogenase, b) FMN is flavin mononucleotide, c) TARF is tetraacetylriboflavin. The accuracy of the reported chemical shifts is ± 0.2 ppm.

	C(2)	C(4)	C(4a)	C(10a)
lip.dh[a] (oxidized)	158.6	163.1	137.8	152.0
FMN[b] in water	159.8	163.7	136.2	152.1
TARF[c] in chloroform	155.2	159.8	135.6	149.1
lip.dh[a] ($2e^-$ reduced)	159.0	163.6	140.5	152.9
	159.0	157.4	101.5	154.3
lip.dh[a] ($4e^-$ reduced)	159.4	157.5	98.9	154.7
$FMNH^-$[b] in water	158.2	157.7	101.4	155.5
$FMNH_2$[b] in water	151.1	158.3	102.8	144.0
$TARFH_2$[c] in chloroform	150.6	157.0	105.2	137.1

FAD in the oxidized complex. Although the data should be regarded with some caution it is suggested that a conformational change takes place by the 4 electron reduction of the protein rendering the reduced $FADH^-$ more accessible to solvent water.

9.3.3.3. The two electron reduced state (EH_2)

It is known that the stability of the two electron reduced state (EH_2) against overreduction is dependent on the source of the protein (Williams, 1976). Recently, it was suggested that also the composition of EH_2 (i.e. the different possibilities of accommodating the reduction equivalents) is dependent on the source of isolation (Wilkinson and Williams (1979)). The EH_2 state of the enzyme from A.vinelandii resembles in stability and absorption spectrum very much the

EH_2 of the protein from pig heart (De Kok et al., 1983, De Kok, personal communication). Therefore, we will compare our results mainly with the results obtained with pig heart lipoamide dehydrogenase.

As with the pig heart enzyme, the red intermediate EH_2 is formed by a stoichiometric titration of the oxidized enzyme with dithionite. The same species is slowly formed in the presence of excess NADPH and is extremely stable under anaerobic conditions (De Kok et al., 1983). Both methods result in similar NMR spectra. Except that the spectrum obtained by NADPH reduction shows also the natural abundance resonances of the pyridine nucleotide (data not shown). In Fig.2 the low field part of the ^{13}C NMR spectra of EH_2 are shown. The spectra were obtained with FAD enriched with ^{13}C at the C(4) and C(10a) position (upper spectrum) and at the C(2) and C(4a) position (lower spectrum). In the upper spectrum four resonances due to the carbon atoms of flavin can be clearly seen, although only two positions are enriched. It should be noted that none of the resonance positions is equal to the resonance positions of either those of the oxidized or 4 electron reduced enzyme. This clearly indicates that one deals with two distinct species in the EH_2 state. The peaks at 163.6 and 157.4 ppm appear at a position, where C(4) is about expected in the oxidized and reduced free isoalloxazine, respectively (Moonen et al., 1984). The peaks at 152.9 and 154.3 ppm are at positions where about the resonance of C(10a) is expected in the oxidized and reduced isoalloxazine, respectively. These results show that EH_2 consists of a mixture of oxidized and reduced isoalloxazine. This is fully confirmed by the lower spectrum, where the resonances at 140.5 and 101.5 ppm resemble the C(4a) position in oxidized and reduced isoalloxazine respectively. Only C(2) does not show a splitting, which is not unexpected because the chemical shift of C(2) hardly differs in isoalloxazine upon reduction. This result closely resembles the result of Wilkinson and Williams (1979), who showed, using stopped flow measurements on the E.coli enzyme, that EH_2 consists of a mixture of species in which FAD and disulfide share the electrons differently. Thus EH_2 of A.vinelandii consists of a species of re-

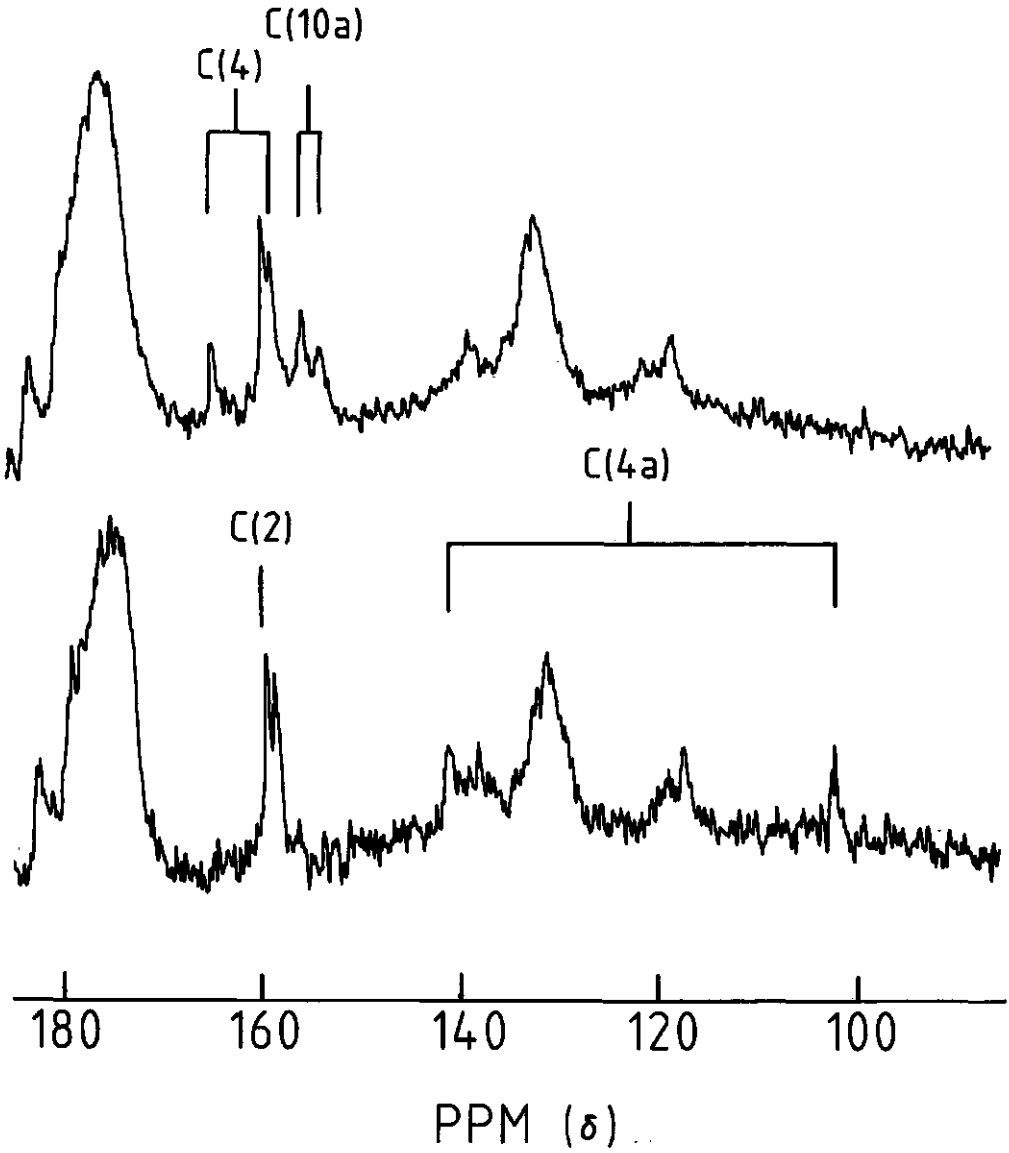


Figure 2. Low field part of the ^{13}C NMR spectra of the two electron reduced (red intermediate) lipoamide dehydrogenase. Reduction was performed by a stoichiometric titration with sodium dithionite (cf. Materials and Methods). Upper spectrum enzyme enriched at the positions C(4) and C(10a). Lower spectrum, enzyme enriched at the positions C(2) and C(4a). For other details see text and Caption of Fig. 1.

duced FAD and oxidized disulfide (EH_2(A)), and a species of oxidized FAD and reduced disulfide (EH_2(B)). To accumulate the two spectra shown in Fig. 2 about 20 h were needed during which time the sample remained anaerobic. The typical red colour of the EH_2 before and after the measurement was the same. No over-reduction nor oxidation occurred during the time course of the experiment as within the signal to noise ratio of the experiment no fully oxidized nor 4 electron reduced enzyme could be detected. The combined intensity of the resonances of the two EH_2 species is equal (±20%) to the oxidized or EH_4 state and indicates that by approximation only the two mentioned EH_2 species constitute the two-electron reduced state of the enzyme. The resonances are summarized in Table 2. The EH_2(B) species shows small but distinct differences with the fully oxidized enzyme. This indicates that either a conformational change has occurred or the reduced disulfide influences the chemical shift position in the EH_2 species. The same can be stated for the difference between the four-electron reduced enzyme and the EH_2(A) species. In the following the electronic structure of the two distinct EH_2 species will be discussed. The differences in chemical shifts due to C(4a) and C(10a) of the oxidized isoalloxazine ring in the fully oxidized enzyme and in the EH_2(B) species are remarkable. In the EH_2(B) species both resonances show a strong downfield shift, which cannot be explained solely by hydrogen bond formation or polarization by a high dielectric medium. In fact the data are consistent only with a negative charge, very close to these atoms. Crystallographic results on glutathione reductase (a flavoprotein with a very similar reaction mechanism and physical properties) show that one thiol of the disulfide moiety is extremely close to C(4a) (Schulz et al., 1978). Therefore, the obvious candidate for the negative charge is the reduced disulfide bond, in agreement with published results (Williams, 1976). The small distance between the thiolate anion and the C(4a) and C(10a) centers of flavin might well cause the 'charge transfer' band in EH_2.

The EH_2 (A) species in which the isoalloxazine is reduced, resembles $FMNH^-$ in water, with the exception that C(2) is slightly downfield and

C(10a) slightly upfield shifted. This shows that the π electron density is enhanced at the C(10a) position at the cost of the π electron density at C(2). The exact reason for this is not yet known. It should be noted, however, that on the basis of the ^{13}C NMR results it cannot be fully excluded that the EH_2 absorption band at 530 nm results from a strong interaction between reduced isoalloxazine and oxidized disulfide, in contrast to the idea that this band originates from an interaction between the thiolate anion and the oxidized isoalloxazine ring.

9.3.4. Mechanistic implication

9.3.4.1. Charge interaction and redox potentials.

The results demonstrate that EH_2 consists of two different species, one with the FAD reduced to $FADH^-$ and the disulfide oxidized, ($EH_2(A)$) the other species with FAD oxidized and the disulfide reduced to SH and S^- ($EH_2(B)$). In both species the two redox centers are very close to each other. The fact that the two species are roughly equally populated (±10%) clearly show that the redox potentials of the couple E_{ox}/EH_2 are almost the same for both species of EH_2. The redox potential of the pig heart enzyme for the couple E_{ox}/EH_2 is -280 mV (pH 7.0) and -346 mV for EH_2/EH_4 (Matthews and Williamson, 1976). As stability and kinetics are rougly the same for the enzyme from pig heart and A.vinelandii it is probably safe to assume that the redox potentials for the latter enzyme are the same. As the redox potential E_{ox}/EH_2 is the same for both EH_2 species and 66 mV more positive than for the couple EH_2/EH_4 it must be concluded that for both the $EH_2(A)$ and the $EH_2(B)$ species the reduction of the disulfide or FAD to form EH_4 from EH_2 requires more energy than the reduction of the same centers in the transition E_{ox}/EH_2. Thus, if the disulfide is reduced, then the reduction of FAD is energetically less favorable than in the case where the disulfide is oxidized. If, on the other hand, FAD is reduced then the reduction of the disulfide is energetically less favorable than if FAD is oxidized. Recalling the discussion on flavodoxin (see 9.2), the obvious reason is a charge repulsion occurring between $FADH^-$ and S^- in the EH_4 state. This conclusion is in full

agreement with the early experiments of Massey and Veeger (1961) using arsenite incubation of the enzyme. These results show that both thiols originating from the reduction of the disulfide can be covalently linked with arsenite. Subsequently FAD is easily reduced by NADH, in contrast to untreated EH_2, indicating that the redox potential of the FAD center is increased by arsenite addition. It is clear that the formation of a complex between arsenite and both thiols results in a neutral complex, and as a consequence, no charge repulsion exists in EH_4. Also methylation of one or both of the thiols results in an increased redox potential of EH_2/EH_4 in the A.vinelandii enzyme (De Kok, personal communication). It should be noted that the charge repulsion in EH_4 is less than the charge repulsion between phosphate and the charge at N(1) in fully reduced flavodoxins, although the distance between the charges in the latter species is probably larger. The reason might be a partial neutralisation of one of the charges in EH_4. There is indeed some indication that the thiolate anion is stabilized by a base (Matthews and Williamson, 1976).

9.3.5. The intramolecular electron transfer and the role of NAD^+

To reiterate the reaction cycle according to Wilkinson and Williams (1979): In the first step dihydrolipoamide becomes oxidized and the electrons enter the active site of the enzyme by reduction of the disulfide. The second step is an equilibration between the different species of EH_2 as shown before. The third step is the reduction of pyridine nucleotide (NAD^+). This third step is probably an electron transfer from reduced FAD to NAD^+ (Williams, 1976). The second step is schematically represented in the following scheme:

This step consists of an intramolecular two-electron transfer from the reduced disulfide to oxidized FAD. It should be noted that this is an essential step in the reaction cycle as electrons enter via the disulfide moiety and leave via the FAD moiety. The kinetics of the intramolecular two-electron transfer has not been implicated in the original kinetic models for the overall reaction and might explain the fact that no kinetic model could sufficiently explain the kinetic data (Williams, 1976). The ^{13}C NMR data actually show the importance of the kinetics of the intramolecular electron transfer as under the conditions of the experiments the lifetime of the electron pair in each EH_2 species is >> 10 ms. This conclusion can be directly derived from the fact that the two EH_2 species are separately observed in the NMR spectra, although the separation of the C(10a) resonances in the two EH_2 species is about 100Hz. Moreover, the linewidth of both resonances hardly differs from linewidths observed in the oxidized state indicating that the exchange frequency hardly causes a linebroadening effect. A broadening > 5Hz would be obvious from the NMR spectra. The exchange rate is thus smaller than 5 s^{-1}. Thus at 26°C in 50 mM potassium phosphate (pH 7.0) the lifetime τ of each species is > 200 ms. This shows that the kinetics of this step is likely of great importance in the overall reaction. The low exchange rate might also be the reason for the slow comproportionation reaction (Wilkinson and Williams, 1979).

Stopped flow experiments have shown that the oxidation of EH_2 by NAD^+ is complete within 3 ms (Massey et al., 1960). It was concluded that the reaction rate is faster than 3300 s^{-1} at saturating substrate concentration. It should be noted that the intramolecular electron transfer, depicted in the above scheme, is necessary for the completion of the oxidation of EH_2 by NAD^+. The combined kinetic data strongly indicate that NAD^+ not only functions as a substrate in the overall reaction, but in addition increases considerably the rate of intramolecular electron transfer. NAD^+ functions thus also as an effector. Matthews et al. (1979) suggested already that oxidized pyridine nucleotide functions as an effector for the disulfide-flavin interaction.

The ^{13}C NMR experiments have shown that this suggestion must be correct. These results offer a good explanation for the kinetics of the reversed reaction. It has been shown by Massey and Veeger (1961) that for the oxidation of NADH by lipoamide dehydrogenase the presence of NAD^+ is required. Our data indicate that without NAD^+ the slow intramolecular electron transfer limits the reduction of lipoamide.

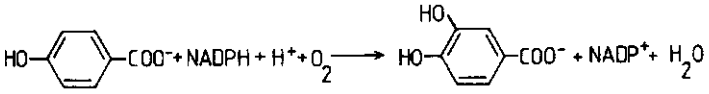
9.3.6. Summary

^{13}C NMR data of lipoamide dehydrogenase reconstituted with FAD, enriched at the positions C(2), C(4), C(4a) and C(10a), allows a rough determination of polarization of the isoalloxazine moiety in the oxidized enzyme (E_{ox}), the two-electron reduced enzyme (EH_2) and the four-electron reduced enzyme (EH_4). The results are consistent with a disulfide, located close to the isoalloxazine ring, which influences (dependent on the redox state) the chemical shifts and redox potential of FAD. It is suggested that charge interactions play an important role in the redox potentials. If FAD is reduced, it is present in the anionic form. It is shown that EH_2 consists of two species, one in which FAD is oxidized a nd the disulfide reduced. In the other one FAD is reduced and the disulfide oxidized. The redox potential of E_{ox}/EH_2 is nearly the same for both EH_2 species. The intramolecular two-electron transfer rate is smaller than $5\ s^{-1}$ under the conditions of the experiments. Comparison with reported kinetic data showed that NAD^+ acts as an effector in this intermolecular transfer process.

9.4. P-hydroxybenzoate hydroxylase

9.4.1. Introduction

p-Hydroxybenzoate hydroxylase (pHBH) EC 1.14.13.2) from Pseudomonas fluorescens is an NADPH dependent, FAD containing monooxygenase catalyzing the hydroxylation of p-hydroxybenzoate (pHB) to form 3,4-dihydroxybenzoate in the presence of molecular oxygen:



Two ternary complexes are involved in the reaction cycle; i.e. the formation of the E(FAD)-pHB-NADPH complex and after reduction of E(FAD)-pHB the formation of $E(FADH_2)$-O_2-pHB. The latter complex is involved in the activation of molecular oxygen to hydroxylate the substrate. Subsequently the product is released and the next catalytic cycle can be executed.(Husain and Massey, 1979).

The binding of pHB to the oxidized state to form a 1:1 complex causes large, characteristic spectral perturbations and fluorescence quenching. The reduction of bound FAD is dramatically enhanced upon substrate binding (Husain and Massey, 1979). The structure of the pHBH-pHB comlex is known by crystallographic results (Wierenga et al., 1979). Significant progress has further been obtained on i) the involvement of some "essential" aminoacids (Wijnands and Müller, 1982; Shoun and Beppu, 1982) ii) the quaternary structure (Müller, et al., 1979). iii) the amino acid sequence (Weyer et al., 1982) and the binding of FAD (Müller and Van Berkel, 1983).

The exact sequence of processes after the formation of the second ternary complex remains still uncertain, although some progress has been booked (Entsch et al., 1980; Claiborne et al., 1982; Entsch et al., 1976; Bruice, 1980; and references therein).

At the moment it is generally accepted that the activation of oxygen proceeds via a flavin C(4a)-hydroperoxide intermediate. Its formation is not fully established. In addition in principle the reaction between singlet flavin and triplet oxygen is spin forbidden. The oxygen transfer reaction to the substrate is unclear. While Entsch et al. (1976) proposed the transfer of one oxygen atom, Kemal and Bruice (1976) proposed a mechanism in which

a substrate-peroxide is formed intermediately.

pHBH from other sources than P.fluorescens appears to be similar with respect to the reaction mechanism and molecular properties.

9.4.2. Materials and Methods

p-hydrobenzoate hydroxylase from P.fluorescens was isolated and purified as described by Müller et al. (1979). Preparation of apoprotein and reconstitution was performed as described by Müller and Van Berkel (1983). Enrichment of FAD by ^{13}C was achieved as described in paragraph 9.3.2.

Samples consisted of 0.6 mM pHBH in 50 mM potassium phosphate (pH 7.0) 10% $^{2}H_2O$ served as a lock for the spectrometer. Dioxan (3µl) was used as an internal reference. 10 mm NMR precision tubes were used. The sample volume was 1.6 ml. Spectra were taken on a Bruker CXP 300 spectrometer operating at 75.5 MHz. Broadband decoupling (0.5 Watt) was used. 30^{o} pulses and a repetition time of 0.6-0.8s were used. Quadrature detection and quadrature phase cycling were applied. Temperature was kept constant at $20^{o}C$. For the reduction and anaerobiosis procedure see 9.3.2.

9.4.3. Results and Discussion

Fig. 3 shows a series of ^{13}C NMR spectra of pHBH in dependence on the redox state and the absence or the presence of substrate. The resonance frequencies of the enriched carbon atoms C(4) and C(10a) are easily determined either directly from the spectra or using difference spectra (see also caption of Fig. 3). The data are summarized in Table 3. In Fig. 4 similar experiments are shown using FAD enriched at the position C(2) and C(4a) (Table 3). For comparison of the chemical shifts with those of free flavin the reader is referred to Table 2 and to a detailed discussion by Moonen and Müller (1984). As in the case of lipoamide dehydrogenase, the results have to be regarded with some caution, as they are based on ^{13}C NMR data only.

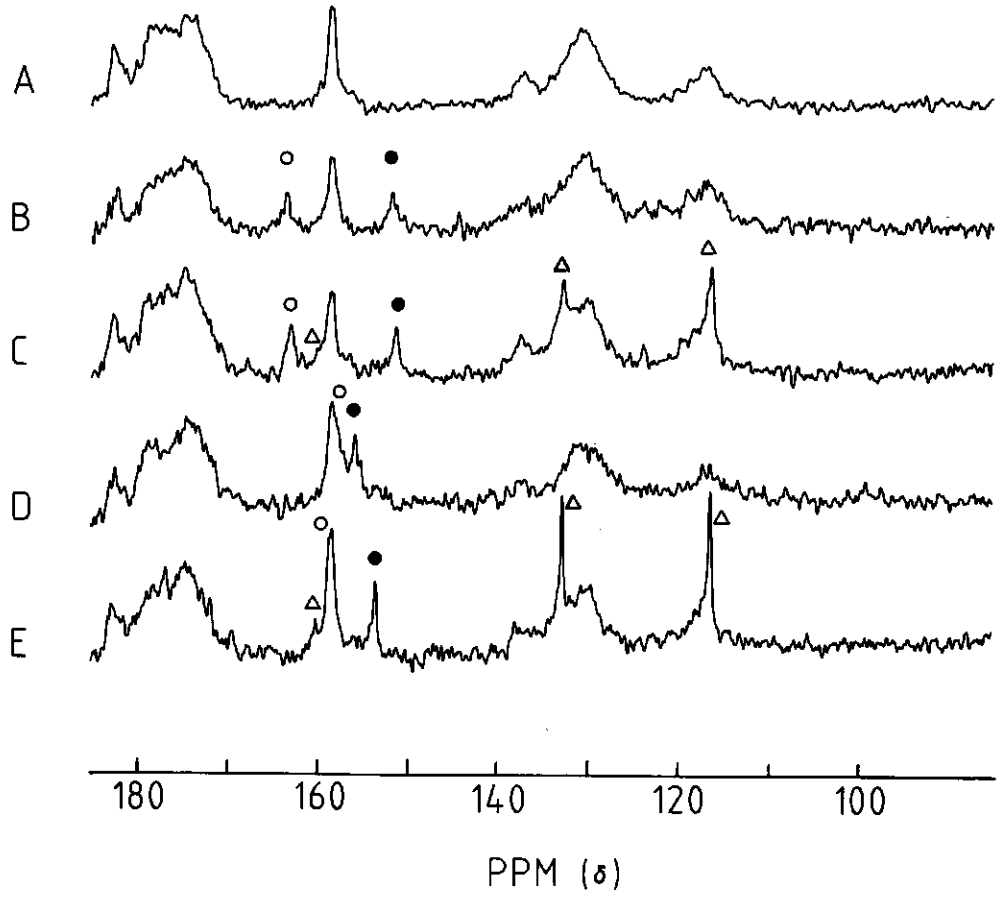


Fig. 3. Low field part of the ^{13}C NMR spectra of p-hydroxybenzoate hydroxylase. Samples contained 0.6 mM enzyme in 50 mM potassium phosphate (pH 7.0). Chemical shifts are referred to TMS. Dioxan (3 µl) served as an internal reference. All spectra were accumulated in 8 to 16 hours. A. Natural abundance spectrum B-E, Enzyme reconstituted with FAD containing ^{13}C isotope (>90%) at the position C(4) and C(10a), B, oxidized state, C, oxidized state in the presence of 5 mM substrate, D, reduced state, E, reduced state in the presence of 5 mM substrate. Resonances marked with Δ represent natural abundance intensity of substrate. Resonances marked O and ● represent the C(4) and C(10a) atom, respectively. In the spectra D and E the resonance due to C(4) is partially obscured by the natural abundance resonance at 158.2, probably representing the C^{ζ} atoms of arginines. The position of C(4) in the spectra D and E was therefore determined by difference spectra between A and D, and between A and E. Data are summarized in Table 3.

The oxidized state

Without substrate, the polarization of the isoalloxazine ring resembles closely free FMN in water, the differences being < 0.5 ppm. Therefore it is proposed that the polar part of the isoalloxazine (i.e. the pyrimidine subnucleus including the N(5) atom) is fairly exposed to solvent. It should be mentioned that hydrogen bonds alone are not sufficient to polarize the isoalloxazine ring in such an extent. A relatively high dielectric constant is needed additionally. The relative exposure to solvent would probably lead to an increased mobility of the isoalloxazine ring, which is in agreement with time-resolved fluorescence depolarization measurements (Visser et al., 1983). The exposure to solvent is also in agreement with the study of Claiborne et al. (1982).

Upon binding of substrate pHBH (more than 90% of ES is present under the conditions of these experiments) a drastic upfield shift of 2.9 ppm for C(2) is observed. The observed value of 156.6 ppm is even close to the value for the corresponding atom in flavin dissolved in chloroform (i.e. 155.2 ppm). This shows that the π electron density at C(2) is increased in the presence of substrate, which indicates that the carbonyl group at C(2) is hardly polarized. The resonance position of C(4) indicates that in the ES complex a fairly strong hydrogen bond exists towards O(4α). The upfield shift of the line due to C(10a) upon substrate binding might be caused by the increased π electron density at C(2) (mesomeric effects). The upfield shift of the resonance due to C(4a) suggests that the π electron density is increased at this position in the ES complex. It is, however, necessary to support this by ^{15}N NMR data. Nevertheless, the changes upon substrate binding clearly show that the interaction between the prosthetic group and the apoprotein is affected by the binding of the substrate. This is in agreement with changes observed in adsorption and fluorescence spectra (Husain and Massey, 1979). Crystallographic data of pHBH are at the moment only available for the enzyme-substrate

complex (Wierenga et al., 1979). From crystallographic data (resolution 0.25 nm) it has been suggested that hydrogen bonds exist to the N(1), O(2α), N(3) and O(4α) atoms of flavin. It should be mentioned that crystallographic data do not allow to determine the strength of the hydrogen bonds, but allow only a rough estimation of the distance between the atoms involved in hydrogen bond formation. In contradiction to the crystallographic results the hydrogen bond to O(2α) must be weak as concluded from ^{13}C NMR data. From crystallographic data it is shown that an α-helix points with its positive pole directly to N(1) in the ES complex (Wierenga et al., 1979). This could be the reason why the C(2) atom accomodates a rather high π electron density. If this is true then the relative position of the α-helix and the isoalloxazine ring is different between the free enzyme and its ES complex. Mechanistic implications will be discussed below.

The reduced state

In the absence of substrate the chemical shift of C(2) and C(10a) (Table 3) clearly show that reduced FAD is present in the anionic form ($FADH^-$). Compared with $FMNH^-$, C(2) and C(4a) are upfield shifted by 1.3 and 1.5 ppm respectively, wheras C(4)and C(10a) resonate at the same frequency as in $FMNH^-$. The upfield shift of C(2) indicates that no or only a weak hydrogen bond exists towards O(2α). The upfield shift of C(4a) is probably caused by a strong π electron donation from N(10), i.e. N(10) is sp^2 hybridized. The flavin in oxidized free enzyme closely resembles free FMN in water, that in reduced free enzyme does not resemble free $FMNH^-$. For the oxidized system it is proposed that the C(2) of flavin gains π electron density upon substrate binding, due to some displacement of the α-helix. If this is true, then it is also probable that the upfield shift of the resonance of C(2) in reduced free enzyme is also caused by the position of the α-helix, i.e. pointing directly towards N(1). This implies that the binding mode of $FADH^-$ in free enzyme is similar to the binding mode of oxidized FAD in the ES complex, but different in the free oxidized enzyme.

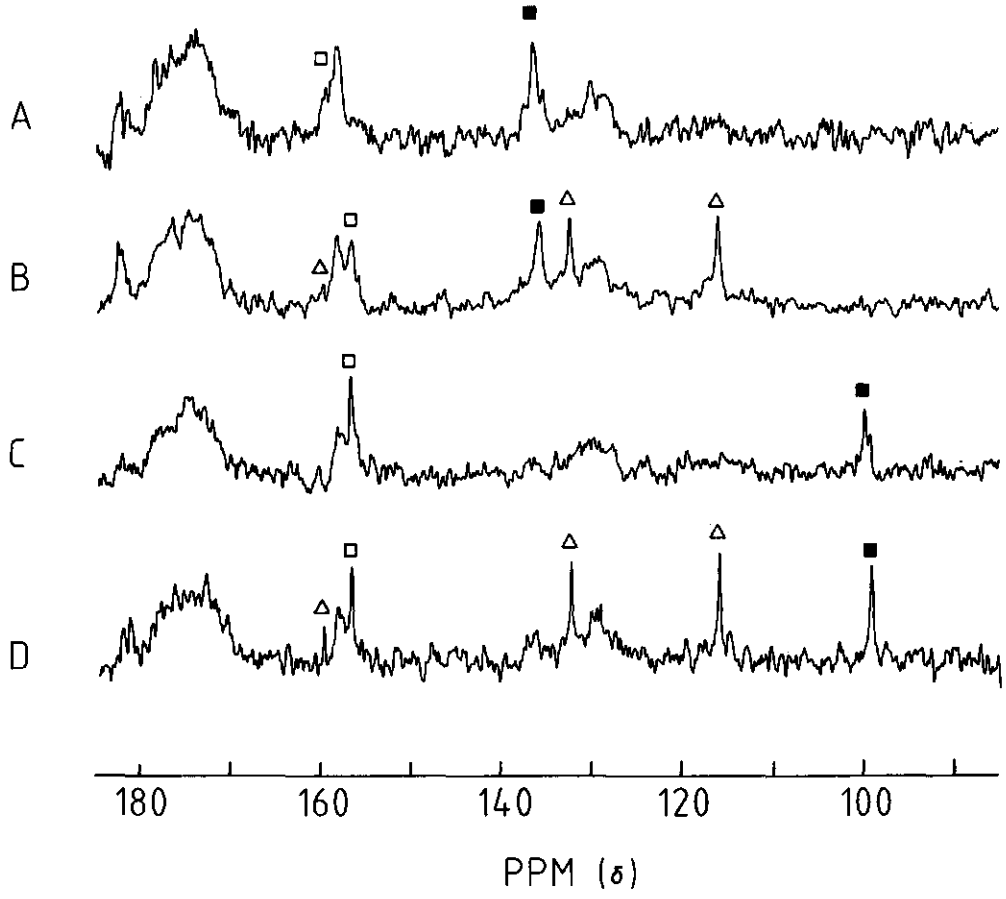


Fig. 4. Low field part of the ^{13}C NMR spectra of p-hydroxybenzoate hydroxylase in which natural FAD was replaced by FAD, isotopically (>90%) enriched with ^{13}C at the position C(2) and C(4a).
A, oxidized state, B, oxidized state in the presence of 5 mM substrate, C, reduced state, D, reduced state in the presence of 5 mM substrate. Resonances marked with △ represent natural abundance intensity of substrate. Resonances marked with □ and ■ represent the C(2) and C(4a) atom, respectively. See caption of Fig. 3 for further explanations.

Table 3. ^{13}C chemical shifts of the carbon atoms C(2), C(4), C(4a) and C(10a) of FAD bound to parahydroxybenzoate hydroxylase in the oxidized (ox) or fully reduced (red) state and in the absence (-) or presence (+) of substrate. Precision of the data is ± 0.2 ppm.

Redox state	Substrate	Carbon atom			
		2	4	4a	10a
ox	-	159.5	163.2	136.4	151.6
ox	+	156.6	162.8	135.7	151.1
red	-	156.9	157.6	99.9	155.6
red	+	156.9	158.1	99.3	153.3

Upon binding of the substrate to the reduced enzyme the C(2) resonance position is unchanged, which supports the above mentioned proposal, i.e. the α-helix causes the upfield shift of C(2) and its relative position to the isoalloxazine ring is unchanged upon binding of the substrate to the reduced enzyme. Upon binding of substrate to the reduced enzyme a remarkable upfield shift of C(10a) is observed (2.3 ppm). According to the theory of Moonen et al. (1984) it is most likely that the upfield of C(10a) is caused by the N(5) atom of flavin which is probably forced into the molecular plane, becoming a strong π electron donor. It should be noted that N(10) is already in the molecular plane in the presence and absence of substrate. As deduced from the chemical shift of C(10a), the hybridization of N(5) in the absence of substrate is similar to that in $FMNH^-$ (i.e. the endocyclic angle of N(5) is about 115-117°). Upon substrate binding the N(5) likely attains full sp^2 hybridization. For C(4a) a slight increase in π electron density is observed upon substrate binding to the reduced enzyme.

9.4.4. Mechanistic Implications

9.4.4.1. Substrate binding

From crystallographic data (Wierenga et al., 1979) it is known that the substrate is bound in the interior of the enzyme, very close to the isoalloxazine ring. If the enzyme structure in the ES complex would be maintained in the free enzyme the formation of the ES complex would be expected to be inhibited due to steric effects. The results suggest that the structure of the ES complex is not maintained in the free enzyme. In contrast, the data suggest a rather dynamic active center, in which the isoalloxazine ring is exposed to solvent. It is proposed that this feature benefits the complex formation between enzyme and substrate.

Contrary to the free oxidized enzyme, the free reduced enzyme likely posesses a structure similar with that of the oxidized and reduced ES complex. If the proposal with respect to the free oxidized enzyme is correct (facilitation of binding of substrate) then it would be expected that binding of substrate to reduced free enzyme is much slower, as the active center conformation is already pre-formed in reduced free enzyme. Indeed, the rate of formation of the ES complex has been determined to be slower by more than 10 000 fold for the reduced enzyme as compared to the oxidized one, whereas the equilibrium constant (K_d) differs only 6-fold (Husain et al., 1980; Entsch et al., 1976). Thus, the combination of kinetic results and ^{13}C NMR data strongly suggest that the conformation of the active site is the same in the oxidized ES complex, the free reduced enzyme and the reduced ES complex, but it differs in conformation and mobility in free oxidized enzyme. The conformational change probably also influences the observed dramatic increase of the reduction of FAD by NADPH upon substrate binding, i.e. a high activation barrier for the redox transition between the oxidized and the hydroquinone state might exist for free enzyme, whereas the conformational change lowers this activation barrier.

9.4.4.2. Oxygen activation

Anionic reduced flavin has generally been regarded as a better oxygen activator than the neutral from (Massey and Hemmerich, 1980; Favaudon, 1977). Reduced pHBH is indeed anionic both in the absence and in the presence of substrate. The attack of dioxygen on the reduced isoalloxazine ring is a remarkable reaction, as it violates the law of spin conservation. Nevertheless, reduced flavins form rather smoothly a flavin hydroperoxide adduct both for free and some protein-boun flavins. Among flavoproteins, however, the reaction rate differs dramatically (cf. Chapter 1 and Massey et al, 1969). Oxygen reacts by an electrophilic reaction. Thus, in principle, C(4a) and C(10a) could be regarded as the reaction sites because the electron density is high at these centers (Moonen and Müller, 1984). Moreover these centers are less involved in resonance stabilization of certain mesomeric structures of reduced flavin than e.g. the carbon atoms of the benzene subnucleus. Indeed, in model systems both adducts are found. However, in flavoenzymes, preferential attack on C(4a) occurs (Müller, 1983, and references therein). As this reaction is an electrophilic reaction it is interesting to compare the oxygen reactivity of various flavoproteins with the π electron density at the C(4a) center as measured by ^{13}C NMR. In Table 4 the flavoproteins investigated up till now are summarized.

The comparison should be regarded with caution, as for example steric effects for the approach of oxygen are not taken into consideration. For example flavodoxin from M.elsdenii flavodoxin is highly reactive towards oxygen yielding O_2^-, although the N(5) and C(4a) are shielded (chapter 11). The C(4a) center in pHBH contains indeed a high π electron density as evidenced by the high field position of the resonance due to C(4a). However, the π electron density at the C(4a) center is even higher in the 4 electron reduced lipoamide dehydrogenase, but the reactivity with oxygen is low. On the other hand, luciferase shows a δ(C(4a)) of 103.2 ppm indicating a decreased π electron density, although this protein reacts fast with oxygen and should in fact be regarded as a hydroxylase. Thus from Table 4, no clear correlation between oxygen reacti-

Table 4: Comparison of reactivity of various reduced flavoproteins towards oxygen versus the chemical shift of C(4a) in the reduced state. As an example for flavodoxin, M.elsdenii flavodoxin is taken, for lipoamide dehydrogenase, the reader is referred to paragraph 9.3, for luciferase from vibrio harveyi the chemical shift is reported by Vervoort et al. (1983), for a hydroxylase, pHBH from Pseudomonas fluorescence is taken. The first product in the reaction of reduced flavodoxin with O_2 is O_2^-. Therefore, for the other enzymes, as far as appropriate, the reaction product in the absence of substrate is given.

Flavoprotein	δ(C(4a)) ppm	Oxygen reactivity	Product
flavodoxin	103.5	+	O_2^-
hydroxylase	99.3	+	H_2O_2
lipoamide dehydrogenase	98.9	-	-
luciferase	103.2	+	H_2O_2

vity and π electron density at the C(4a) center can be deduced.

It has been pointed out by some authors that electron transfer to oxygen, yielding flavin radical and superoxide radical, precedes the formation of a covalent adduct (Kemal and Bruice, 1979). If this is the case then indeed addition to C(4a) would be expected by a radical recombination reaction, as C(4a) possesses a high spin density in the semiquinone state (Müller, 1983). It should be noted that the semiquinone (radical) state of FMN is planar (Müller et al., 1982). Therefore, it is anticipated that a planar hydroquinone state has a higher reactivity towards oxygen than a bent one. If from a bent flavin an intermediate semiquinone state is formed during the reaction with oxygen a nuclear rearrangement must occur to accomodate the planar semiquinone state. This nuclear rearrangement would probably cause an activation barrier

similarly to that as already discussed for the redox transitions in flavodoxin (Moonen and Müller, 1982, 1984). In this respect it is interesting to note that N(5) in reduced pHBH becomes more planar upon substrate binding. As also N(10) is almost sp^2 hybridized the reduced flavin in the ES complex is essentially planar. From this it is postulated that the main feature of the reduced isoalloxazine ring to activate oxygen is not the π electron density of the C(4a) center, but rather the sp^2 hybridization of the N(5) and N(10) atoms. The latter atom is already essentially sp^2 hybridized in $FMNH^-$, but the N(5) is only partially sp^2 hybridized in $FMNH^-$ ($\sim$70%). As the hybridization of N(5) is hardly influenced upon polarization of the flavin molecule, (Moonen and Müller, 1984) full hybridization of the N(5) atom can probably only be achieved by steric effects. It is interesting to note that it has been proven that in reduced flavodoxin from M.elsdenii N(5) is forced into the molecular plane (Franken et al., 1983). This flavodoxin shows indeed a high reactivity towards oxygen, yielding superoxide anion, which is prevented by steric effects to form a covalent adduct with protein-bound flavin. In luciferase the degree of hybridization of N(5) is not known due to lack of sufficient NMR data. In lipoamide dehydrogenase, it is estimated from the ^{13}C NMR data that the N(5) atom is hybridized as in $FMNH^-$, which could explain the lowered reactivity towards oxygen, according to the postulate presented. The hybridization of N(5) could be of importance for the problem of spin conservation in the reaction between reduced flavin and triplet oxygen. The reaction would be spinallowed if not the singlet state, but the triplet state of reduced flavin would be involved. It is however not known if a low lying triplet state in reduced flavin exists. It would be of great interest to solve this problem and to elucidate the influence of the various conformations of reduced flavin on the triplet state.

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Chapter 10

A PROTON NUCLEAR MAGNETIC RESONANCE STUDY AT 500 MHZ ON MEGASPHAERA ELSDENII FLAVODOXIN

A study on the stability, proton exchange and the assignment of some resonance lines.

Chrit T.W. Moonen and Franz Müller

SUMMARY

^1H NMR studies were performed on the three redox states of M.elsdenii flavodoxin. The results show that the protein is remarkably stable, as concluded from amide proton exchange studies. Some amide protons are still present in the ^1H NMR spectrum even after one month in ^2H_2O at 33°C (pH 8.3). The reactivity of the exchangeable protons can be grouped into three catagories, i.e. $t_{\frac{1}{2}} >> 5$ min, 10s $\lesssim t_{\frac{1}{2}} \lesssim$ 5 min, and $t_{\frac{1}{2}} <<$ 10s.
The amide proton exchange reactions are hardly dependent on the redox state. Optimal resolution of ^1H NMR spectra is obtained at 33°C, independent of the redox state. No conformational change of the protein is observed in the pH range between 6 and 8.5. Assignments of resonances to protons of flavin and of some amino acid residues are established in both the oxidized and the hydroquinone state using chemically and isotopically substituted flavins and the driven nuclear Overhauser technique. Preliminary two-dimensional ^1H-^1H correlated spectra show that the protein is amenable to two-dimensional NMR techniques. Previous assignments are confirmed by this technique.

INTRODUCTION

The flavodoxins form a class of small proteins (M_r 15.000-23.000) containing flavin mononucleotide (FMN) as the prosthetic group and serve as redox carriers in the living cell [1]. During in vitro redox reactions the prosthetic group shuttles between the quinone (oxidized), semiquinone (radical) and hydroquinone (1,5-dihydro or fully reduced) states. The redox potential of the different redox states is strongly altered as compared with free FMN. This can probably be ascribed to the influence of charges present in the active site (Moonen and Müller, unpublished results). The crystallographic structure of flavodoxin from Clostridium MP in its three redox states is known [2-4]. The flavodoxin from M.elsdenii is strongly related to the former flavodoxin in many chemical and physical aspects [1].

M.elsdenii flavodoxin, due to its easy availability in large quantities, has been subjected to various NMR techniques. It has been shown that specific hydrogen bonds are formed on binding of the prosthetic group to the apoprotein [5,6]. The isoalloxazine ring (i.e. the redox-active moiety) of FMN appears to have negligible motional freedom in the complex [7]. Moreover, it has been shown, that a tryptophan residue (Trp-91) is located near the prosthetic group in the oxidized complex. Van Schagen et al. [8] and Moonen et al. [9] assigned all resonances of this tryptophan residue and showed that the internal flavin is shielded from solvent by this tryptophan. The NMR studies also showed that the active centers of M.elsdenii and Clostridium MP flavodoxins are identical with respect

to the structural arrangement of the flavin and the tryptophan residue [9].

A long recognized fact from the interchangeability with ferredoxins is that only the transition between the semiquinone and the hydroquinone state in Clostridium MP and M.elsdenii flavodoxin is of biological relevance [1]. Moonen and Müller [10] showed that this is related to the fact that the activation energy for the transition between the oxidized and the semiquinone state is larger than that between the semiquinone and the hydroquinone state. According to the crystallographic studies of Ludwig et al. [4] on Clostridium MP flavodoxin the different activation barriers can be rationalized by a minor conformational change in the transition from the oxidized to the semiquinone state.

Although the crystallographic data of Clostridium MP flavodoxin have provided detailed information on the structure, one has to be careful with regard to the dynamics of the given structures, especially since the flavin is located near the surface of the protein. In addition protein-protein contacts in crystals can influence the structure of proteins, as has been shown for the basic pancreatic tryspin inhibitor by ^{1}H NMR [11]. The recent enormous advances in two-dimensional NMR techniques have even increased the potentialities of ^{1}H NMR [12-15]. At present the ^{1}H NMR techniques even compete with crystallographic methods in elucidating the structure of small proteins. The major advantage of the ^{1}H NMR methods may lie in the fact that they are in principle multi-parameter methods by which structural and dynamic data can be acquired at the same time [16]. This prompted us to undertake a detailed ^{1}H NMR study on M.elsdenii flavodoxin. In this paper we describe the exchangeability of amide protons against deuterons yielding information about the structural stability of the protein.
In addition some advanced techniques were used to assign resonance lines due to protons of groups in or close to the protein active center. Some priliminary results were reported elsewhere [17].

MATERIALS AND METHODS

Flavodoxin from M.elsdenii was isolated and purified according to published procedures [18]. Reduction and reoxidation experiments were conducted by the addition of the desired amount of a dithionite solution to the anaerobic solution of flavodoxin. 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 reduced solution followed by gentle mixing. Apoflavodoxin was prepared as described by Wassink and Mayhew [19]. The reconstitution was performed at pH 7 by addition of an excess of FMN followed by exhaustive dialysis. Wilmad 5 mm tubes of highest precision were used for all ^{1}H NMR experiments. Wilmad 10 mm precision tubes were used for the ^{15}N NMR measurements. The ^{15}N NMR measurements and the experiments using the Redfield pulse sequence were performed on a Bruker CXP-300 spectrometer. DOUBTFUL experiments were done on a Bruker HX 360 spectrometer. All other spectra were recorded on a Bruker WM 500 spectrometer. Normally 8K data points were used unless otherwise stated. ^{1}H Chemical shifts are reported relative to trimethylsilylpropionate (TSP).

Samples contained 3 mM flavodoxin in 100 mM potassium phosphate buffer (pH 8.3), except for the samples used for the two-dimensional technique and the ^{15}N NMR measurements which contained 8 mM flavodoxin in the same buffer. Measurements were performed at 33^{o}C, except for the temperature dependent studies. To reduce the water line in the NMR spectra, 100% $^{2}H_2O$ was used as a solvent in most experiments. When observation of labile protons was desired, a mixture of 10% $^{2}H_2O$ and 90% H_2O was used. In this mixed solvent the water line was diminished using two different techniques depending on the kind of experiment: i) continuous irradiation at the frequency of the water line except during the acquisition time, ii) by the Redfield pulse sequence [20] resulting in a 'hole' in the excitation spectrum at the frequency of the water line. The latter technique was slightly

modified for our experiments in that the normal sequence 2(+x)1(-x)4(+x)1(-x)2(+x) was improved by optimizing both the length and the phase of the -x pulse. This slight modification resulted in a drastically decreased excitation of the water hydrogens. A further advantage of the method is the disappearance of phase distortions in the resulting spectra.

Amide proton exchange was measured by following the decrease of the intensity of the amide protons after dissolving the protein in $^{2}H_{2}O$. At least eight spectra were used for the determination of the rate constant of the exchange reation.

Driven NOE difference spectra were recorded using the method of Dubs et al. [21]. An attenuation of 33 dB from 0.2 Watt was used for the saturation of a particular resonance line.

DEPT spectra were recorded using the method of Doddrell et al. [22], optimized for the $^{1}J(NH)$ coupling constants reported by Franken et al. [6]. The selective decoupling power after the DEPT pulse sequence was 60 dB attenuated from 20 Watt. The selective enrichment of FMN by the ^{15}N isotope at the positions N(1), N(3) and N(5) was achieved as described previously [23]. DOUBTFUL measurements were performed as described by Hore et al. [24]. The two dimensional COSY spectrum was obtained using the pulse sequence described by Aue et al. [25]. Groups of 16 recordings with different phases were added for each value of t_1 as described by Nagayama et al. [26]. The spectrum of Fig. 6 was obtained from 512 spectra of 2K data points with an increment in t_1 of 80 μs. The carrier frequency was at the low field end of the spectrum. The solvent resonance irradiation (15 dB attenuation from 0.2 Watt) was applied at all times, except during data acquisition. Before Fourier transformation of the time domain spectra, free induction decays were multiplied by a sine function (both in the t_1 and the t_2 direction). Furthermore, the time domain spectra were expanded to 2048 points in the t_1 direction and 4096 points in the t_2 direction by zero-filling to end up with a 1024x 1024 data matrix in the relevant frequency domain. The spectrum is shown in the

absolute value presentation. The spectrum was further improved by a symmetrization procedure in which the values of two corresponding points in the spectrum were replaced by the lowest value.

RESULTS AND DISCUSSION

The ^{1}H NMR spectra of oxidized and reduced M.elsdenii flavodoxin in H_2O and of the oxidized protein in $^{2}H_2O$ are shown in Fig. 1. Several resonance lines which are all shifted from the random-coil position are observed at the extremes of high and low field. These can therefore be regarded as conformational probes. At first glance most of the resonance lines remain at the same position in both redox states. This indicates that no gross conformational change occurs upon two-electron reduction of the protein, as already concluded from previous ^{1}H NMR measurements [27,28]. A closer inspection of the spectra shows that some resonance lines appear at a different field when going from the oxidized to the reduced state of the protein. Considering that the ring current effect of the bound flavin is undoubtedly different in the two redox states, one cannot conclude that the change in resonance position of some lines is caused by a conformational change. Such a conclusion requires a more detailed analysis of the NMR spectra. In the semiquinone state several resonance lines broaden beyond detection due to the paramagnetic center [10]. This observation will be used below to assign resonance lines due to groups in the active center.

Although the spectra of Fig. 1 show a remarkable resolution for a protein of this size (M_r = 15.000) it is evident that several resonance lines overlap. This renders specific assignments rather difficult. It is therefore not surprising that some previous assignments to flavin protons are incorrect [28], as will be shown below. Due to strong overlap of some lines we endeavoured to acquire the spectra at conditions optimized for the resolution, i.e. at the optimum temperature and the highest available magnetic fields.

Temperature and pH-dependent studies

The temperature dependence of the ^{1}H NMR spectrum of oxidized flavodoxin in H_2O

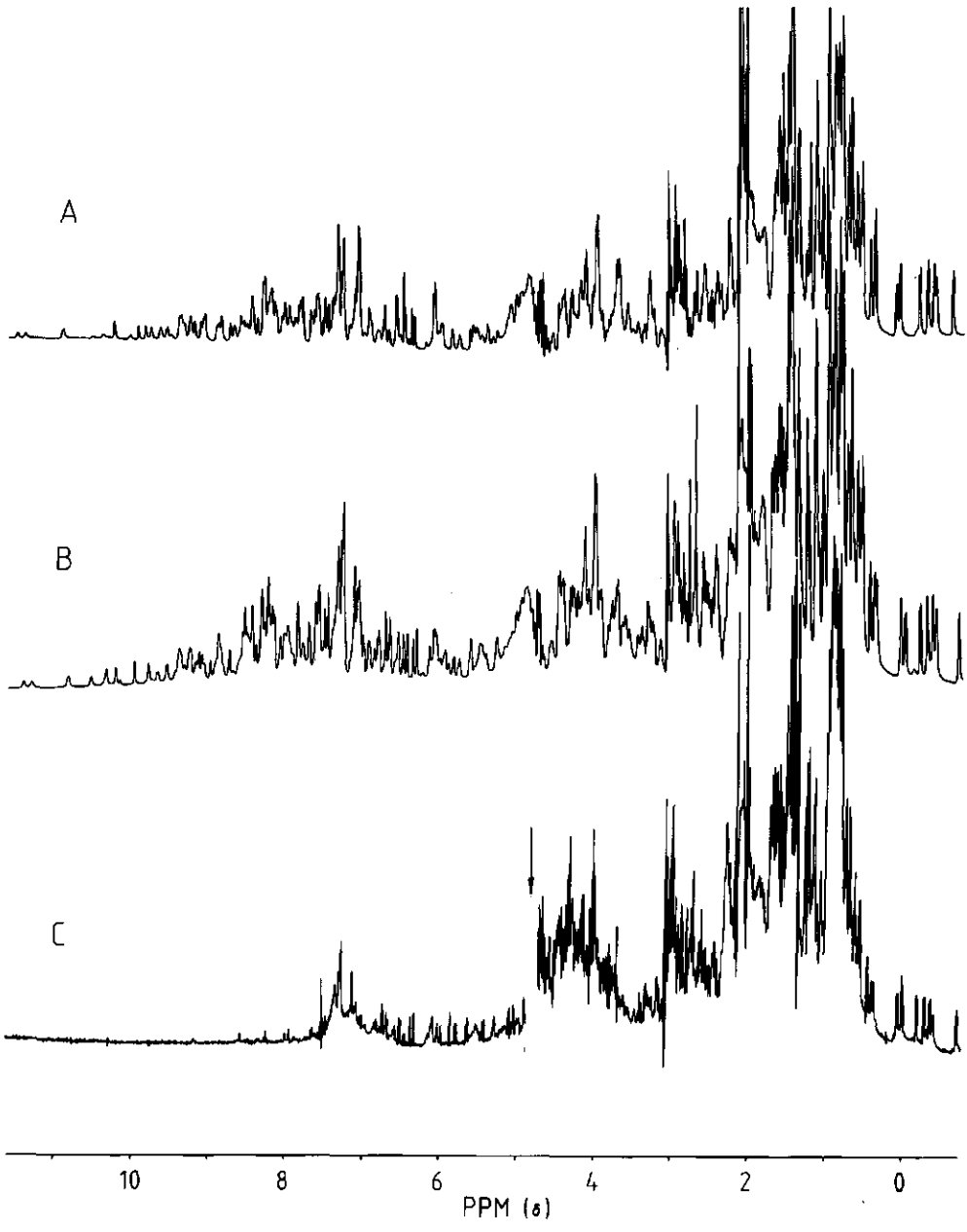

Fig. 1
^{1}H NMR spectra at 500 MHz of 8 mM oxidized (B) and reduced (A) M.elsdenii flavodoxin in 0.1 M phosphate buffer (pH 8.3) at 33°C (solvent is H_2O).
Spectrum C represents the oxidized protein in ^2H_2O (see text). All labile protons have been exchanged prior to the experiment. The arrow indicates the residual water line, which is omitted from the spectrum.
The resolution of all spectra was enhanced by a Lorentzian to Gaussian transformation. The small baseline distortion around 5 ppm is due to the water line, which is not ideally saturated.

was investigated between 8^{o} and 40^{o}C. Several resonance lines were followed throughout this temperature range. No change in chemical shift position could be detected for most resonances due to aliphatic and aromatic protons clearly indicating that there are no gross conformational changes in the temperature region investigated. Almost all amide resonances shift upfield upon an increase in temperature, however. The upfield shift varies with the different amide resonances, with a maximal shift of < 0.15 ppm for a temperature increase from 8^{o} to 40^{o}C. This behaviour is expected for amide resonances involved in hydrogen bonds [29] and does not indicate conformational changes. In the aromatic part of the spectrum no gross changes could be detected at all. In basic trypsin inhibitor, for example, temperature increase has been shown to cause a simplification of some resonance patterns due to tyrosine residues, i.e. from a AA'BB' to a simple AB pattern [30]. This phenomenon has been explained as an increase of the rotational motion around the $C(\beta)$ -$C(\gamma)$ axis of the tyrosine residue. The fact that we do not observe such changes for flavodoxin indicates that this rotational motion is either slow or fast throughout the temperature region investigated. A characteristic, observed throughout the whole spectra of the three redox states, is a change in apparent T_2 relaxation as determined from the linewidth. Increasing the temperature from 8^{o} to 33^{o}C causes a sharpening of the resonances, which is likely caused by a decrease of the rotational correlation time of the protein. Above 33^{o}C the resonance lines broaden with increasing temperature suggesting that conformational averaging influences the T_2 relaxation rates. Consequently most experiments were performed at 33^{o}C yielding an optimal resolution.

Varying the pH value of the solution from 6 to 8.5 has no influence on the position of individual resonances. It should be noticed that M.elsdenii flavodoxin contains no histidine residues [31,32].

Amide proton exchange

The NH protons exchange against deuterons of the solvent dissolving flavodoxin in $^{2}H_2O$. The rate constants of this exchange reaction give an indication on the

intramolecular fluctuations in the protein, since a partial opening of the structure of the protein is required for the exchange reaction to occur [33].

About 60% of the total amide protons present in M.elsdenii flavodoxin was calculated to exchange slowly enough to be observed after 5 min of dissolution of the protein. This number is close to values expected for globular stable proteins [29]. The number of 60% was derived by integration of the low field part (> 7.5 ppm) of a spectrum recorded immediately after dissolution of the protein in ^2H_2O. The integrated area of the NH peaks was subsequently normalized with the integral of the well resolved resonance line due to the methyl group resonating at -0.74 ppm.

In the oxidized as well as in the reduced state there are several NH groups which show a remarkable activation barrier against exchange. Some NH protons are still observed even after one month in ^2H_2O and at 33°C (pH 8.3). Spectrum C in Fig. 1 was in fact obtained after exchange of the labile protons in the apoprotein and subsequent reconstitution of the protein. These facts suggest that M.elsdenii flavodoxin is a remarkable stable protein.

Due to considerable overlap (Fig. 1) it was not possible to trace the individual exchange reactions of all NH protons. However, dissolving the protein in ^2H_2O leaves four well resolved NH proton resonances located at the extreme of the low field part (> 10 ppm) in the NMR spectrum (data not shown). The corresponding NH groups are hardly affected upon change of the redox state. The exchange rate was carefully measured by integrating the peak area at different times, after dissolution of the protein in ^2H_2O. The results are summarized in Table 1 and lead to the conclusion that the exchange rates for resonances 3 and 4 are similar in the two redox states, whereas the rates are different for the resonances 1 and 2. In addition it cannot be excluded that the relative positions of peaks 1 and 2 are reversed in the two redox states.

It has been shown that all NH protons exchange rapidly in apoflavodoxin [34]. Of interest is that the association constant for apoflavodoxin and FMN is about 20 times higher for the quinone than for the hydroquinone state. This difference in association constants is clearly not reflected in the NH exchange rates for

the two redox states. Apparently we are predominantly measuring the exchange rates of the complexes rather than those of the apoflavoprotein.

Recording the ^{1}H NMR spectrum in a mixture of $^{2}H_2O/H_2O$ (1:9, by vol.) instead of in pure $^{2}H_2O$ allows the detection of labile protons, which are already exchanged

Table 1. The rates of exchange of labile protons against deuterons of the solvent in oxidized and reduced M.elsdenii flavodoxin at 7°C and $p^{2}H$ = 8.3. The exchange rate is expressed as the time needed to reduce the original intensity of the resonances by 50%. The accuracy is ± 20%. The $t_{\frac{1}{2}}$ values <10s are extrapolated from saturation transfer experiments at 33°C and pH 8.3. Chemical shifts of all resonances are reported for 33°C for the sake of comparison with future experiments. Resonances indicated by an asterisk are located within 1 nm from the isoalloxazine ring, as deduced from the spectrum of the paramagnetic semiquinone state.

Resonance line	Oxidation state, chemical shift and exchange rates			
	Oxidized		Hydroquinone	
	ppm (δ)	$t_{\frac{1}{2}}$	ppm(δ)	$t_{\frac{1}{2}}$
1	11.41	3.0 h	11.50	5.6 h
2	11.31	10.8 h	11.40*	2.1 h
3	10.85	13.1 h	10.92	13.3 h
4	10.33	29.7 h	10.35	28.0 h
5	10.55	< 5 min	10.26*	< 5 min
6	10.21	< 5 min	10.02	< 5 min
7	10.00	< 5 min	9.97*	< 5 min
8	10.33	< 10 s	10.23	< 10 s
9	10.16	< 10 s	10.33	< 10 s
10	--	--	10.52	< 10 s

within 5 min after dissolution of the protein in ^2H_2O. In this way three NH protons, exchanging with a $t_{\frac{1}{2}} < 5$ min, could be studied. These protons together with the slow ones, are seen in Fig. 1, where well resolved resonances are present around 10 ppm (Table 1) which are absent in the spectrum recorded in ^2H_2O (data not shown).

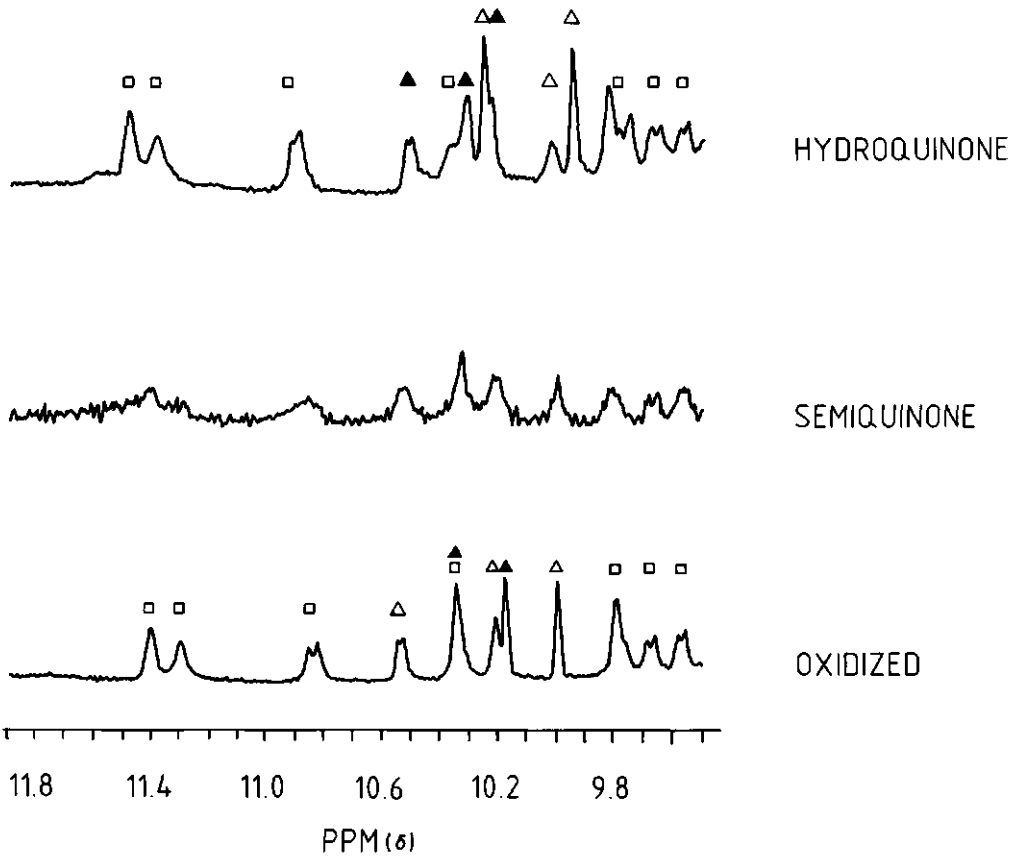

Fig. 2.
Low field part of the ^1H NMR spectra of 3 mM M.elsdenii flavodoxin in the three indicated redox states in 0.1 M phosphate buffer (pH 8.3) at 33°C (solvent is H_2O). The spectra were obtained by the modified Redfield pulse sequence (cf. Materials and Methods). Resonances marked with □, △ and ▲ represent labile protons exchanging with a $t_{\frac{1}{2}}$ of >> 1hr $\lesssim$ 5 min and <10s, respectively, (see also Table 1).

If labile protons exchange rather fast, i.e. with a lifetime even approaching the T_2 values, the resonances disappear from the spectrum by chemical exchange due to saturation transfer from the irradiated water protons [16]. The excitation of the water protons can be avoided by use of the Redfield pulse sequence [20] (cf. Materials and Methods) allowing the observation of the fast exchanging labile protons ($t_{\frac{1}{2}}$<10s). This is demonstrated in Fig. 2 for the well resolved low field part of the ^{1}H NMR spectrum. Compared with Fig. 1, three additional lines are seen in Fig. 2. The results are summarized in Table 1. It cannot be excluded that some resonances crossed in the spectrum upon reduction, which renders the connections difficult.

Reduction by one electron (i.e. formation of the paramagnetic semiquinone form) broadens some resonances due to labile protons beyond detection (Fig. 2). These resonances are marked in Table 1 and are due to labile atoms localized close (<1nm) to the flavin ring system.

Assignments

The analysis of the active center of M.elsdenii flavodoxin by ^{1}H NMR spectroscopy would be facilitated if the resonance frequency of the hydrogen atoms of the bound FMN was known. The resonances due to the C(6)H and C(9)H atoms are singlets in the quinone as well as in the hydroquinone state which should somewhat facilitate their assignments. As the prosthetic group can be easily removed from the holoprotein and the reconstituted protein yields identical ^{1}H NMR spectra, unambiguous assignments should be aided by reconstitution with modified flavins. Thus on substitution of the natural prosthetic group by 7-methyl-10-ribityl-isoalloxazine-5'-monophosphate (i.e. replacement of the methyl group at C(8) by a proton) the singlet at 7.12 ppm disappeared from the spectrum and the difference spectrum between spectra of native and reconstituted oxidized flavodoxin showed two doublets at 7.12 and 7.37 ppm and a singlet at 6.75 ppm. These doublets (^{1}J ~ 7Hz) likely originate from C(9)H and C(8)H of the flavin. With respect to the free flavin the resonances of C(9)H and C(8)H are shifted upfield by 0.61 and 0.42 ppm, respectively. These upfield shifts are in agreement with crystal-

lographic data on the related flavodoxin from Clostridium MP [1] from which it can be deduced that the ring current effect from Trp-90 is larger for C(9)H than C(8)H. In addition the upfield shifts of C(9)H for flavodoxin containing the natural and modified prosthetic groups are very similar, supporting the assignments. The previous assignment of the line at 6.68 ppm to C(9)H is therefore incorrect [28]. The difference spectrum also shows non-vanishing intensity for all resonances of Trp-91 for the frequencies, see [9]) and of a few other resonances. This effect is ascribed to a slight difference between the ring current effects of the two isoalloxazine rings and shows that Trp-91 is indeed located very close to the isoalloxazine ring, as demonstrated previously [9]. This effect renders however, assignments in rather crowded regions difficult. The resonance line at 6.75 ppm is therefore tentatively assigned to C(6)H. The assignment of the 7α and 8α methyl groups is, on the other hand, easy because in the region where these resonances are expected [35] the ^{1}H NMR spectrum of M.elsdenii flavodoxin is not very crowded. In the region between 2.6 and 2.8 ppm two sharp singlets are observed. Significantly the singlet at 2.77 ppm is absent in the NMR spectrum of flavodoxin containing the modified flavin. The resonances at 2.77 and 2.69 ppm are therefore assigned to the 8α and 7α methyl groups of the isoalloxazine ring, respectively (Table 2). This also means that the previous assignment of the resonances at 2.36 and 2.32 ppm to these methyl groups must be incorrect.

To obtain NMR spectra of M.elsdenii flavodoxin in the hydroquinone state care must be taken that no trace of semiquinone is present in the solution because the electron exchange rate between the semiquinone and the hydroquinone state can be very fast, as is the case under the conditions the spectra in this paper were recorded. In the semiquinone state the hydrogen atoms of the isoalloxazine ring are expected to have linewidths above 1kHz due to the paramagnetic center [10]. Therefore, the presence of only 1% semiquinone will increase the linewidth of the resonances of isoalloxazine hydrogen atoms with at least 10 Hz.

The reduction was performed with dithionite which redox potential decreases

with decreasing pH values [36]. It was therefore necessary to keep the samples above or at a pH value of 8.0. To avoid the ambiguities arising from the difference in ring current effects between the natural and modified protein-bound flavin we replaced the natural prosthetic group in M.elsdenii flavodoxin by [1,3,5-$^{15}N_3$] FMN with an isotopic enrichment of >90 atom %. Some assignments are subsequently established by ^{15}N NMR (Fig. 3). It has been shown [6] that the exchange frequency of the protons at N(3) and N(5) of flavin is slow in reduced flavodoxin as deduced from the observation of ^1J (^{15}N-^1H) coupling constants Although ^{15}N NMR is a rather insensitive method, due to the low gyromagnetic ratio, the existence of a clear coupling of the ^{15}N atom with the attached covalently bound hydrogen atoms should enable polarization of these nitrogen atoms using the DEPT method (cf. Materials and Methods). This approach appeared to enhance the signal to noise ratio appreciably. The spectra of Fig. 3 were obtained within a measuring time of one hour using the DEPT pulse sequence. The frequencies of the covalently bound protons were identified using specific irradiation during the detection period after the DEPT pulse sequence. First high power off resonance decoupling was applied to roughly identify the proton frequencies. Subsequently the irradiation frequency was varied in steps of 10 Hz around the roughly determined frequency. An attenuation of 60 dB from 20W was applied in the latter experiments. The spectra shown in Fig. 3 were obtained under optimal decoupling conditions of the N(3) and N(5) atoms of reduced flavodoxin. The N(3)H and N(5)H resonances are thus determined to be at 9.38 and 5.67 ppm, respectively (Table 2). In Fig. 3 a broad resonance line of small intensity is also observed near 120 ppm, a frequency where peptide nitrogens resonate [37]. This broad line most likely represents the natural abundance resonance of peptide groups. The N(1) atom of reduced protein-bound flavin carries no hydrogen atom and can therefore not be observed in the ^{15}N NMR spectra using the DEPT pulse sequence. It should be noted that the DEPT pulse sequence was unsuccessful for the assignment of the N(3)H group in oxidized flavodoxin in at pH 8.3 and 33°C, since

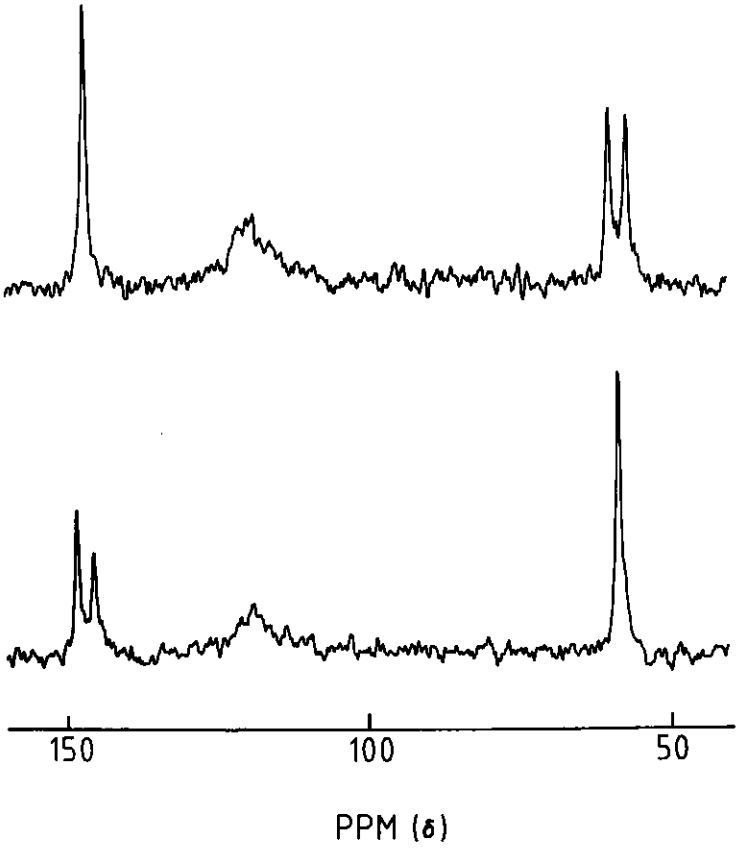

Fig. 3
^{15}N NMR spectra of reduced M.elsdenii flavodoxin (8 mM) in 0.1 M potassium phosphate buffer (pH 8.3) at 33°C (solvent is H_2O). Spectra were obtained with the DEPT pulse sequence (cf. Materials and Methods). FMN was enriched (>90%) with the ^{15}N isotope at the positions N(1), N(3) and N(5). In the upper spectrum the $^1J(^{15}N(3)-^1H)$ was specifically decoupled and in the lower spectrum that of $^1J(^{15}N(5)-^1H)$. The ^{15}N chemical shifts are reported relative to NH_3. For further details see text.

the ^{15}N NMR line due to the N(3) atom appeared as a broad singlet owing to a high exchange rate of the proton at N(3) with solvent water [6].

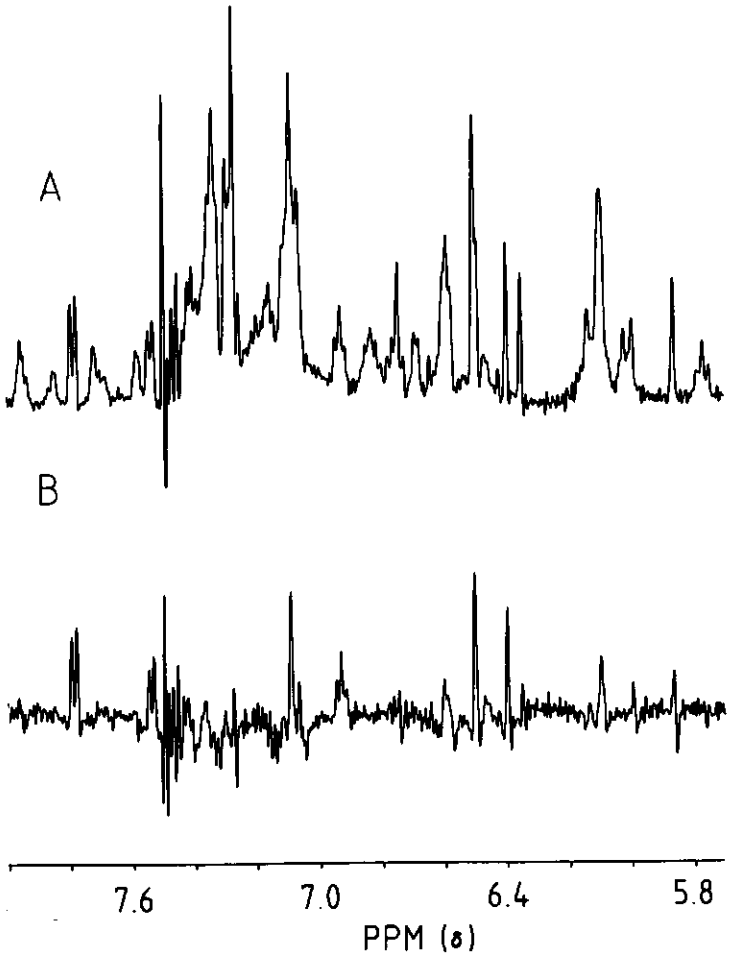

Fig. 4
The aromatic part of ^1H NMR spectra of the hydroquinone state of 3 mM M.elsdenii flavodoxin in 0.1 M potassium phosphate buffer (p^{2}H 8.3) at 33°C (solvent is ^2H_2O) is shown in the upper spectrum. The lower spectrum represents a difference spectrum between the upper spectrum and a similar spectrum from a solution consisting of about 2% semiquinone and of 98% of hydroquinone. For further details see text.

In an attempt to assign resonance lines to other protons of the reduced protein-bound flavin we made use of the very fast electron exchange reaction between the semiquinone and the hydroquinone state [10] and recorded difference NMR spectra between flavodoxin in the reduced form and mixtures of reduced flavodoxin containing a few percent of semiquinone. Such a difference spectrum is shown in Fig. 4 for only the low field part of the spectra. Only a few resonances remain in the difference spectrum. The protons due to these resonances must be located within about 0.8 nm from the paramagnetic flavin. Two sharp singlets remain in the difference spectrum at 2.10 and 2.09 ppm (data not shown). These resonances are tentatively assigned to the methyl groups at position 8α and 7α, respectively, of the prosthetic group. The resonances due to C(6)H and C(9)H are singlets and are expected to appear in the region between 6 and 7 ppm. In Fig. 4B singlets are observed at 7.09, 6.50, 6.39 and 6.09 ppm.

Further discrimination between the four resonances can be made by the determination of interproton resonances by measuring the cross-relaxation rate, manifested by the nuclear Overhauser effect (NOE). Although steady state ^1H-^1H NOE effects in proteins are rather unspecific due to spin diffusion [21], Kalk and Berendsen [38] have shown that the initial build up of the NOE is inversely proportional to the sixth power of the distance between the irradiated and the observed hydrogen atom. 'Driven NOE' experiments [39] on oxidized M.elsdenii flavodoxin are shown in Fig. 5. The resonance at -0.74 ppm is irradiated in these spectra. This line has already been assigned to one of the δ-methyl groups of Leu-62 [28,10]. Specific NOE effects are observed in the difference spectra. Applying irradiation times >1s renders the effects less specific. The closest hydrogen atoms appear at -0.23 ppm (the other δ-methyl of Leu-62 [28]), at 1.10, 4.01 and 6.32 ppm. Upon performing the same experiments for the hydroquinone state the resulting difference spectra show an NOE pattern extremely similar to that of the oxidized state, although the resonance positions are slightly changed, i.e. at -0.23, 1.06, 4.17 and 6.39 ppm. This observation also indicates that the

Table 2. Assignments of isoalloxazine and some other hydrogen atoms in the ^1H NMR spectra of oxidized and reduced M.elsdenii flavodoxin. Chemical shifts marked with an asterisk are tentative assignments.

Proton(s)	Chemical shifts (ppm) in M.elsdenii flavodoxin	
	Oxidized	Reduced
FMN		
N(3)H	?	9.38 ± 0.05
N(5)H	-	5.67 ± 0.05
C(6)H	6.75*	6.50*
C(9)H	7.12	6.09*
$CH_3(8\alpha)$	2.77	2.10*
$CH_3(7\alpha)$	2.69	2.09*
Others		
C(2)H, Trp-96	6.32	6.39
C(2)H, Trp-91	-	7.09*
δ-CH_3, Leu-62	-0.74	
δ-CH_3, Leu-62	-0.23	-0.23
γ-CH , Leu-62	1.10	1.06
α-CH , ?	4.01	4.17
C(4)H, Trp-91	7.61	--
C(5)H, Trp-91	6.80	--
C(6)H, Trp-91	5.57	--
C(7)H, Trp-91	6.41	--

conformation of the protein represented by the groups of these resonances is similar in both redox states. It should be noted that the small difference in frequency for the corresponding hydrogen atoms in the two redox states can be explained by an attenuated ring current effect of the flavin upon reduction [28]. In a previous paper [10] the distance between the δ-methyl group of Leu-62

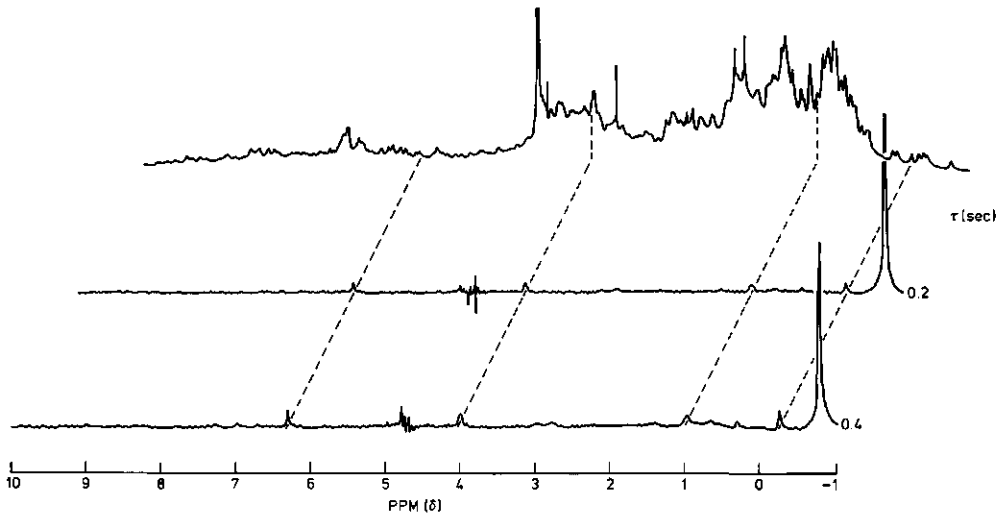

Fig. 5
Driven nuclear Overhauser ^1H NMR spectra of 3 mM M.elsdenii flavodoxin in the oxidized state in 0.1 M potassium phosphate buffer (p^2H 8.3) at 33°C (solvent is ^2H_2O). Upper spectrum represents a conventional ^1H NMR spectrum. The lower spectra are difference spectra resulting from the subtraction of a ^1H NMR spectrum in which the high field resonance was irradiated for a time τ indicated in the Figure and a similar ^1H NMR spectrum in which the irradiation frequency was shifted 200 Hz upfield from the first spectrum. Note that in the original spectrum in ^2H_2O several amide protons are present although the sample was kept 24 hours at 33°C (p^2H 8.3).

(-0.74 ppm) and the paramagnetic flavin was determined to be 1.02 nm. As a direct NOE effect can only be expected for hydrogen atoms at distances of < 0.3 nm from the irradiated group it can be concluded that the singlet at 6.39 ppm in the hydroquinone and the corresponding singlet at 6.32 ppm in the oxidized state cannot represent the C(6)H or the C(9)H atoms of flavin. A possible candidate for the resonance at about 6.3 ppm would be the C(2)H atom of Trp-96. This as-

signment is in accordance with the three-dimensional structure of the related flavodoxin from Clostridium MP [2-4]. The resonance is, as expected, also present in the difference spectrum of Fig. 4. The correctness of the assignment is further supported by the fact that the resonances belonging to Trp-91 have recently been assigned [9]. None of these resonances appear around 6.3 ppm. Based on similar arguments the lines at 1.1 ppm and at 4.01 and 4.17 ppm in the spectrum of oxidized and reduced flavodoxin respectively, are assigned to γ-CH of Leu-62 and a yet unidentified α-CH group of an amino acid residue respectively. These assignments reduce the possible candidates for the three residual singlets at 7.09, 6.50 and 6.09 ppm in reduced flavodoxin (Fig. 4). The resonance at 7.09 ppm most probably belongs to C(2)H of Trp-91, in line with previous assignments [9]. The remaining two resonances are tentatively assigned to C(9)H and C(6)H of the prosthetic group (Table 2).

Apart from the knowledge of interproton distances, the identification of the resonances due to the different amino acids must be known for a successful study of the structure [12]. As the spin system of an amino acid side chain is rather specific for each amino acid, the identification can be done by the determination of the through-bond 'J' connectivities throughout the side chain. Van Schagen and Müller [28] have shown that specific irradiation (decoupling) can be succussfully applied to achieve this goal. By this technique it has been shown [28] that the γ-CH group of Leu-62 resonates at about 1.1 ppm. However, since that time powerful two-dimensional NMR methods have been developed to arrive at a map of through-bond connectivities of the whole ^{1}H NMR spectrum in one two-dimensional spectrum. In Fig. 6 an example of a so-called COSY spectrum (cf. Materials and Methods) is shown of oxidized M.elsdenii flavodoxin in H_2O to demonstrate the applicability of this method. All off-diagonal peaks ('cross peaks') show J-coupling between the resonances obtained by horizontal and vertical projection of the cross peaks onto the diagonal, which represents the normal one-dimensional NMR spectrum. The clear pattern observed in Fig. 6 is encouraging

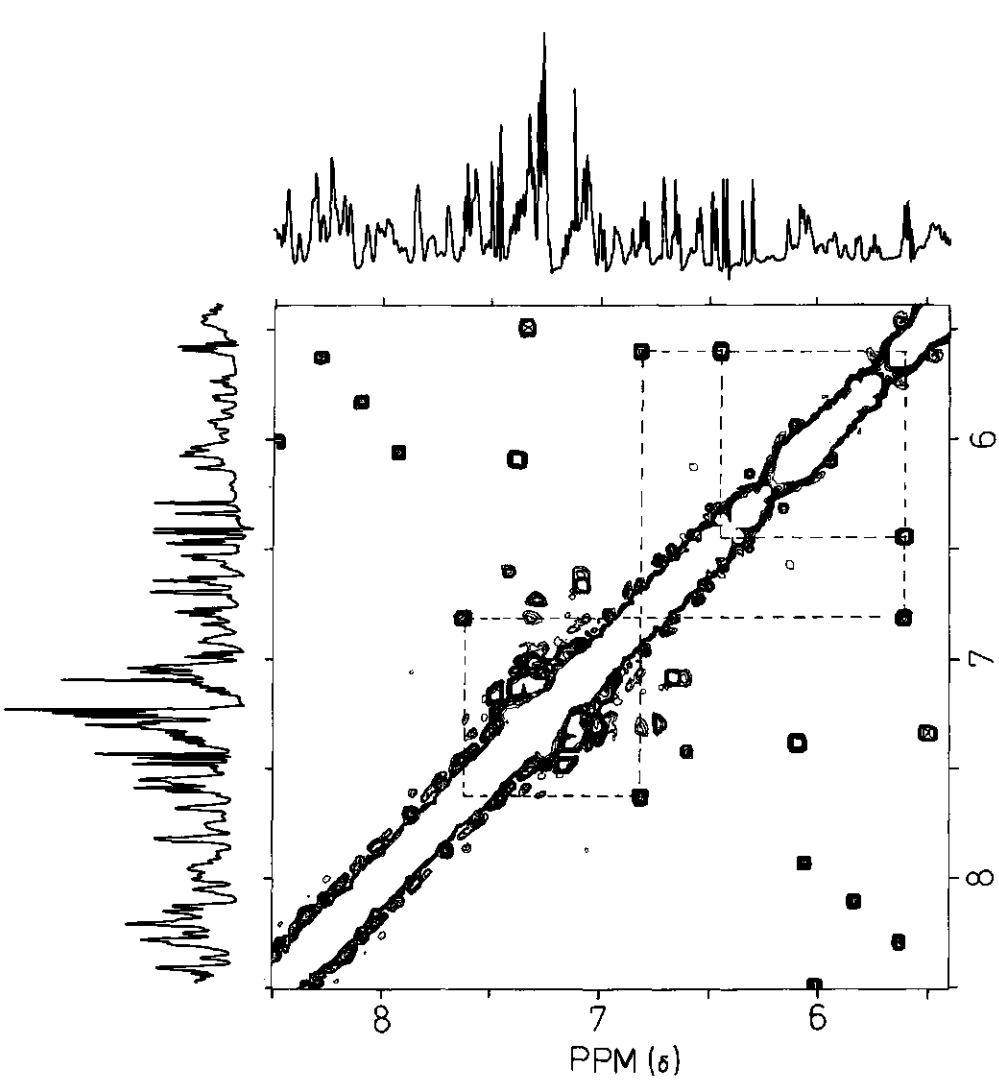

Fig. 6
Aromatic part of the contour plot of the COSY ^{1}H NMR spectrum of oxidized M.elsdenii flavodoxin in 0.1 M potassium phosphate buffer (pH 8.3) at 33°C (solvent is H_2O) (cf. Materials and Methods). For comparison the one-dimensional NMR spectra are also shown at the upper and left part of the figure. These one-dimensional spectra are optimally resolution-enhanced by a Lorentzian to Gaussian transformation. For experimental details see Materials and Methods. The outlined connectivity pattern represents the benzene part of the indole ring of Trp-91 (for the assignment, see [9]. The diagonal represents the conventional one-dimensional spectrum.

to start a detailed NMR study on this protein. This result was not obvious, beforehand, as line widths are larger than for the proteins investigated with these methods so far. During the transfer of magnetization by J-coupling, loss of magnetization due to T_2 relaxation is inevitable. Therefore, on increasing the size of the protein (and thus the T_2 relaxation times) the loss of magnetization during the COSY pulse sequence is also increased. Nevertheless, with this flavodxin it appears to be possible to arrive at strong cross peaks. In Fig. 6 the low field part of a COSY spectrum is shown which is chosen to compare it with results obtained earlier by a different technique [9]. The feature seen in Fig. 6 is the coupling pattern of the benzene part of the indole moiety of Trp-91 (Table 2), which is in perfect agreement with results obtained by a time-resolved photochemically induced dynamic nuclear polarization (CIDNP) study [9]. Trp-91 was shown to be at the surface of the protein but interacting strongly with the bound flavin, thereby shielding the isoalloxazine ring from solvent water. This explains the unusual shifts of the hydrogen atoms of the indole ring of Trp-91. Furthermore, the connectivity between the resonances near 6.66 and 7.06 ppm likely belong either to one or two tyrosine residues . This connectivity is confirmed by the DOUBTFUL technique (data not shown), which is based on double quantum coherence. In addition this tyrosine residue is not polarized by the CIDNP technique. Whether the C(3)H and C(5)H atoms of this tyrosine residue are magnetically slightly inequivalent, due to a limited rotation around the $C_\beta - C_\gamma$ axis, or whether two tyrosine residues are overlapping could not be determined unambiguously.

The 'Driven NOE' experiments and the preliminary COSY experiment show that this flavodoxin is amenable to a detailed two-dimensional ^1H NMR study which will be reported later. We hope to be able to solve the structure of the active cluster of M.elsdenii flavodoxin in detail by these techniques and to report the results in the near future.

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Chapter 11

THE USE OF TWO-DIMENSIONAL NMR SPECTROSCOPY AND TWO-DIMENSIONAL DIFFERENCE SPECTRA IN THE ELUCIDATION OF THE ACTIVE CENTER OF *MEGASPHAERA ELSDENII* FLAVODOXIN

Chrit T.W. Moonen, Ruud M. Scheek, Rolf Boelens and Franz Müller

SUMMARY

^1H-^1H "through bond" correlated (COSY) and ^1H-^1H "through space" (NOESY) two-dimensional NMR techniques were applied to study the structure of M.elsdenii flavodoxin in the oxidized and reduced state. It is shown that two-dimensional NOESY difference spectra between spectra of flavodoxin in the reduced and semiquinone state result in a spectrum representing only the active center of the fully reduced state. The sphere of the active center observed in the difference spectra can be varied easily by changing the relative amount of flavodoxin semiquinone in the second sample. The difference NOESY spectra simplified the analysis of the complex spectra. Resonances could be assigned to Ala-56, Tyr-89 and Trp-91, which are located in the direct vicinity of the protein-bound flavin. The relative positions and side chain dihedral angles of these residues are compared for the two redox states. Ala-56 and Tyr-89 show identical relative positions and dihedral angels in the two redox states, although the rotational motion of Tyr-89 is enhanced in the oxidized state. In both redox states Trp-91 is immobilized and extremely close to the prosthetic group. However, a small displacement of Trp-91 towards the N(5) atom of the flavin occurs upon reduction. The results obtained for Trp-91 are in excellent agreement with crystallographic results of the related flavodoxin from Clostridium MP. However, the latter studies showed a somewhat different position of the tyrosine residue compared with our results.

INTRODUCTION

Flavodoxin from Megasphaera elsdenii has a molecular weight of 15.000 daltons. The amino acid sequence is known (Tanaka et al., 1973, 1974). Flavodoxins contain riboflavin-5'-monophosphate (FMN) as a prosthetic group and function as electron carriers in biological reactions (Mayhew and Ludwig, 1975). The protein-bound flavin can occur in the oxidized, the semiquinone and the hydroquinone state. In biological reactions, however, flavodoxins do not make use of the oxidized state, owing to a high activation barrier between the oxidized and the semiquinone state (Moonen and Müller, 1982a, 1983a).

In a previous study we have shown by NMR methods that M.elsdenii flavodoxin is remarkably stable in the three redox states over a long period of time (Moonen and Müller, 1983b). It was concluded from these data that this flavodoxin appeared to be amenable to two-dimensional NMR spectroscopy. Recent developments in two-dimensional NMR spectroscopy (Wüthrich et al., 1982; Billeter et al., 1982; Wagner and Wüthrich, 1982; Wider et al., 1982) have shown that detailed structural information can be obtained for small proteins by these techniques (Steinmetz et al., 1981). In this paper we report on a two-dimensional ^1H NMR study with the aim to elucidate the structure of the active center of M.elsdenii flavodoxin. Although these two-dimensional NMR techniques have up to now only been applied to proteins of a molecular weight less than 10.000 daltons our results show that these techniques can also be applied succesfully to larger proteins, in spite of the higher complexity of the ^1H NMR spectra. Since the protein-bound flavin forms a stable radical we used this property of the protein in combination with difference NMR spectra to obtain information with regard to the structure of the active center of the protein.

MATERIALS AND METHODS

Flavodoxin from Megasphaera elsdenii was isolated and purified as previously described (Mayhew and Massey, 1969). Wilmad 5 mm precision tubes were used. All samples contained 8 mM flavodoxin in 0.1 M potassium phosphate, pH 8.3. A mixture of 10% ^2H_2O/90% H_2O was used as solvent. Anaerobiosis was achieved by carefully flushing the sample with argon for 20 min. The desired degree of reduction was achieved by adding an appropriate volume of a dithionite solution (Mayhew, 1978). Reduced or partially reduced samples were sealed after the reduction.

^1H-^1H correlated spectra were obtained by the "COSY" pulse sequence $(90^o\text{-}t_1\text{-}90^o\text{-}t_2)_n$ with an appropriate phase cycling to eliminate experimental artefacts (Nagayama et al., 1980).

^1H-^1H NOESY spectra were recorded with the sequence $(90^o\text{-}t_1\text{-}90^o\text{-}\tau_M\text{-}90^o\text{-}t_2)$ and the phase cycling as described by Anil Kumar et al. (1980). Magnetization ex-

change occurs during the mixing time τ_M for which normally 0.1 s was choosen, unless otherwise stated.

All spectra were recorded on a Bruker WM 500 spectrometer operating at 500 MHz. Quadrature detection was used for data acquisition. The spectral width was 12500 Hz with the carrier frequency at the left extreme. Normally 512 spectra of 1024 points were accumulated. Specific irradiation of 13-17 dB attenuation from 0.2 Watt was applied to saturate the protons due to water. The water protons were irradiated at all times, except during data acquisition. The repetition time needed to saturate the water protons sufficiently was 2.3 s. The acquisition of a two-dimensional spectrum lasted 30 to 50 h. The temperature was 33°C.

After data acquisition, all further data handling was carried out on a CYBER 170/760 computer of the University of Groningen, Groningen, The Netherlands. Zero-filling was applied to end up with a 512x512 point data matrix in the frequency domain. Before Fourier transformation, the time domain data matrix was multiplied with a sine bell for the COSY spectra and with a 45° shifted sine bell for the NOESY spectra, both in the t_1 and the t_2 direction. The length of the sine bell was adjusted so as to reach zero at the last experimental data point. After Fourier transformation the absolute value spectrum was calculated for the COSY spectra, whereas pure absorption spectra were obtained for the NOESY spectra after phase adjusting as described by States et al. (1982).

For the two-dimensional difference spectra, a baseline correction was applied first, omitting a region of about 0.3 ppm around the water line. Then the two spectra were normalized on the basis of an "off-diagonal peak" which represents a through space connectivity far from the active center. Subsequently, the subtraction was performed.

RESULTS AND DISCUSSION

Reduced flavodoxin

A COSY spectrum of two-electron reduced M.elsdenii flavodoxin is shown in Figure 1. The information in the region around 4.7±0.2 ppm is lost due to the

high power used to saturate the water protons. Several cross peaks are evident in the NH_i-αCH_i region of the spectrum. In principle each amino acid residue gives rise to one such peak, with the possible exception of proline and glycine, and residues which are exposed to solvent. Some spin systems can be identified from Figure 1. The doublet at 0.11 ppm for example has already been assigned to the methyl group of Ala-56 (Moonen and Müller, 1983a). From the COSY spectrum we find the α-CH resonance at 2.66 ppm and that of the amide proton at 6.47 ppm. The doublets at -0.65 and -0.23 ppm have previously been assigned to the δ-CH_3 groups of Leu-62 (Van Schagen and Müller, 1981). These methyl groups couple with the same proton resonating at 1.06 ppm. The doublets (methyl groups) at 0.03 and -0.39 ppm also couple with a common proton at 0.90 ppm. The triplet (one methyl group) at -0.43 ppm probably represents the δ-CH_3 group of a Ile residue as a coupling is observed with two non-equivalent hydrogen atoms at 0.73 and 1.51 ppm. In the conventional ^1H NMR spectrum (see Figure 1) three doublets and one triplet are observed between -0.15 and -0.45 ppm. Interestingly, the COSY spectrum clearly shows that two additional resonances are present in this region, i.e. at -0.19 ppm, coupled with a hydrogen atom resonating at 1.37 ppm, and a group at -0.36 ppm, coupled with a hydrogen atom resonating at 1.20 ppm. These additional resonances are "hidden" in the conventional NMR spectrum. Although several coupling patterns can be demonstrated in Figure 1 unambiguous assignment of the complete spin system of a particular amino acid residue appeared to be possible only for simple spin systems, e.g. the Ala-56. This is due to the fact that the region between 2.4 and 0.8 ppm is overcrowded, even in the two-dimensional spectrum (see below). We expected to observe the coupling pattern of the indole moiety of Trp-91 in the aromatic region of the spectrum (Figure 1), as previously observed for the oxidized protein by a different NMR technique (Moonen et al., 1982b). This pattern is not found in Figure 1.

To aid further assignments the results of Figure 1 are complemented by results obtained by through-space connectivities as observed by the determination of cross-correlation rates by the nuclear Overhauser effect (Anil Kumar et al., 1980). The NOESY spectrum (Figure 2), obtained with a mixing time of 0.2 s, shows that

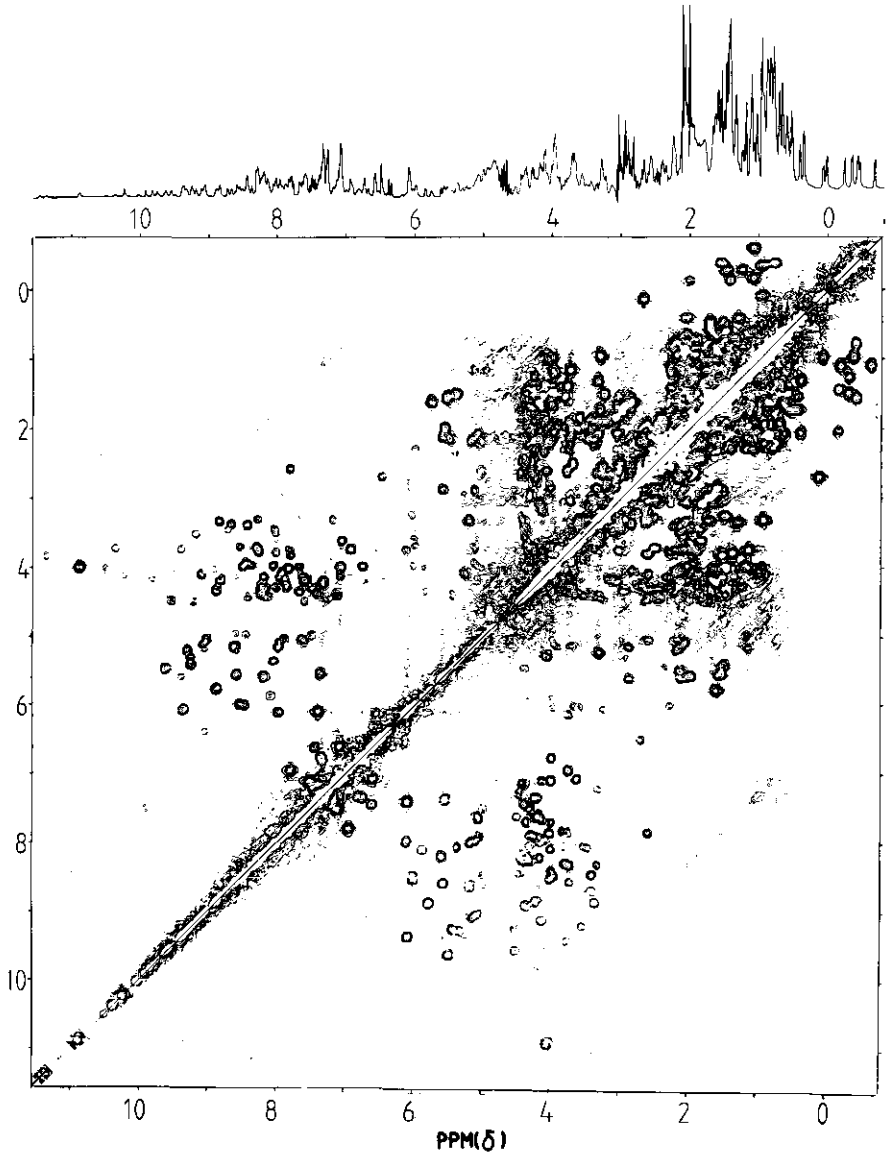

Figure 1: Absolute value "contour" plot of a ^{1}H-^{1}H correlated (COSY) spectrum of the hydroquinone state of M.elsdenii flavodoxin in 0.1 M potassium phosphate buffer (pH 8.3). The solvent consists of 90% H_2O and 10% $^{2}H_2O$. The diagonal represents the normal one-dimensional spectrum. All off-diagonal "cross-peaks" represent a through-bond J-connectivity between the resonances found by horizontal and vertical projection onto the diagonal. For comparison the one-dimensional spectrum (resolution enhanced by a Lorentzian to Gaussian transformation) is shown in the upper part of the spectrum. For further details, see Materials and Methods.

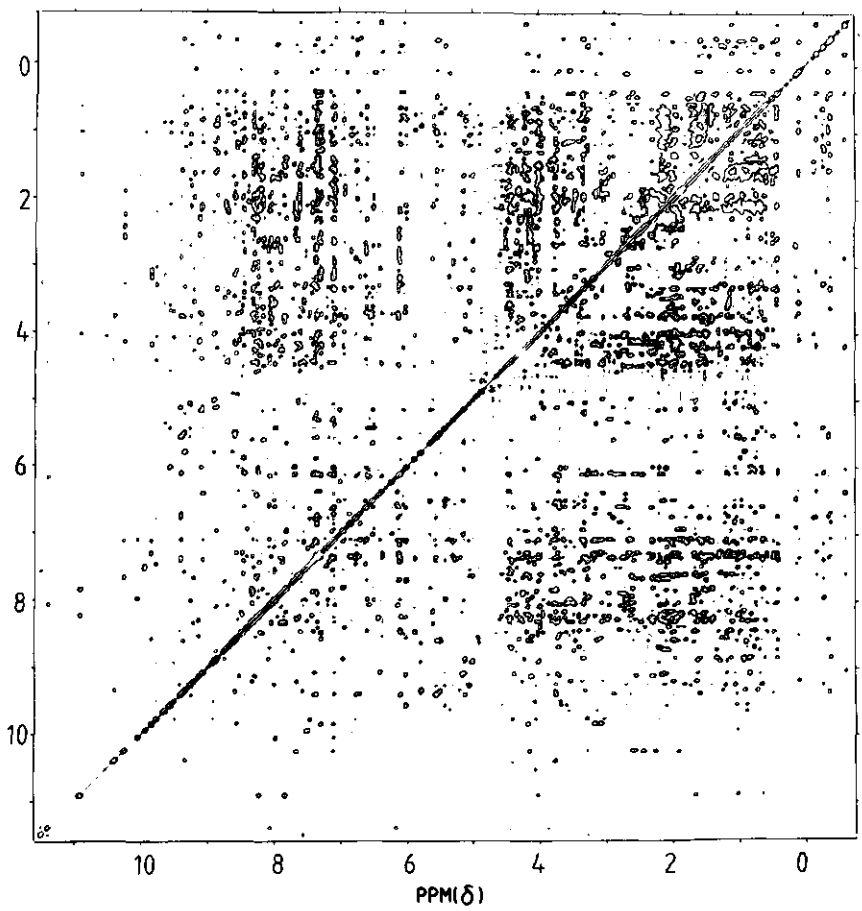

Figure 2: Pure absorption (contour) plot of a ^1H-^1H NOESY spectrum of the hydroquinone state of M.elsdenii flavodoxin in 0.1 M potassium phosphate buffer (pH 8.3). Solvent consists of 90% H_2O and 10% ^2H_2O. A mixing time τ_M of 0.2s was used. Cross peaks represent ^1H-^1H through-space NOE effects between the resonances found by horizontal and vertical projection on the diagonal. For further details, see Materials and Methods.

the pure absorption representation results in a much better resolution than the absolute value representation of the COSY spectra (Figure 1). Similar through space contacts for well resolved resonances were also observed by conventional driven NOE measurements (Moonen and Müller, 1983b) for some well resolved resonance lines and appear to be identical in the spectra obtained by both techniques. In Figure 2 a large number of connectivities is observed, as expected for a pro-

tein of this size. For example, the β-CH_3 (0.11 ppm) of Ala-56 transfers its magnetization to the α-CH (2.66 ppm) and the NH group (6.47 ppm) of the residue. Sequential assignments are rather difficult however, again because of severe overlapping of resonance lines. All the same a dramatic simplification of the spectra can be achieved by utilizing the kinetic properties i.e. the negligible activation energy of the electron exchange reaction between the semiquinone and the hydroquinone states (Moonen and Müller, 1983a). In addition the structures of the two redox states are very similar.

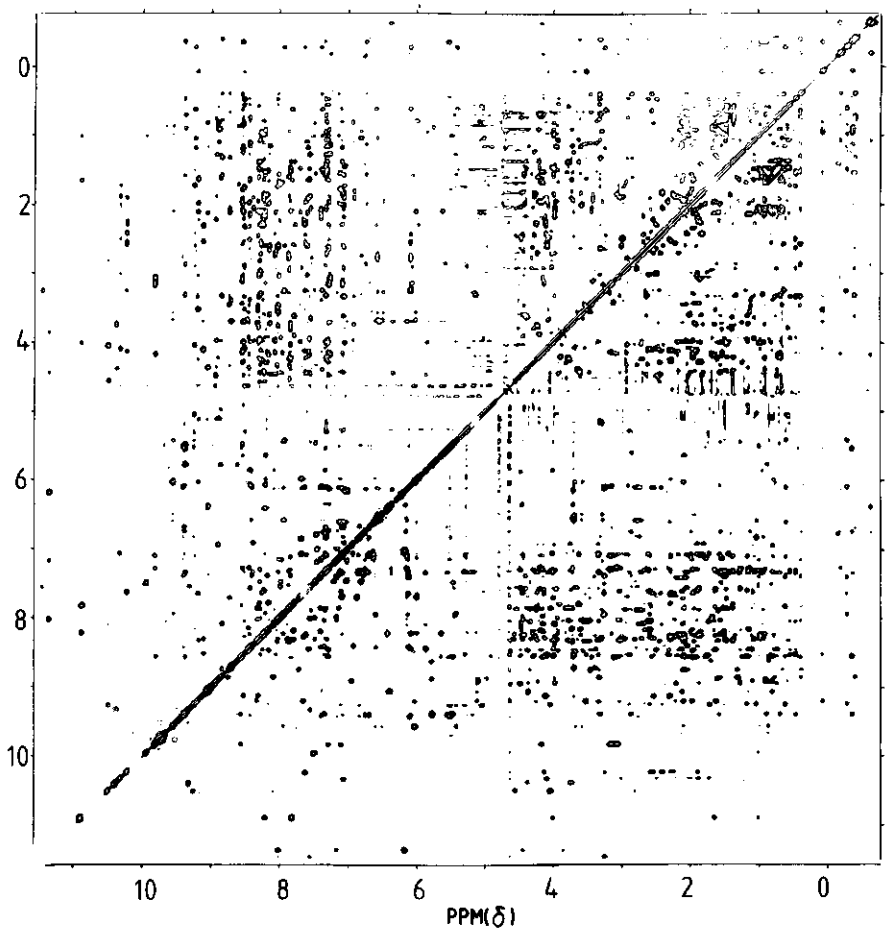

Figure 3: Pure absorption (contour) plot of a ^1H-^1H NOESY spectrum of approximately 90% hydroquinone and 10% semiquinone state of M.elsdenii flavodoxin in 0.1 M potassium phosphate buffer (pH 8.3). Solvent consists of 90% H_2O and 10% ^2H_2O. A mixing time τ_M of 0.1 s was applied. For further details, see Materials and Methods.

In flavosemiquinone most of the spin density is confined to the pyrazine moiety of the isoalloxazine ring (Müller, 1983). Owing to the paramagnetism of the one-electron reduced protein-bound prosthetic group the resonances due to protons in the vicinity of the semiquinone will be broadened. Since the electron exchange reaction is diffusion determined the presence of for instance 10% of the semiquinone in the solution studied, has the same effect on the resonances as placing 0.1 of an electron on the flavin of each protein molecule. In Figure 3 a NOESY spectrum is presented of a solution of M.elsdenii flavodoxin consisting of about 90% hydroquinone and about 10% semiquinone. Despite the presence of the paramagnetic center, it appeared to be possible to sufficiently saturate the water protons, indicating that the flavin is fairly well shielded from the solvent. Most of the connectivities observed in Figure 2 are also present in Figure 3. Other contacts are clearly removed from the spectrum. In fact, Figure 3 represents a NOESY spectrum of the hydroquinone state minus the environment of the flavin in the paramagnetic state. Two different mechanisms cause the disappearance of the active center protons in Figure 3, i.e. broadening and a leakage mechanism occurs due to the paramagnetism. The latter competes with the magnetization transfer for hydrogen atoms near the flavin during the mixing time. This renders the effects of the paramagnetism more pronounced in the NOESY spectrum than in the COSY spectrum (data not shown). In Figure 4 a difference two-dimensional NOESY spectrum is shown resulting from a subtraction of a spectrum of the hydroquinone state and of the spectrum presented in Figure 3. Both experiments were performed under identical conditions (c.f. Materials and Methods). Figure 4 is thus a NOESY spectrum of only the active center of the hydroquinone state of M.elsdenii flavodoxin. It is estimated that interproton connectivities in Figure 4 extend to a limit of about 1 nm from the paramagnetic center. This distance can be adjusted by varying the concentration of the semiquinone. In this way a series of difference spectra can be produced containing different structural information. At any rate it can be seen from Figure 4 that the problem of overcrowded regions is greatly reduced by this technique and that assignments are facilitated. For example,the proton at N(5) of flavin resonates at 5.67±0.05 ppm (Moonen and Müller, 1983b). A strong NOESY connectivity is found

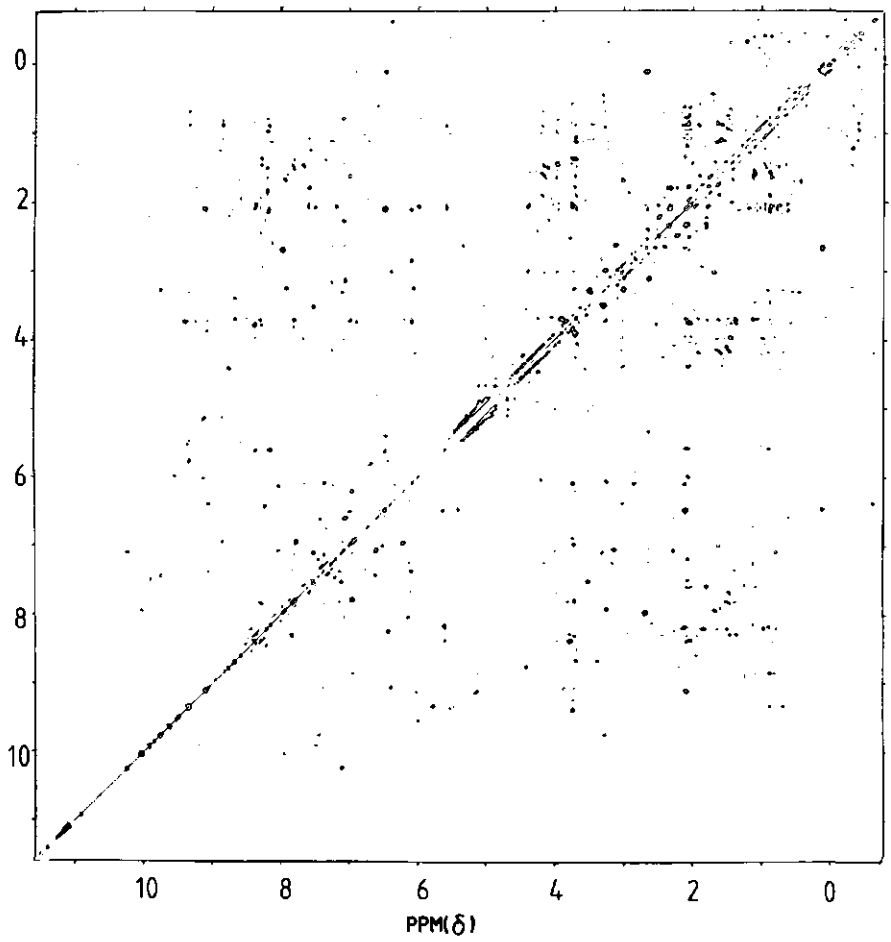

Figure 4: Difference ^{1}H-^{1}H NOESY spectrum (contour plot) in pure absorption phase, representing the NOESY spectrum of only the active center of the hydroquinone state of M.elsdenii flavodoxin (sphere of the active center is estimated to be about 1 nm). The spectrum is obtained by a subtraction of a NOESY spectrum of 100% reduced and the NOESY spectrum presented in Figure 3. The two spectra were recorded under the same experimental conditions. Mixing time τ_m was 0.1 s for both spectra. For further details, see Materials and Methods.

between the resonance at 5.63 ppm and a singlet at 6.50 ppm. The proton at N(5) of flavin is undoubtedly very close (<0.25 nm) to the proton at C(6) of flavin. The singlet at 6.50 ppm is therefore assigned to C(6)H of the prosthetic group. Using a mixing time of 0.2 s a connectivity is also observed between the resonance at 5.63 ppm and that at 7.79 ppm, which is a doublet. This doublet is assigned to

C(2)H of a tryptophan residue, it shows a strong coupling (Figure 1) and a strong NOE connectivity to a resonance at 6.94 ppm (a well resolved triplet). The triplet, on the other hand, exhibits NOE connectivities with the line at 7.79 ppm and a resonance line at 7.10 ppm. The latter is obscured by several overlapping resonances. In Figure 1 the COSY connectivity between the resonances at 6.94 ppm and 7.10 ppm is partially obscured by the broad diagonal (due to the absolute value presentation). The obscured resonance line at 7.10 ppm shows clear COSY and NOE connectivities with the well resolved doublet at 7.53 ppm. Thus the doublet at 7.79, the triplet at 6.94, the multiplet at 7.10 and the doublet at 7.53 ppm are assigned to Trp-91 (see Table 1). These assignments are further supported by the weak NOE connectivity between the resonances at 6.94 and 7.53 ppm, when a mixing time of 0.2 s is applied (Figure 2). Moreover from the presence of the NOE connectivities in Figure 4 and previously published one dimensional difference spectra (Moonen and Müller, 1983b) it must be concluded that the tryptophan residue is close to the isoalloxazine ring system. Other resonances of Trp-91 can be assigned from Figures 2 and 4 . The group due to the resonance at 7.53 ppm exhibits a strong NOE connectivity with the resonance line at 3.50 ppm. Using a mixing time of 0.2 s a weak NOE connectivity to the line at 3.28 ppm is observed. The two resonances at 3.50 and 3.28 ppm show a strong mutual NOE connectivity, also present in Figure 4, indicating a small distance to the flavin ring. In addition they show a NOE connectivity to a resonance at 5.04 ppm which in turn is close to the group resonating at 7.53 ppm (Table 1). Thus, the resonances at 3.50 and 3.28 ppm represent the two nonequivalent C(β) protons and the resonance at 5.04 ppm represents the C(α) proton of Trp-91. The resonance line at 7.08 ppm is assigned to the C(2)H of Trp-91 as the line at 3.28 ppm shows a strong NOE effect on that at 7.08 ppm. The C(α)H group also exhibits a small NOE effect on the resonance at 7.08 ppm, though only with a 0.2 s mixing time. It should be noticed that no COSY connectivity for C(α)H-C(β)H was observed (Figure 1). The absence of such connectivities is unfortunately a rather common feature for the technique used in Figure 1 (cf. below).

The intraresidue NOESY connectivities allow a rough determination of the dihedral angles χ_1 and χ_2 of the side chain of Trp-91, like previously calculated

from time-resolved photochemically induced dynamic nuclear polarization spectra (Moonen et al., 1982). The results agree most with $\chi_1 = 60^oC$ and $\chi_2 = \pm 90^oC$.

Further NOE connectivities are observed between the line at 6.59 ppm and the resonances at 7.06 and 7.42 ppm (Figure 4). The resonances at 6.59 and 7.42 ppm are doublets, whereas the multiplet pattern of the resonance at 7.06 ppm could not be determined due to severe overlapping. As both connectivities are also found in the COSY spectrum (Figure 1) the resonances probably belong to a tyrosine residue with non-equivalent C(2,6) protons and two overlapping C(3,5) protons. Another strong NOE connectivity is found between the resonance line at 7.06 ppm and a resonance at 3.12 ppm, and a weak one with the line at 2.63 ppm. The latter two resonances show strong mutual NOE and COSY connectivities, indicating that they represent the two non-equivalent C(β) atoms of the same tyrosine residue. The corresponding C(α)H is found at 5.33 ppm which shows a strong NOE connectivity with one of the C(β) protons (2.63 ppm) and a weak one with the other C(β) proton (3.12 ppm). The assignments are confirmed by the fact that these resonances are absent in Fig. 3 indicating the close distance to the flavin. The assignments are summarized in Table 1. Again unfortunately, no COSY connectivity could be detected between C(α)H and the $C(\beta)H_2$. The dihedral angles of the side chain can be estimated from the NOESY intraresidue connectivities, i.e. χ_1 must be close to 180^o or 60^o, and χ_2 is likely close to 0^o.

The resonances due to Ala-56 have been assigned above, based on the results of Figure 1 (Table 1). The intraresidue NOE connectivities for this residue are also clearly observed in Figure 4, supporting the assignments and the conclusion that this residue is located close to the flavin. Interestingly, Ala-56 is also very close to the above mentioned tyrosine residue as clear connectivities are found between the β-methyl group of Ala-56 (0.11 ppm) and the C(α)H of the tyrosine residue. In addition a weak connectivity is found between the β-methyl of Ala-56 and the C(2,6) protons of the tyrosine residue. Combining these observations and the crystallographic data on the related flavodoxin from Clostridum MP it is justified to assign these resonances to Tyr-89.

Having assigned three spin systems to specific amino acid residues in the immediate vicinity of the flavin, one would like to give the sequential backbone

assignments, as established by Wüthrich et al. (1982). Although several so-called d_1, d_2 and d_3 connectivities can be observed in Figure 4, the analyses are hampered by the fact that almost no clear COSY connectivities could be established for more complicated spin systems, i.e. when two nonequivalent C(β) protons are present, as shown by Nagayama and Wüthrich (1981). In some cases some probable connectivities could be determined from NOE effects, but assignments to specific amino acid residues would be too speculative and are, therefore, not further explored in this paper.

Table 1. Assignments of resonances observed in two-dimensional ^1H NMR spectra of M.elsdenii flavodoxin in the oxidized and reduced state.

Residue	Assignment and chemical shift in ppm		
	Atom(s)	Reduced	Oxidized
Ala-56	NH	6.47	6.31
	α-CH	2.66	2.32
	β-CH_3	0.11	-0.04
Leu-62	δ-CH_3	-0.65	-0.74
	δ-CH_3	-0.23	-0.23
	γ-CH	1.06	1.10
Tyr-89	NH	n.o.	n.o.
	α-CH	5.33	5.26
	β-CH_2	2.63, 3.12	2.90, 3.14
	C(2,6)H_2	7.06, 7.42	7.06
	C(3,5)H_2	6.59	6.66
Trp-91	NH	n.o.	n.o.
	α-CH	5.04	4.88
	β-CH_2	3.28, 3.50	3.38
	C(2)H	7.08	7.09
	C(4)H	7.53	7.61
	C(5)H	7.10	6.80
	C(6)H	6.94	5.57
	C(7)H	7.79	6.41
Isoalloxazine	N(5)H	5.63	--
	C(6)H	6.50	n.o.

n.o. = not observed.

On the basis of the crystal structure of the related flavodoxin from Clostridium MP (Burnett et al., 1974) the resonance at -0.65 ppm was already assigned to one of the C(δ) methyl groups of Leu-62 (Moonen and Müllller, 1983b). No safe assignments could be established for the whole side chain of Leu-62 using the combined NOESY and COSY results. The resonance at -0.65 ppm however gives a clear NOE connectivity to the singlet at 6.38 ppm, which in turn shows a NOE connectivity to the C(α)H of Ala-56, indicating a small distance between the methyl group of Leu-62 and the β-CH_3 of Ala-56. To arrive at reliable sequential assignments, additional experiments are needed, such as double and triple quantum coherence experiments of flavodoxin in pure ^2H_2O.

Oxidized flavodoxin

The assignments of resonances to Ala-56, Tyr-89 and Trp-91 in the hydroquinone state of the protein facilitate the assignments of resonances to these amino acid residues in the oxidized state of the protein, because most resonances are fortunately well resolved. The COSY-spectrum of oxidized flavodoxin shows the complete spin system of Ala-56 (Table 1), although the COSY connectivity between the C(α)H and the NH is considerably weaker than in the hydroquinone state. The connectivities are also clearly observed in the NOESY spectrum (Figure 5). The β-CH_3 of Ala-56 shows in addition a NOE connectivity to a resonance at 5.26 and a doublet at 7.06 ppm. This doublet belongs to Tyr-89 (Table 1). The resonance at 5.26 ppm shows a strong NOE connectivity with the resonance at 2.90 ppm. This in turn exhibits strong NOE connectivities with the lines at 7.06 and 3.14 ppm. A strong NOE connectivity is also present between the lines at 3.14 and 7.06 ppm. Therefore, the resonances at 2.90 and 3.14 ppm are the two non-equivalent C(β)H atoms and the resonance at 7.06 probably represents the two equivalent C(2,6)H atoms of Tyr-89. The C(3,5)H atoms are easily identified from the strong NOE and COSY connectivities between the resonance at 7.06 and 6.66 ppm. Thus the C(3,5)H atoms are also equivalent. Weak NOE connectivities are found between the C(β)H atoms and the doublet at 6.66 ppm (Table 1). No COSY connectivity was found between the C(α)H and one of the C(β)H atoms in the quinone state either, as already discussed for the hydroquinone state.

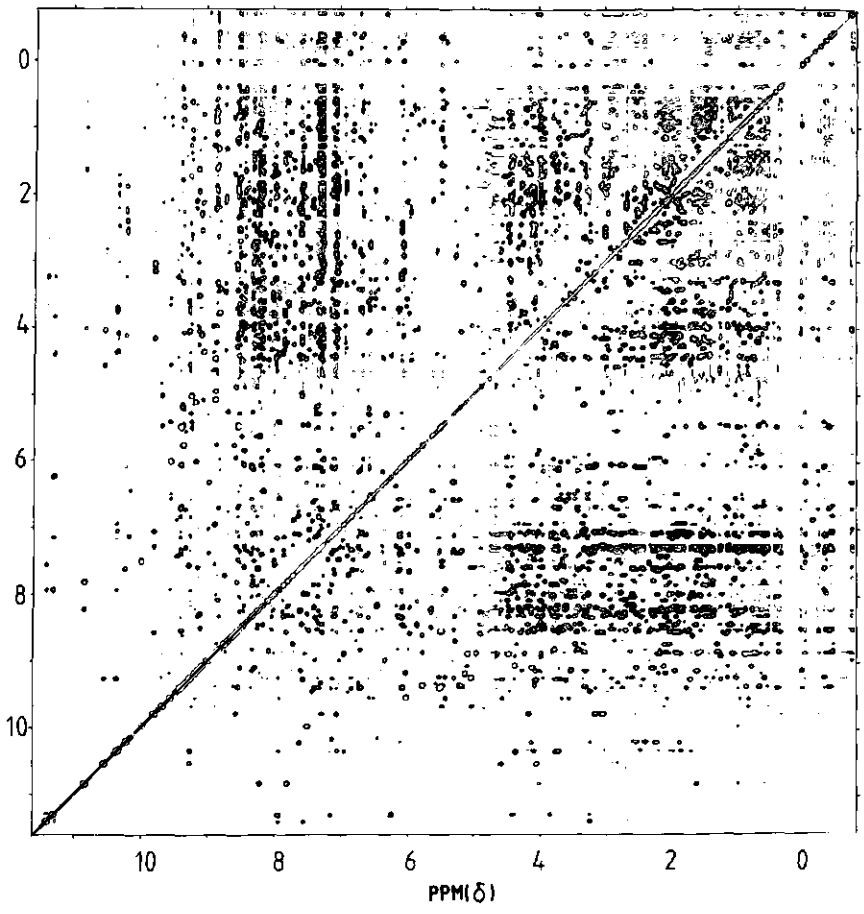

Figure 5: Pure absorption (contour) plot of a ^{1}H-^{1}H NOESY spectrum of the oxidized state of M.elsdenii flavodoxin in 0.1 M potassium phosphate buffer (pH 8.3), solvent consists of 90% H_2O and 10% $^{2}H_2O$. Mixing time τ_M was 0.1 s. For further details, see Materials and Methods.

The complete spin system of Trp-91 has been assigned previously using another technique (Moonen et al., 1982b). The assignments are confirmed by both NOE and COSY connectivities. Here again no COSY connectivity was observed between the C(α)H and one of the C(β)H atoms of Trp-91.

It is now possible to discuss the changes in structure and mobility upon reduction for the three assigned residues and a detailed comparison is allowed with

the crystallographic results of the related flavodoxin form Clostridium MP (Burnett et al., 1974; Smith et al., 1977; Ludwig et al., 1976). It has already been shown (Moonen et al., 1982) that Trp-91 in the quinone state is immobilized and that its conformation in solution is identical to that described for the crystal structure of Clostridium MP flavodoxin. This is confirmed in this study. The strong NOE connectivities in the hydroquinone state show that Trp-91 is also immobile. For Clostridium MP flavodoxin (Burnett et al., 1974) it has been demonstrated that the C(7)H atom of Trp-90 is the nearest atom to the N(5) atom of the flavin. This also holds for Trp-91 in M.elsdenii flavodoxin in solution, as evidenced by the NOE connectivity between N(5)H and C(7)H of Trp-91. The chemical shift of the different atoms of Trp-91 indicates their relative position with respect to the flavin, as deduced previously (Moonen et al., 1982). However, it should be noted that the ring current effect of flavin in the hydroquinone state (especially of the pyrazine subnucleus) is decreased as compared with that of the oxidized state. This fact should be considered when comparing the chemical shifts of the two states. Nevertheless, the C(7)H of Trp-91 differs by 1.38 ppm in the two redox states, i.e. a strong upfield shift occurs in the oxidized state and a small downfield shift in the hydroquinone state as compared to that of free tryptophan. This shows that the atom in question must be right above the isoalloxazine ring in the oxidized state and somewhat displaced in the hydroquinone state, i.e. towards the N(5) atom of flavin as deduced from NOE effects. The combined results show that the indole ring of Trp-91 moves towards the N(5) atom of flavin on reduction but the angle between the indole ring and the isoalloxazine ring is hardly affected. The observed displacement, the relative position of the two planes and the side chain dihedral angles are in excellent agreement with the crystallographic data of Clostridium MP flavodoxin (Smith et al., 1977). In the latter study it was pointed out that Trp-90 is particularly susceptable to the influence of intermolecular contacts. Nevertheless the results presented in this paper give strong evidence that the position of Trp-91 in M.elsdenii flavodoxin in solution is identical to Trp-90 in crystallized MP Clostridium flavodoxin.

In both redox states the NOE connectivity found between the NH and the C(β) protons of Ala-56 is in accordance with the crystal structure of Clostridium MP

flavodoxin. This is also true for the NOE connectivity between the C(α)H of Tyr-89 and the C(β) protons of Ala-56. However, the side chain dihedral angle of Tyr-89 differs from that of Tyr-90 in Clostridium MP flavodoxin. A dihedral angle χ_1 of about 60^o is predicted for Tyr-89 from the crystal structure of MP Clostridium flavodoxin. This implicates that the NOE connectivity between the C(α)H and both C(β)H atoms should be nearly identical. Our study demonstrates, however, that one connectivity is very strong and the other is absent, indicating a dihedral angle of 180^o or -60^o. A similar analysis shows that χ_2 is probably 0^o, in contrast to the value of $\pm 90^o$ in the crystal structure. Moreover the NOE connectivity between the C(β) protons of Ala-56 and one or both of the C(2,6)H atoms of Tyr-89 in the hydroquinone state is not obvious, considering the crystal structure of Clostridium MP. Our study indicates that the relative position of Ala-56 and the side chain of Tyr-89 differ from that in crystals of Clostridium MP flavodoxin. In addition, we have shown that the relative position of the two residues are identical in the quinone and the hydroquinone state, but the rotational motion around the C(β)-C(γ) axis differs in the two redox states. Equivalent C(3,5)H atoms are found in the quinone state and non-equivalent ones in the hydroquinone state. This indicates that the "flipping" rate in the quinone state must be much faster than in the hydroquinone state. As strong NOE connectivities are found between one of the C(β) protons and the C(2,6)H atoms the frequency of the rotational motion must be within the range of 10^3 and 10^9 Hz for the quinone, and $<<10^3$ Hz for the hydroquinone state.

Although incomplete due to the absence of several C(α)H and C(β)H_2 COSY connectivities it is demonstrated that two-dimensional NMR experiments allow a detailed analysis of some residues in the active center of M.elsdenii flavodoxin. Besides several close agreements between the crystal structure of Clostridium MP flavodoxin and the solution structure of M.elsdenii flavodoxin, some small distinct differences are found. An extension of this study, using other two-dimensional NMR experiments, is currently under active study in our laboratory and the results will be reported elsewhere in the near future.

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Summary

^{1}H, ^{13}C, ^{15}N and ^{31}P NMR was applied to a study of free and protein-bound flavins in order to obtain a better insight into the mechanism by which the function of the flavin coenzyme is „tuned" upon binding to apoflavoproteins. A thorough ^{13}C and ^{15}N investigation of free flavins provided a detailed view on the effects of sterical hindrance and polarization on the structure of oxidized and reduced flavins. Moreover, this study resulted in a semi-empirical basis for the interpretation of NMR data obtained on protein-bound flavins. Remarkable results of the study of free flavins are the fact, that oxidized isoalloxazine is not fully planar in an apolar medium, and that fully reduced isoalloxazine is more planar than believed up till now. Unsubstituted reduced isoalloxazine in water has an almost fully sp^2 hybridized N(10) and an N(5) atom which is approximately 70% sp^2 hybridized. Upon modification the hybridization of N(5) and N(10) can be modulated rather independently due to steric hindrance. The so-called "butterfly" motion has a low activation barrier, which is probably an important feature for the mentioned tuning mechanism.

Flavodoxin from Megasphaera elsdenii is the protein which is most extensively studied in this thesis. By a ^{13}C relaxation study it was shown that the isoalloxazine ring is strongly immobilized upon binding to apoflavodoxin. Also the phosphate group of FMN is strongly immobilized in the interior of the protein. Moreover, the phosphate group is dianionic in the complex, regardless of the redox state of bound FMN. Based on the NMR data, it was shown that the redox potential for the transition semiquinone/hydroquinone is mainly governed by charge interactions. For the first time a quantification of the redox potential modification by the apoprotein could be established, thereby showing that, contrary to literature reports, the redox potential modulation is not governed by steric effects exerted on the isoalloxazine moiety. Both by ^{31}P NMR and ^{1}H NMR it was shown that M.elsdenii flavodoxin acts as a one-electron transferring protein shuttling between the semiquinone and hydroquinone state, because the oxidized state is ruled out as a biologically relevant redox state in this protein due to the introduction of a high activation barrier between the oxi-

dized and semiquinone state. Moreover, the electron transfer mechanism is of an outer sphere type. Based on the NMR data some arguments are presented that a specific complex formation between M.elsdenii flavodoxin and the electron donor or acceptor is not needed for an effective electron transfer. An important part of the active center of M.elsdenii flavodoxin was elucidated using time resolved photochemically induced dynamic nuclear polarization (CIDNP) and modern two-dimensional NMR techniques. The use of the paramagnetic semiquinone state in combination with 2D NMR techniques allowed the generation of NMR spectra of only the active center. Complete assignments were given in both the oxidized and the hydroquinone state for Trp-91, Ala-56 and Tyr-89, which are all very close to the isoalloxazine ring. It was shown that the relative position of Trp-91 with respect to the prosthetic group is slightly different in the two redox states. The active center of flavodoxin from M.elsdenii and Clostridium MP appear to be similar. The only small difference between results of the crystallographic study on Clostridium MP flavodoxin and ^{1}H NMR results on M.elsdenii flavodoxin was observed for the relative position of Ala-56 and Tyr-89.

A detailed ^{13}C and ^{15}N NMR study was performed on the complex of riboflavin and Riboflavin Binding Protein from the egg yolk and egg white. Subtle information on hydrogen bonding, conformation of the isoalloxazine ring and solvent accessibility were obtained in oxidized and reduced state. As far as the results could be compared with reported binding studies of riboflavin analogues, the results appear to be in excellent agreement. The pyrimidine ring is exposed to solvent, except for O(4α) in both redox states. N(10) is forced into the molecular plane, which in turn probably causes N(1) to be somewhat out of the plane. The NMR results are consistent with a partial opening of the protein at pH 6 and a more stable conformation at pH 9. Based on the NMR results a possible function for the complex in the embryonic development is suggested.

As an example of the class of dehydrogenases, lipoamide dehydrogenase from Azotobacter vinelandii was studied by ^{13}C NMR. In the oxidized state hydrogen bonds exist to O(2α) and O(4α), but the polarization of the isoalloxazine ring probably does not extend to the N(10) atom. The 4 electron reduced protein

(both the essential disulfide and the flavin are reduced) contains an essentially planar N(10) atom, and again hydrogen bonds to O(2α) and O(4α). The 2 electron reduced protein (EH_2) appeared to be particularly interesting. It consists of an equal mixture of protein in which the disulfide is reduced and the flavin oxidized, and protein in which the disulfide is oxidized and the flavin reduced. The results allowed a detailed description of the electronic structure of the two-electron reduced protein. From the NMR results it is concluded that the redox potential of both centers is roughly the same. The results show that if the disulfide is reduced one thiol group is present as an anionic thiol group and extremely close to the C(4a) atom of the flavin. The exchange of reduction equivalents between the two redox centers in EH_2 is slow ($<5\ s^{-1}$). In combination with reported kinetic data, it became evident that NAD^+ accelerates considerably the exchange of reduction equivalents between the disulfide and the flavin. The results show that this transfer must be implemented in the reaction cycle and offer a nice explanation of some „anomalous" kinetic data.

As an example of the class of hydroxylases, p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens was studied by ^{13}C NMR. Without substrate the isoalloxazine ring is probably exposed to solvent. Upon substrate binding a drastic change of the polarization of C(2) occurs, which is in accord with the published enzyme-substrate complex as revealed by crystallographic methods. It is suggested, that the active center without substrate is rather mobile in order to facilitate the binding of substrate. A conformational change accompanies the binding of substrate. The active center, as present in the oxidized enzyme-substrate complex, is probably already essentially formed upon reduction of the enzyme in the absence of substrate, which explains the drastically decreased binding rate of substrate after reduction. Upon complex formation with substrate in the reduced state the N(5) is forced into the molecular plane of flavin. Some arguments are presented suggesting that the sp^2 hybridization degree of N(5) and N(10) are the main factors which govern the reactivity (or „activation") of oxygen, but not the π electron density at the C(4a) center

of flavin. An electron transfer from the hydroquinone state towards oxygen probably precedes the formation of a C(4a) peroxyflavin by radical pair combination.

Samenvatting

^{1}H, ^{13}C, ^{15}N en ^{31}P NMR werd toegepast op een studie van flavines, vrij in oplossing en gebonden aan apoflavoëiwitten. Het doel van deze studie was het verkrijgen van meer inzicht in het mechanisme, waarmee de funktie van de flavine coënzymen door binding aan de apoflavoëiwitten a.h.w. wordt afgestemd op de uiteindelijke funktie in het eiwit. Een diepgaande ^{13}C en ^{15}N studie van vrije flavines leidde tot een grondig inzicht in de effekten van sterische hindering en polarisatie op de struktuur van geoxideerde en gereduceerde flavines. Bovendien voorzag deze studie in de behoefte aan een semi-empirische theorie ter verklaring van NMR gegevens verkregen van flavines gebonden aan eiwitten. Opmerkelijke resultaten waren dat geoxideerde isoalloxazine niet volledig vlak is in een apolair medium en dat gereduceerd isoalloxazine in feite vlakker is dan tot dusverre werd aangenomen. Ongesubstitueerd gereduceerd isoalloxazine in water heeft een vrijwel sp^2 gehybridiseerde N(10), terwijl de N(5) ongeveer 70% sp^2 gehybridiseerd is. Modifikatie blijkt de hybridisatie van deze beide stikstof atomen onafhankelijk te kunnen beïnvloeden d.m.v. sterische hindering. Bovendien werd duidelijk, dat de aktiveringsenergie van de zgn. vlinderbeweging laag is, wat waarschijnlijk van belang is voor het eerder genoemde „afstem" mechanisme van de flavin coënzymen.

Het flavodoxine van Megasphaera elsedenii is het meest grondig onderzochte eiwit in deze dissertatie. Door een ^{13}C relaxatiestudie werd duidelijk, dat de isoalloxazine ring stevig gebonden zit aan het eiwit en in feite geïmmobiliseerd is. Ook de fosfaatgroep van FMN is sterk verankerd in het eiwit en bovendien steeds dianionisch, onafhankelijk van de redox toestand. De NMR resultaten maakten duidelijk, dat interaktie van ladingen de redox potentiaal bepalen van de overgang semiquinon naar hydroquinon. Voor het eerst kon de modifikatie van de redox potentiaal door het eiwit gequantificeerd worden. Duidelijk is, dat eertijds gepubliceerde resultaten omtrent de oorzaken van deze modifikatie onjuist zijn. Zowel ^{31}P als ^{1}H NMR toonden aan dat dit flavodoxine werkt als een één-electron overdragend eiwit, waarbij het eiwit pendelt tussen de semiquinon en de hydroquinon toestand. Dit werkingsmechanisme

is gebaseerd op het feit, dat de geoxideerde toestand uitgeschakeld wordt door de invoering van een hoge aktiveringsberg in the overgang tussen de geoxideerde en semiquinon vorm. Het mechanisme van electronoverdracht is waarschijnlijk van het „outer sphere" type. Bovendien lijken de resultaten er op te wijzen, dat een specifieke binding tussen het flavodoxine en een electron donor of acceptor niet nodig is voor een effektieve electronoverdracht. De struktuur van het aktieve centrum werd gedeeltelijk bepaald door middel van tijd-opgeloste CIDNP (fotochemisch geïnduceerde dynamische kern polarisatie) en moderne twee-dimensionale NMR technieken. Van groot belang in deze studie bleek het gebruik van de paramagnetische semiquinon toestand. Toekenningen werden gedaan voor Trp-91, Ala-56 en Tyr-89 in zowel de gereduceerde als geoxideerde toestand. De drie aminozuren bevinden zich zeer dicht bij de isoalloxazine ring. De relatieve positie van Trp-91 t.o.v. de isoalloxazine ring verschilt enigszins in de twee genoemde redox toestanden. Het aktieve centrum van dit eiwit gelijkt zeer sterk op dat van het verwante flavodoxine uit Clostridium MP. De enige kleine verschillen tussen de röntgenstudies van het laatste eiwit en de NMR resultaten van M.elsdenii flavodoxine werden waargenomen in de relatieve positie van Ala-56 en Tyr-89.

De interaktie tussen riboflavine en Riboflavine Bindend Eiwit werd onderzocht d.m.v. ^{13}C en ^{15}N NMR. Subtiele informatie werd verkregen over waterstofbruggen, conformatie van de isoalloxazine ring, en toegankelijkheid van oplosmiddel. In zoverre de resultaten vergeleken konden worden met bindingsstudies van analoga van riboflavine, bleken de studies in zeer goede overeenstemming. De pyrimidinering is blootgesteld aan water behalve O(4α). De N(10) wordt a.h.w. in het moleculaire vlak geduwd, terwijl N(1) enigszins uit het vlak is. De resultaten zijn consistent met het idee, dat bij pH 6 het eiwit een meer open struktuur aanneemt dan bij pH 9. De beschreven informatie gaf aanleiding tot enkele suggesties omtrent de funktie van het complex in de embryonale ontwikkeling.

Als voorbeeld uit de klasse van dehydrogenases, werd lipoamide dehydrogenase uit A.vinelandii bestudeerd m.b.v. ^{13}C NMR. Waterstofbruggen naar

O(2α) en O(4α) bestaan in het geoxideerde eiwit, maar de polarisatie reikt niet tot N(10). In de 4 elektronen gereduceerde vorm, waarin zowel de disulfide als het FAD gereduceerd zijn, is N(10) vrijwel vlak en opnieuw bestaan er waterstofbruggen naar O(2α) en O(4α). Vooral de 2 electronen gereduceerde toestant (EH_2) bleek erg interessant te zijn. Het bleek te bestaan uit een mengsel van enerzijds eiwit, waarin disulfide gereduceerd en FAD geoxideerd is, en anderzijds eiwit, waarin disulfide geoxideerd en FAD gereduceerd is. De electronische struktuur van de twee soorten werd beschreven. De redox potentiaal van de beide redox groepen is vrijwel gelijk. Indien disulfide is gereduceerd, dan is één thiolgroep anionisch. De overdracht van elektronen tussen de twee redoxgroepen is langzaam ($<5\ s^{-1}$). Vergelijking met gerapporteerde kinetische data leerde, dat NAD^+ deze overdracht aanzienlijk versnelt. Deze kinetisch belangrijke stap moet toegevoegd worden aan het reaktiemechanisme en verklaart enkele vreemde kinetische data.

Als voorbeeld uit de klasse van hydroxylases werd p-hydroxybenzoaathydroxylase uit Pseudomonas fluorescens bestudeerd m.b.v. ^{13}C NMR. Zonder substraat is de isoalloxazine ring blootgesteld aan oplosmiddel. Na binding van substraat treedt een verandering in polarisatie van C(2) op, wat in overeenstemming is met het enzymsubstraat complex zoals dat gevonden is m.b.v. röntgenstudies. Zonder substraat lijkt het aktieve centrum mobiel om de binding van substraat te vergemakkelijken. Het aktieve centrum, zoals aanwezig in het ES complex is waarschijnlijk ook gevormd na reduktie van het vrije enzym zonder substraat. Bij binding van het substraat in de gereduceerde toestand wordt N(5) in het moleculaire vlak geduwd. Enkele argumenten worden aangedragen, dat de sp^2 hybridisatie van N(5) en N(10) de belangrijke faktoren kunnen zijn die de reaktiviteit (of aktivering) van zuurstof bepalen en niet de π electronen dichtheid van het C(4a) atoom. Waarschijnlijk gaat een electronoverdracht van hydroquinon naar zuurstof vooraf aan de vorming van een C(4a) peroxyflavin door combinatie van het radikaal paar.

List of Abbreviations

Ac_4rf	tetraacetylriboflavin
CIDNP	chemically induced dynamic nuclear polarization
COSY	through bond correlated 2 DNMR spectroscopy
CSA	chemical shift anisotropy
2DNMR	two dimensional nuclear magnetic resonance
EDTA	ethylenediaminotetraacetic acid
FAD	flavin adenine dinucleotide
FID	free induction decay
FMN	riboflavin 5'-phosphate
2'FMN	riboflavin 2'-phosphate
3'FMN	riboflavin 3'-phosphate
4'FMN	riboflavin 4'-phosphate
$FMNH^-$	anionic 1,5-dihydro-FMN
$FMNH_2$	neutral 1,5-dihydro-FMN
$pHBH^-$	para-hydroxybenzoate hydroxylase
HOMO	highest occupied molecular orbital
Lipdh	lipoamide dehydrogenase
LUMO	lowest unoccupied molecular orbital
MeOH	methanol
Me_3IA	3,7,10-trimethylisoalloxazine
MeIMN	7 methyl-10-ribity-isoalloxazine-5'-phosphate
$MeIMNH^-$	anionic 1,5-dihydro-MeIMN
$MeIMNH_2$	neutral 1,5-dihydro-MeIMN
$MeLfl^2$	3-methyllumiflavin
$MeTARFH_2$	3-methyl-1,5-dihydrotetraacetylriboflavin
$MeTARI^2$	7-methyl-10-tetraacetylribityl-isoalloxazine
Me_2TARI	3,7-dimethyl-10-tetraacetylribitylisoalloxazine
$MeTARIH_2$	1,5-dihydro-MeTARI
Me_2TARIH_2	1,5-dihydro-Me_2TARI
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser enhancement
NOESY	through space (NOE) correlated 2DNMR spectroscopy
RBP	riboflavin binding protein
RBPW	riboflavin binding protein from egg white
RBPY	riboflavin binding protein from egg yolk
TARF	tetraacetylriboflavin
$TARFH_2$	1,5-dihydro-tetraacetylriboflavin
TMS	tetramethylsilane
Tris	tris (hydroxymethyl) aminomethane
TSP	trimethylsilylpropionate
τ	lifetime

Curriculum Vitae

Chrit Moonen is geboren op 30 september 1955 in Stramproy. In 1973 werd het diploma gymnasium β aan het Bisschoppelijk College te Weert behaald. In hetzelfde jaar werd de studie Moleculaire Wetenschappen aan de Landbouwhogeschool in Wageningen begonnen. Het doctoraal examen bevatte een onderzoek op de vakgroep Virologie (3 mnd), een onderzoek op de vakgroep Biochemie (3 mnd), een onderzoek op de vakgroep Moleculaire Fysica (hoofdvak), een onderzoek op de vakgroep Fysische Chemie (3 mnd) en een tweede hoofdvak Biofysica (praktijk) aan de ETH in Zürich, onder leiding van prof. Wüthrich. De studie Moleculaire Wetenschappen werd in januari 1980 cum laude afgerond. Vanaf eind januari 1980 werd aan deze dissertatie gewerkt, in eerste instantie in dienst van de Stichting voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.). Vanaf maart 1982 was hij in dienst van de Landbouwhogeschool Wageningen. Sedert januari 1983 was hij in vaste dienst aan de Landbouwhogeschool verbonden. Tijdens de werkzaamheden aan deze dissertatie was hij betrokken bij onderwijs in het vak „Maatschappelijke functies van de N-4 richtingen" en in het vak „enzymregulering".