The interactions between apoflavoproteins and their coenzymes as studied by nuclear magnetic resonance techniques

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Proefschrift

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van de landbouwhogeschool te Wageningen.

ISN 244899.

"Niet het leren is het voornaamste maar het doen"

Spreuken der Vaderen

Aan mijn ouders

Aan Flory

LANDBOUWUNIVERSITEIT
WAGENINGEN

NN08201,1079

LAN... WAGENINGEN

Stellingen

1. De door Ghisla et al. toegekende piek in het ^{13}C NMR spectrum van het luciferase-gebonden C(4a)-peroxyflavine is niet die van dit intermediair maar van een verontreiniging in het oplosmiddel.
   Ghisla, S., Hastings, J.W., Favaudon, V. en Lhoste, J.-M. (1978) Proc.Natl.Acad.Sci. USA 75, 5860-5863.
   Hoofdstuk 6, dit proefschrift.

2. Arbeidstijdverkorting leidt tot verlies aan werkgelegenheid.

3. Genetic engineering is tegenwoordig een "must".
   Een van de belangrijkste motivaties voor het bedrijfsleven is dat de concurrent actief is op dit onderzoeksgebied.

4. Het is zeer onwaarschijnlijk dat intermediair II in de door para-hydroxybenzoaat hydroxylase gekatalyseerde reactie een 6-amino-5-oxo-3H, 5H-uracil molekuul is zoals Schopfer et al. postuleren.
   Schopfer, L.M., Wessiak, A. en Massey, V. in Flavins and Flavoproteins (Bray, R.C., Engel, P.C. en Mayhew, S.G., eds.) Walter de Gruyter, Berlijn, 1984, pp. 781-784.

5. Het veelvuldig voorkomen van charge-transfer banden in absorptiespectra in eiwit-gebonden flavine-NAD(P)H complexen is niet in overeenstemming met de in die eiwitten gepostuleerde hydride transfer.
   Massey, V. (1985) 13th International Congress of Biochemistry, Amsterdam, pp. 427.

6. De schaalvergroting in de biotechnologie vindt meer plaats op het gebied van de toegekende subsidie dan op het gebied van werkelijke innovatie.

7. De conclusie dat molecuulgewichten van de natieve vorm van biologisch actieve eiwitten met behulp van CTAB-PAGE bepaald kunnen worden is voorbarig. Para-hydroxybenzoaat hydroxylase is als monomeer zichtbaar in plaats van als dimeer.
   Aking, D.T., Schapira, R. en Kinkade, J.M. 81985). Anal.Biochem. 145, 170-176.

8. De conclusie van Juhl et al. dat lage concentraties pentachloorfenol schadelijker zijn voor de gezondheid dan hoge concentraties is onjuist. Uit hun proeven blijkt eerder het omgekeerde.
Juhl, U., White, I. en Butte, W. (1985). Bull.Environ.Contam.Toxicol. 35, 596-601.

9. Voor een objectievere beoordeling van een ingezonden wetenschappelijk artikel zou het wenselijk zijn dat de referee niet op de hoogte gebracht wordt van de namen van de auteurs.

10. Een biochemicus die niet kan zuiveren is geen zuivere biochemicus.

11. Een academische titel is geen garantie voor wetenschappelijke kwaliteit.

Jacques Vervoort

The interactions between apoflavoproteins and their coenzymes as studied by nuclear magnetic resonance techniques.

Wageningen, 26 maart 1986

Voorwoord

Aan allen, die aan de totstandkoming van dit proefschrift hebben bijgedragen, betuig ik mijn hartelijke dank.
In het bijzonder wil ik noemen:

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Chrit Moonen, die door de vele discussies heeft bijgedragen tot het creëren van een plezierig academische werksfeer. De samenwerking heb ik als erg prettig ervaren.

Willy van den Berg en Willem van Berkel voor het isoleren en karakteriseren van een groot aantal eiwitten. Zij hebben mij een "post-academische cursus" biochemie gegeven.

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I am very grateful to John Lee, Mustaq Ahmad and Dennis O'Kane who purified almost more luciferase than I could use for the NMR experiments. Dear John, your inspiring participation and constructive criticism has been very pleasant.

I also want to thank Lena Sahlman and Steve Mayhew who contributed and are still contributing to part of this work.

Zonder NMR machines die altijd in prima conditie verkeerden en verkeren zou dit onderzoek niet mogelijk zijn geweest. Adrie de Jager was daarbij onmisbaar voor de Bruker CXP 300, Cees Haasnoot en Pieter van Dael voor de Bruker WM500 en Bruker WH200.

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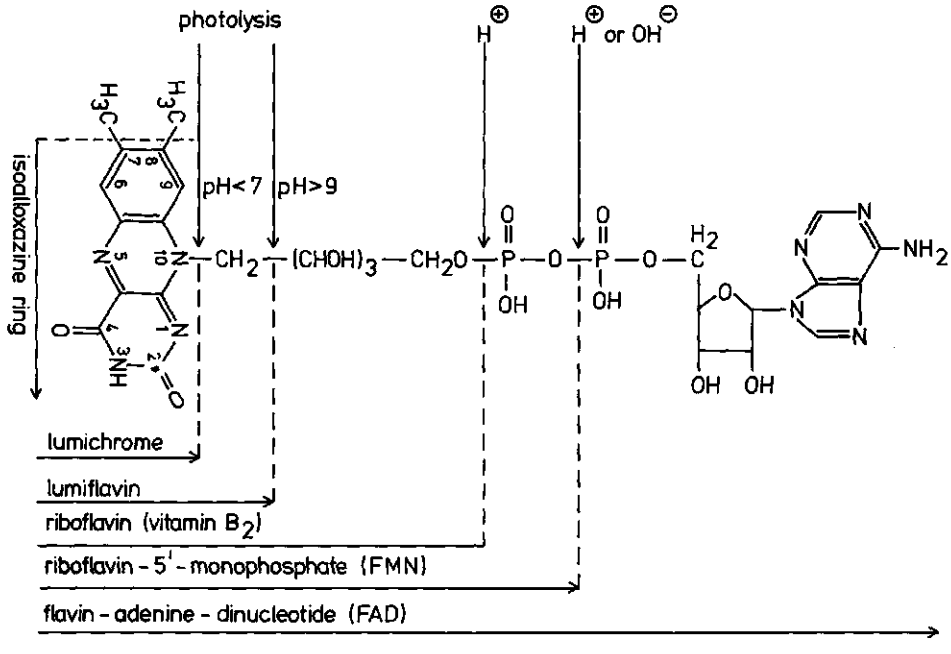
Contents

Chapter 1. Introduction

The term "flavin" relates to yellow pigments (latin: flavus = yellow) which were first isolated from various biological sources (e.g. eggs, milk). About 50 years ago the yellow colour of these pigments was proven to be of a tricyclic system, isoalloxazine and the structure was established as a ribose derivative of isoalloxazine rendering the name riboflavin (1,2). Riboflavin is being synthesized by all plants and many microorganisms but not by higher animals.

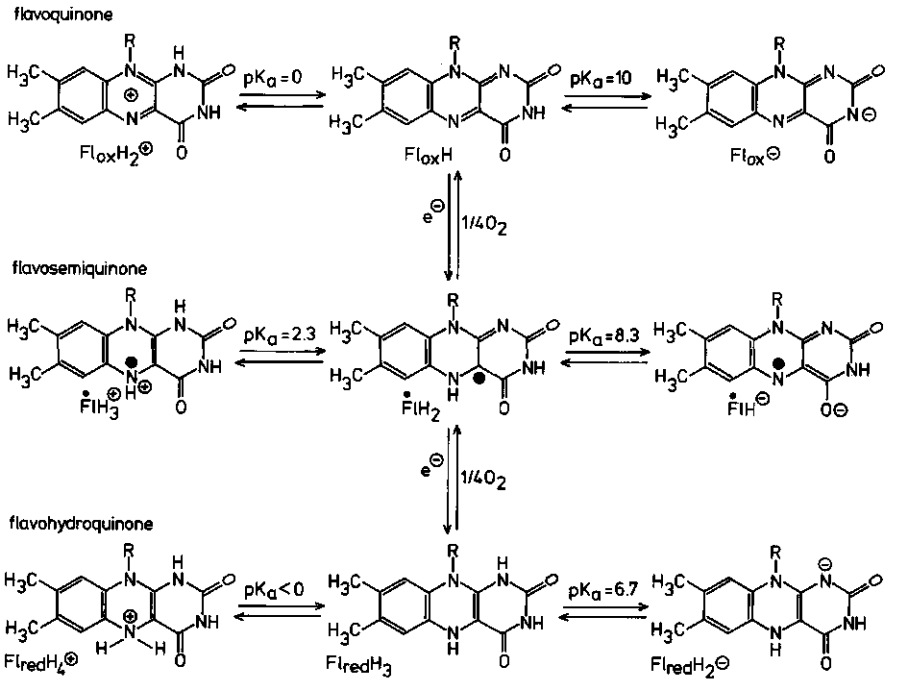
It was also found about 50 years ago that riboflavin is an essential nutrient of the food supply for higher animals and hence it was called Vitamin B_2. A deficiency of the vitamin causes numerous types of diseases e.g. dermatitis, eye disorders, lesions of mouth and tongue. The riboflavin molecule functions in metabolism as part of the coenzymes FMN and FAD. In scheme 1 the most common flavin coenzymes are shown including the internationally accepted numbering system.

The flavin nucleotides function as prosthetic groups of oxidation-reduction enzymes known as flavoproteins. In most flavoproteins the flavin coenzymes are tightly but non-covalently bound to the protein. However, during the last decade more and more flavoproteins were found which contain a covalently bound flavin molecule (3). The most prominent feature of the biochemical properties of flavin is its redox properties. Flavins can exist in three redox states: the oxidized state (flavoquinone), the one-electron reduced state (flavosemiquinone) and the two-electron reduced state (flavohydroquinone). The flavoquinone and flavohydroquinone state are diamagnetic, the flavosemiquinone state is paramagnetic. The possible cationic, neutral and anionic forms of each redox state are outlined in scheme 2. Only five of these possible structures are found in flavoproteins because the proteins are not stable at pH < 2 or pH > 9.



Scheme 1

The uniqueness of flavins is that they can undergo both one- and two-electron redox transitions, a feature not shared by any other redox coenzyme. For instance, in electron-transferring flavoproteins the protein shuttles between the flavosemiquinone and the flavohydroquinone state during catalysis, whereas flavoprotein oxidases shuttle between the flavoquinone and flavohydroquinone state avoiding (= destabilizing) the flavosemiquinone state. The important property in the regulation of the redox potential of a flavoprotein and also in determining either the one- or two-electron redox transition, is the specific interaction between the flavin coenzyme and the apoprotein in the different redox states. If the flavocoenzyme is bound more tightly to the reduced than to the oxidized state then the redox potential of this transition will be more positive than that



Scheme 2

for the free flavin-dihydroflavin couple. On the other hand if the flavin molecule is bound more tightly in the oxidized than in the reduced (or semiquinone) state then the redox potential will be more negative than that for the free coenzyme. In the latter case the flavoprotein will be a less powerful oxidizing agent and a better reducing agent than free flavin. The differences in redox potential of flavoproteins can amount up to more than 650 mV.

In general each flavoprotein accepts electrons from a substrate which is being oxidized and passes these electrons onto another substrate, which is being reduced. The enormous diversity which exists among flavoproteins (up to now more than 100 different flavoproteins have been found) is not only related to differences in redox potential but also to differences in

substrate. Nevertheless, many flavoproteins share common features. The most prominent feature is that flavoproteins are highly reactive towards reductive oxygen metabolism. This property is used in many different reactions catalyzed by flavoproteins. When molecular oxygen is the electron acceptor, it can be converted to H_2O_2 (oxidases), or to H_2O and one oxygen atom incorporated in the product (hydroxylases, oxidases-decarboxylases) or both oxygen atoms end up in the product (dioxygenases). When oxygen is not being used as electron acceptor the flavoproteins are either classified as dehydrogenases or electron transferring proteins e.g. flavodoxins. It is also possible to classify the flavoproteins according to the structural type of organic substrate oxidized. However, the first categorization scheme is favoured by most flavin biochemists. The reader is referred to Walsh and references therein (4) for more detailed information.

Flavoproteins catalyze a large variety of biological reactions. The question how one and the same chemical entity, the isoalloxazine ring system, can be involved in so many different reactions is still not answered sufficiently. It was proposed by Müller (5) that the coenzyme is "tuned" by means of a specific hydrogen bonding network to its actual function in a particular flavoprotein.

Hemmerich and Massey (6) by using this idea talked about a N(1) and a N(5) block, depending on the mode of action of a given flavoprotein. However, such a proposal is a very rough one.

Several questions are not answered so far, although attempts have been made. The most important question is how the redox potential is regulated in flavoproteins. Until recently it was proposed that the conformation (degree of planarity) of the reduced, protein-bound prosthetic group is the main factor in the determination of the redox potential. It has been known for quite a time that free flavins in the 1,5-dihydro state are nonplanar (7). This finding was confirmed by crystallography. However, the crystal structure of the reduced state of Clostridium MP flavodoxin revealed an almost planar isoalloxazine ring system (8). Therefore it was proposed that the low redox potential in flavodoxins are caused by steric constraints put on to the reduced isoalloxazine ring system. Recent NMR results showed this hypothesis to be too simple (9). So the question how redox potential

regulation is brought about was reopened again. Moonen et al. (10) proposed that charge-charge interactions are probably much more important in the regulation of redox potentials in flavoproteins.

The aim of this study was to arrive at a more detailed explanation with respect to the tuning mechanism of protein-bound flavin. A very suitable technique to get a detailed view into the molecular and submolecular structure of the flavin molecule is nuclear magnetic resonance (NMR) spectroscopy. ^{13}C and ^{15}N NMR chemical shifts can reveal both π electron density and the presence of specific hydrogen bonds at the carbon and nitrogen atoms investigated. Unfortunately, natural abundance ^{13}C and ^{15}N NMR spectroscopy is hampered by the fact that the ^{13}C and ^{15}N isotopes have a natural abundance of 1.1% and 0.4%, respectively. Therefore we used isoalloxazine selectively enriched with ^{13}C and ^{15}N at all carbon and nitrogen atoms of the isoalloxazine ring. The electronic and conformational structure of the protein-bound flavin was studied by using these ^{13}C and ^{15}N enriched flavins. After removal of the natural coenzyme, the apoprotein was reconstituted with the ^{13}C or ^{15}N enriched coenzyme. In this way one obtains a flavoprotein, which does not differ in any chemical property from the original flavoprotein. This approach gives a detailed view of a single atom (of the isoalloxazine ringsystem) of the flavincoenzyme in the various redox states. In fact it is even possible to monitor the changes during catalysis of a particular atom i.e. to measure intermediates formed (chapter 6).

The ^{13}C and ^{15} N NMR results obtained can be interpreted in terms of hydrogen bonds, structure, π electron density and mobility.

^{31}P NMR is not hampered by the fact that isotopic enrichment would be necessary because ^{31}P has an abundance of 100% in nature. ^{31}P NMR is 4 times more sensitive than ^{13}C NMR and even 66 times more sensitive than ^{15}N NMR for an equal number of nuclei. It is evident that ^{31}P NMR spectra are much easier to obtain than ^{13}C or ^{15}N NMR spectra. ^{31}P NMR studies applied to flavoproteins revealed not only information on the specific interaction between the apoprotein and the phosphate group(s) of the flavocoenzyme but also, sometimes, the presence of a covalently bound phosphate residue (11). A remarkable result of the ^{31} P NMR studies on Azotobacter vinelandii and Megasphaera elsdenii fla-

vodoxin was that the phosphate group of FMN is bound in the dianionic form with no positively charged amino acids present in the phosphate binding region (12,13).

A part of this thesis deals with the specific types of interaction between apoflavodoxins and FMN. Flavodoxins are small flavoproteins (15000 D < M_r < 22000 D) which contain one molecule of FMN as prosthetic group. The flavodoxins function as electron carriers in low potential oxidation-reduction reactions (14). The physico-chemical properties of flavodoxins have been studied extensively (15) and the crystal structures of the flavodoxins from Clostridium MP (8), Desulfovibrio vulgaris (16) and Anacystis nidulans (17) are known. The small size of these proteins and the ease of preparation of stable apoenzymes make them very suitable for NMR studies.

The interaction between the flavin coenzyme and relatively large proteins (compared to flavodoxins) has been studied also in this thesis: One FMN binding protein, luciferase, and two FAD containing proteins, para-hydroxybenzoate hydroxylase and mercuric reductase. All three proteins are of bacterial origin.

Luciferase is a protein in light emitting bacteria e.g. from the Vibrio or Photobacterium species. The in vitro reaction is a luciferase-catalyzed, oxidation of $FMNH^-$ and an aliphatic aldehyde by molecular oxygen with the concomitant emission of light. No other flavoprotein, known nowadays, produces light during a reaction. During the course of the reaction several intermediates can be observed (18). The intermediate which received the most attention was claimed to be a luciferase-bound 4a-hydroperoxide-flavin (19). No real proof was obtained so far, in spite of ^{13}C NMR "evidence" (19). The real ^{13}C NMR evidence will be shown in chapter 6. The literature on bacterial luciferase has received great attention and the field is still growing also due to applications in immuno-assay techniques. The reader is referred to a recent review article for more detailed information (21).

The FAD containing proteins para-hydroxybenzoate hydroxylase and mercuric reductase are classified as a hydroxylase and a dehydrogenase, respectively. Both enzymes are NADPH dependent. Para-hydroxybenzoate hydroxylase is a well studied protein of which the crystal structure

in the enzyme-substrate complex in the oxidized state is known at 0.25 nm resolution. It catalyzes the hydroxylation of an aromatic compound, para-hydroxybenzoate, to 3,4-dihydroxybenzoate. Oxygen is also needed as a substrate. This in contrast to mercuric reductase, which catalyzes the reduction of toxic mercuric compounds to the volatile Hg°. No oxygen is needed for this reaction. The reader is referred to the literature (22, 23) for more details.

This thesis is just a snap-shot of a rapidly moving object. From the very fast advancing methods and instrumentation it can be expected that nuclear magnetic resonance techniques will yield a better understanding of the molecular basis of flavoprotein catalysis.

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Chapter 2.

Carbon-13 and nitrogen-15 nuclear-magnetic-resonance investigation on *Desulfovibrio vulgaris* flavodoxin

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Desulfovibrio vulgaris apoflavodoxin has been reconstituted with ^{15}N and ^{13}C-enriched riboflavin 5'-phosphate. For the first time all carbon atoms of the isoalloxazine ring of the protein-bound prosthetic group have been investigated. The reconstituted protein was studied in the oxidized and in the two-electron-reduced state. The results are interpreted in terms of specific interactions between the apoprotein and the prosthetic group, and the chemical structure of protein-bound FMN.

In the oxidized state weak hydrogen bonds exist between the apoprotein and the N(5), N(3) and O(4α) atoms of FMN. The N(1) and O(2α) atoms of FMN form strong hydrogen bonds. The isoalloxazine ring of FMN is strongly polarized and the N(10) atom shows an increased sp^2 hybridisation compared to that of free FMN in aqueous solution. The N(3)-H group is not accessible to bulk solvent, as deduced from the coupling constant of the N(3)-H group.

In the reduced state the hydrogen bond pattern is similar to that in the oxidized state and in addition a strong hydrogen bond is observed between the N(5)-H group of FMN and the apoprotein. The reduced prosthetic group possesses a coplanar structure and is ionized. The N(3)-H and N(5)-H groups are not accessible to solvent water. Two-electron reduction of the protein leads to a large electron density increase in the benzene subnucleus of bound FMN compared to that in free FMN.

The results are discussed in relation to the published crystallographic data on the protein.

Flavoproteins catalyze a large variety of biological reactions. Although many of these enzymes have been studied in great detail by kinetic and physical techniques, it is still not understood how, for instance, the redox potential of a particular flavoprotein is regulated by the apoprotein. It has been suggested that specific hydrogen bonds between the apoflavoprotein and its prosthetic group [1] and the conformation (degree of planarity) of the reduced, protein-bound prosthetic group [2, 3] are the main factors determining the redox potential of a particular flavoprotein. To unravel the possible contributions of the two factors mentioned in the regulation of the redox potential in flavoproteins we have set up an NMR study on these proteins using ^{1}H, ^{13}C ^{15}N and ^{31}P NMR techniques [4–8]. These results combined with recent NMR studies on free flavins [9, 10] revealed that in general the protein-bound flavin exhibits a high degree of planarity in the reduced state. This factor can therefore no longer be considered a major factor for the regulation of the redox potential. Instead it was proposed that charge-charge interactions are probably much more important in the regulation of redox potentials in flavoproteins [11].

As a continuation of the research outlined above we decided to study some flavodoxins by NMR techniques in more detail in order to test the hypothesis in depth. Flavodoxins are particularly useful proteins for such an approach because they possess a low molecular mass (≈15–20 kDa), exhibit different redox potentials, and are easily available in large quantities [12]. In this paper we report on the electronic structure of oxidized and reduced FMN bound to the apoflavodoxin from *Desulfovibrio vulgaris* as revealed by ^{13}C and ^{15}N NMR techniques. In contrast to previous studies [4, 7, 8], we also used FMN derivatives selectively enriched with ^{13}C in the benzene subnucleus. In this way it was possible to investigate all carbon atoms of the isoalloxazine ring of FMN in dependence of the redox state. In addition, the study also allowed us to investigate in more detail the electronic perturbation of the isoalloxazine moiety of FMN, as compared to free FMN, upon binding to the apoflavodoxin. Preliminary results have been published elsewhere [13].

MATERIALS AND METHODS

FMN and other flavin derivatives selectively enriched with ^{13}C at positions 2, 4, 4a and 10a were prepared as described previously [4]. The synthesis of ^{15}N-enriched flavins has been described elsewhere [8, 14]. The preparation of flavins enriched in the benzene subnucleus of flavin will be reported

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Abbreviations. NMR, nuclear magnetic resonance; FMN, oxidized riboflavin 5'-phosphate; $FMNH^-$, two-electron-reduced ionized riboflavin 5'-phosphate; Ac_4rF, oxidized tetraacetylriboflavin; Ac_4rFH_2, two-electron-reduced tetraacetylriboflavin; DEPT, distortionless enhancement by polarization transfer; Me_4Si, tetramethylsilane; $MeAc_4rF$, *N*(3)-methyltetraacetylriboflavin in the oxidized state; $MeAc_4rFH_2$, *N*(3)-methyltetraacetylriboflavin in the two-electron-reduced state.

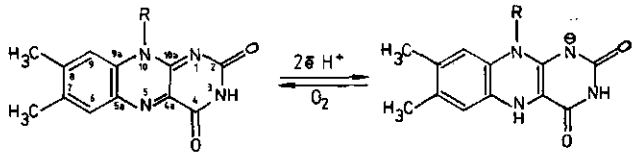

Fig. 1. *The structures of oxidized and two-electron-reduced flavin.* R is ribityl 5′-phosphate

elsewhere (Bacher and Sedlmaier, unpublished results). The enrichment of the isotope was 90–95 atom%.

Flavodoxin from *Desulfovibrio vulgaris*, strain Hildenborough, was isolated as previously described [15]. The apoprotein was prepared by the trichloroacetic acid precipitation method [16]. The reconstitution of the apoflavodoxin with the ^{13}C or ^{15}N-labeled prosthetic group was carried out in neutral, buffered solutions at 4°C. Excess of FMN was removed on a Bio-Gel P6DG column. The protein-FMN complex was then concentrated by means of lyophilization.

Wilmad 10-mm precision NMR tubes were used. The samples contained 1–5 mM flavodoxin in 100 mM potassium phosphate buffer, pH 6.0–7.5 and 100 mM potassium pyrophosphate buffer, pH 7.5–8.5, both in the presence of 10% ^2H_2O. The sample volume was 1.6 ml. All measurements were performed on a Bruker CXP 300 spectrometer operating at 30.4 MHz for ^{15}N NMR and at 75.6 MHz for ^{13}C NMR measurements. Broadband decoupling, when applied, of 1.0 W was used for ^{13}C and ^{15}N measurements on the protein samples. In case of free FMN 2-W broadband decoupling was used. All spectra were recorded using 30° pulses and a repetition time of 0.8–1.3 s.

Distortionless enhancement by polarization transfer (DEPT) spectra were recorded using the method of Doddrell et al. [17], optimized for the $^1J(^{15}N\text{-}^1H)$ coupling constants as reported by Franken et al. [8]. Dioxane (3 µl) served as an internal standard for ^{13}C NMR measurements. Chemical shift values are reported relative to Me_4Si ($\delta_{dioxane} - \delta_{Me_4Si} = 67.84$ 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. [18]. Chemical shift values are reported relative to liquid NH_3 at 25°C ($\delta_{CH_3NO_2} - \delta_{NH_3} = 381.9$ ppm for the magnetic field parallel to the sample tube [18]). Values are reported as true shieldings, i.e. corrected for bulk volume susceptibilities [18]. The accuracy of the reported values is about 0.1 ppm for ^{13}C NMR and about 0.3 ppm for ^{15}N NMR chemical shift values. The temperature of the samples was 26 ± 2°C. Reduction of the samples was conducted by the addition of the desired amount of a dithionite solution to the anaerobic solution of either flavodoxin or FMN. Anaerobiosis was achieved by carefully flushing the solution in the NMR tube with argon for about 15 min. The NMR tube was sealed with a serum cap.

RESULTS AND DISCUSSION

Flavodoxins function in nature as electron-transferring proteins in low-potential redox reactions. In these reactions flavodoxins shuttle between the semiquinone and hydroquinone state. For obvious reasons we cannot investigate the protein in the semiquinone state, confining our study to the oxidized and the two-electron-reduced states (Fig. 1). In addition, since the three-dimensional structure of only the oxidized form of *Desulfovibrio vulgaris* flavodoxin has been published [19], comparison of the NMR results with X-ray data is limited.

For convenience and a better understanding of the interpretation of the results the ^{13}C and ^{15}N chemicals shifts of free FMN and Ac_4rF in the two redox states are given in order to demonstrate the difference between the chemical shifts of free and protein-bound flavin. In this context it should be kept in mind that the results of oxidized and reduced Ac_4rF in $CHCl_3$ represent the flavin molecule in the absence (or almost absence) of hydrogen bonds. The interpretation of the ^{13}C chemical shifts is based on the fact that a linear relationship exists between the ^{13}C chemical shift of the carbon atom in question and its π electron density [20]. This fact led recently to a more detailed interpretation of the structure of oxidized and reduced free flavin [9].

Studies on the oxidized state

The previously published ^{13}C chemical shifts for oxidized FMN in aqueous solutions were determined at concentrations larger than 10 mM, except for the ^{13}C chemical shifts of the C(2), C(4), C(4a) and C(10a) atoms for which ^{13}C-enriched derivatives were available [9]. The ^{13}C chemical shift of the latter four atoms were found to be dependent on concentration. Since then FMN derivatives selectively ^{13}C-enriched at the other atoms of the isoalloxazine ring of FMN have become available (see Materials and Methods). The ^{13}C chemical shifts of these atoms were therefore redetermined at concentrations of about 0.1 mM where stacking of FMN molecules is slight or negligible [21]. The chemical shifts of C(5a) and C(9a) undergo a downfield shift of 1.0 ppm and 2.0 ppm, respectively, as compared with those of FMN in concentrated solutions, similar to that found for C(2), C(4), C(4a) and C(10a). Only the C(6) atom shifts upfield by 1.0 ppm upon dilution. The C(7), C(8) and C(9) atoms are very insensitive to concentration effects, i.e. the variation of the chemical shift of these atoms is within the accuracy of the measurements. The results are collected in Table 1.

Some selected spectra of apoflavodoxin reconstituted with ^{13}C-enriched FMN derivatives are shown in Fig. 2. The good signal-to-noise ratio and the rather weak intensities of the natural abundance signals due to the apoprotein in the spectra allows us to assign all resonances to the carbon atoms of the isoalloxazine ring of FMN without further treatment of the data, i.e. difference spectroscopy. The natural abundance resonance lines originate from peptide carbonyl groups and carboxylic side chains (170–180 ppm), Arg C(ζ) atoms (158 ppm) and Trp, Tyr, Phe and His (110–140 ppm) [22, 23].

Binding of FMN to *D. vulgaris* apoflavodoxin causes a downfield shift of most resonances as compared to those of free FMN (Table 1). The largest downfield shift (2.3 ppm) is observed for C(8). The C(7) atom undergoes a downfield shift of 1.6 ppm. The downfield shifts of the other atoms amount to about 1 ppm or less. The C(4), C(4a), C(9) and C(9a) atoms

Table 1. *^{13}C and ^{15}N chemical shifts of various selectively enriched free and protein-bound FMN derivatives in 100 mM potassium pyrophosphate, pH 8.0*
^{13}C chemical shifts are relative to Me_4Si. ^{15}N chemical shifts are relative to liquid NH_3. Values for FMN and $FMNH^-$ are taken in part from [9]. Values for Ac_4rF and Ac_4rFH_2 were determined in $CHCl_3$

Atom	Shift in					
	oxidized state			reduced state		
	D. vulgaris flavodoxin	FMN	Ac_4rF	*D. vulgaris* flavodoxin	$FMNH^-$	Ac_4rFH_2
	ppm					
C(2)	159.7	159.8	155.2	157.5[a]	158.2	150.6
C(4)	162.4	163.7	159.8	154.0	157.7	157.0
C(4a)	134.3	136.2	135.6	102.7	101.4	105.2
C(5a)	137.4	136.4	134.6	134.6	134.2	136.0
C(6)	132.5	131.8	132.8	114.5	117.3	116.1
C(7)	142.0	140.4	136.6	130.7	133.0	133.6
C(7α)	20.5	19.9	19.4	19.4	19.0	18.9
C(8)	154.0	151.7	147.5	126.7	130.3	129.0
C(8α)	23.3	22.2	21.4	20.3	19.4	18.9
C(9)	117.2	118.3	115.5	114.7	116.8	118.0
C(9a)	131.9	133.5	131.2	129.1	130.9	128.2
C(10a)	152.3	152.1	149.1	155.0[a]	155.5	137.1
N(1)	188.0	190.8	199.9	186.6[a]	182.6	116.7[b]
N(3)	159.9	160.5	159.8	148.3[a]	149.3	145.8[b]
N(5)	341.1	334.7	344.3	62.1[a]	57.7	60.4[b]
N(10)[b]	165.6	164.6	150.2	98.4	97.2	72.2

[a] Independent of pH in the range 6.0–8.5.
[b] [1,3,5,10-$^{15}N_4$]7-Methyl-10-ribityl-isoalloxazine-5′-phosphate.

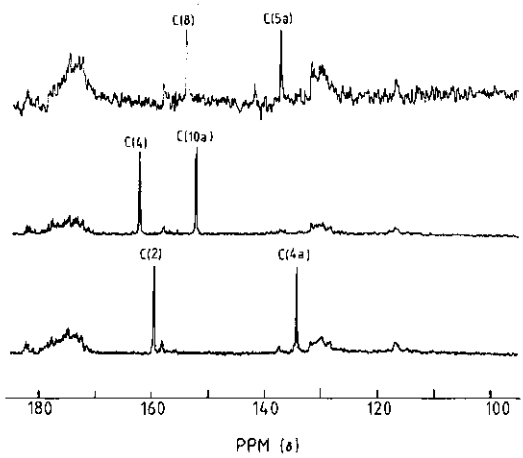

Fig. 2. *^{13}C NMR spectra of* D. vulgaris *apoflavodoxin reconstituted with [4,10a-$^{13}C_2$] FMN (50690 acquisitions), [2,4a-$^{13}C_2$] FMN (54060 acquisitions) and [5a,8-$^{13}C_2$]FMN (51000 acquisitions) in the oxidized state in 100 mM potassium pyrophosphate, pH = 8.0*

exhibit an upfield shift as compared to those of free FMN. C(4a) and C(9a) exhibit the largest upfield shifts of 1.9 ppm and 1.6 ppm, respectively. When the chemical shifts of apoflavodoxin-bound FMN are compared with those of Ac_4rF, all carbon atoms except for C(4a) and C(6) are seen to appear at lower fields. This means that FMN in *D. vulgaris* flavodoxin is strongly polarized. The polarization even exceeds that of FMN in aqueous solution [9], as manifested, for instance, by the large downfield shift of C(8). For convenience the ^{13}C chemical shifts of free and protein-bound FMN are presented in a correlation diagram in Fig. 3.

The formation of a hydrogen bond between the apoflavodoxin and the O(2α) atom of FMN leads to a rather strong polarization of the isoalloxazine ring of FMN. This effect is reflected by a downfield shift of the C(8), C(6) and C(10a) atoms. A comparison of the chemical shifts of C(2) in free

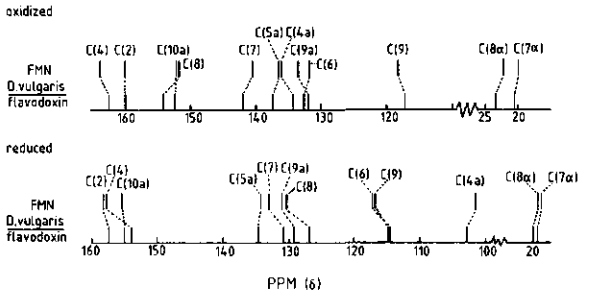

Fig. 3. *Correlation diagram of* ^{13}C *NMR chemical shifts of FMN and* D. vulgaris *flavodoxin in the oxidized and the reduced state*

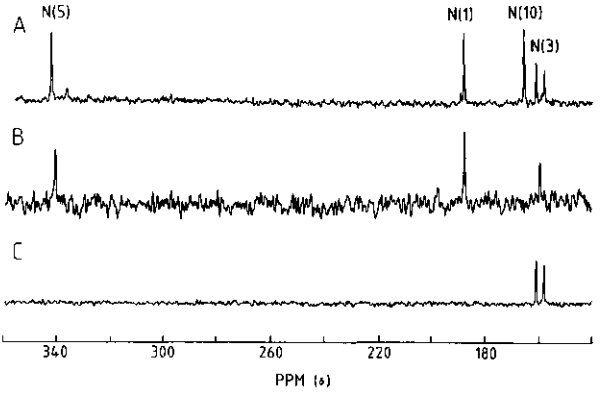

Fig. 4. ^{15}N *NMR spectra of* D. vulgaris *apoflavodoxin reconstituted with [1,3,5,10-*$^{15}N_4$*]7-methyl-10-ribityl-isoalloxazine 5'-phosphate (A) (54560 acquisitions) and [1,3,5-*$^{15}N_3$*]FMN (B,C) in the oxidized state in 100 mM potassium pyrophosphate, pH 8.0.* Spectrum B (70310 acquisitions) was taken under broad-band proton decoupling and spectrum C (51410 acquisitions) was obtained applying the DEPT pulse sequence

and protein-bound FMN suggests that the strength of the hydrogen bond to the C(2) carbonyl group is about the same. But the strong downfield shift of C(8) as compared to that of the same atom in free FMN, leaves no doubt that FMN bound to *D. vulgaris* apoflavodoxin must be more strongly polarized. This seems in contradiction with the chemical shifts of C(2) and C(10a), which would be expected to resonate at a lower field than observed. This apparent discrepancy can be explained assuming that a strong hydrogen bond is also formed with the N(1) atom of protein-bound FMN. Such a hydrogen bond would counteract the downfield shift of the ^{13}C resonances of the atoms in question to some degree. Support for this idea comes from ^{15}N NMR experiments (see below).

The O(4α) atom of FMN in *D. vulgaris* flavodoxin only forms a weak hydrogen bond with the apoflavodoxin. As outlined in a previous paper [9], this leads to a π electron density increase at C(4a) as the sp^2 hybridization of N(10) increases. The large upfield shift of the resonance line due to C(4a), compared both to free FMN and Ac_4rF, suggests that N(10) in protein-bound FMN is more sp^2-hybridized than in free FMN in aqueous solution [9]. That this is the case follows from the ^{15}N chemical shift of the N(10) atom (see below). This explanation is also in line with the observed downfield shifts of C(7) and C(5a) (Table 1, Fig. 3). These atoms accommodate some of the partial positive charge created on the N(10) atom [9]. In this context a downfield shift for C(9) was expected as well. This is not observed. Similarly C(9a) shows a stronger upfield shift than expected [9]. As mentioned above for the concentration dependence of the ^{13}C chemical shifts of free FMN, C(9a) is rather sensitive to the stacking of FMN, i.e. with increasing concentration an upfield shift occurs. In *D. vulgaris* flavodoxin Trp-60 is located in such a way that the C(9) and C(9a) atoms of FMN are under the influence of Trp-60 [19, 24, 25]. We tentatively propose as an explanation the upfield shifts of C(9) and C(9a) are a result of ring current effects of Trp-60.

^{15}N NMR spectra of apoflavodoxin reconstituted with ^{15}N-enriched flavin derivatives are shown in Fig. 4. The ^{15}N chemical shifts are given in Table 1 and compared with those of free FMN in aqueous solution and Ac_4rF in $CHCl_3$. With respect to the ^{15}N chemical shifts of Ac_4rF, upfield shifts are observed for the N(1) and N(5) atoms in free and protein-bound FMN, whereas the N(10) atom shows a large and the N(3) atom a small downfield shift. As pointed out by Witanowski et al. [18], the nitrogen atoms in heteroaromatic compounds can be classified as pyridine-type and pyrrole-type nitrogen atoms as an aid in the interpretation of the ^{15}N chemical shifts. N(1) and N(5) of flavin belong to the former class whereas N(3) and N(10) belong to the latter. Deprotona-

Table 2. *^{13}C-^{1}H and ^{15}N-^{1}H coupling constants of free and protein-bound flavins in the oxidized and reduced state*
The accuracy of the coupling constants is ± 1 Hz for ^{13}C measurements and ± 1.2 Hz for ^{15}N measurements. tetraacetylriboflavin ($MeAc_4rF$) was measured in $CHCl_3$; FMN and *D. vulgaris* flavodoxin were measured in 100 mM potassium pyrophosphate, pH 8.0

Coupling	Coupling constant in					
	oxidized state			reduced state		
	D. vulgaris flavodoxin	FMN	$MeAc_4rF$	*D. vulgaris* flavodoxin	$FMNH^-$	$MeAc_4rFH_2$
	Hz					
$^{1}J[^{13}C(6)\text{-}^{1}H]$	164.6	164.5	164.5	152.0	155.4	153.5
$^{1}J[^{13}C(7\alpha)\text{-}^{1}H]$	131.0	128.5	128.0	125.8	127.6	126.0
$^{1}J[^{13}C(8\alpha)\text{-}^{1}H]$	125.8	127.5	128.0	123.0	127.6	126.0
$^{1}J[^{13}C(9)\text{-}^{1}H]$	161.0	162.8	161.8	153.5	157.0	165.2
$^{1}J[^{15}N(3)\text{-}^{1}H]$	90.6	–	92.7	89.8	–	93.1
$^{1}J[^{15}N(5)\text{-}^{1}H]$	–	–	–	86.2	–	87.5

tion of a pyrrole-type nitrogen gives rise to a downfield shift, while protonation of a pyridine-type nitrogen causes an upfield shift. The ^{15}N chemical shifts of FMN in *D. vulgaris* flavodoxin indicate that a rather strong hydrogen bond exists with the N(1) atom. This hydrogen bond is stronger than that in FMN in aqueous solution. As judged from the difference in the chemical shifts of the N(5) atom in FMN and Ac_4rF, in *D. vulgaris* flavodoxin only a weak hydrogen bond exists with the N(5) atom. The strong hydrogen bond to N(1) supports the interpretation of the ^{13}C chemical shifts of C(2) and C(10a) as proposed above.

The ^{15}N chemical shift of the N(3) atom in apoflavodoxin-bound FMN is almost identical with that observed in Ac_4rF, but it is at higher field than that in FMN. It must be concluded from this that the N(3)H group of FMN forms at best a weak hydrogen bond with the apoflavodoxin. The fact that the $^{1}J[^{15}N(3)\text{-}^{1}H]$ coupling can be observed (Fig. 4) indicates that the proton does not exchange with protons of the bulk solvent; i.e. the exchange rate is slow with respect to the NMR time scale (see also below). Broad-band proton-decoupling (Fig. 4B) proves that the observed doublet is due to the one-bond N-H coupling. However, the decoupled signal is not much different in intensity from the coupled signal, which shows that almost no nuclear Overhauser enhancement is observed. This can only be the case when the isoalloxazine ring is bound tightly to the apoprotein. The long rotational correlation time of the protein (about 5.5 ns) causes the nuclear Overhauser effect to be very small.

As previously discussed in detail [9] the downfield shift of the resonance position of the N(10) atom in free FMN as compared to that in Ac_4rF indicates that the N(10) in FMN is more sp^2-hybridized than in Ac_4rF. This means that the N(10) atoms lies more in the molecular plane in FMN than that in Ac_4rF. In *D. vulgaris* flavodoxin the chemical shift of N(10) appears at even lower field than in FMN indicating a further increase in sp^2 hybridization.

It is worthwhile discussing briefly the difference between the ^{15}N chemical shifts of the nitrogen atoms of FMN bound to *D. vulgaris* apoflavodoxin and *Megasphaera elsdenii* apoflavodoxin. The chemical shifts of the latter protein have been published previously [8]. Based on these results it can be stated that the hydrogen bonds to N(1) and N(3)H of FMN in *M. elsdenii* flavodoxin are stronger than those in *D. vulgaris* flavodoxin. On the other hand the N(5) atom of FMN in *M. elsdenii* flavodoxin is not involved in the formation of a hydrogen bond at all, whereas in *D. vulgaris* flavodoxin it forms a weak hydrogen bond. In addition it seems that the microenvironment of N(5) in *M. elsdenii* flavodoxin is more hydrophobic than in *D. vulgaris* flavodoxin. The N(10) atoms show about the same degree of sp^2 hybridization in both proteins. These results demonstrate that the ^{15}N NMR technique is able to reveal small but significant differences between the electronic structure of FMN bound to different apoflavoproteins and the specific interaction between the prosthetic group and the apoflavoprotein. The investigation of other flavoproteins by these NMR techniques is in progress and we hope that these results will allow us to deepen the insight into the factors 'tailoring' the flavin for a certain biocatalysis.

The one-bond coupling constants $^{1}J(^{13}C\text{-}^{1}H)$, $^{1}J(^{13}C\text{-}^{13}C)$ and $^{1}J(^{15}N\text{-}^{1}H)$ are collected in Tables 2 and 3. There are two structural features which control the magnitude of these coupling constants. These are (a) the percentage of s character of the hybrid orbital of the carbon or nitrogen atoms involved in the C-H, C-C or N-H bond and (b) electron-withdrawing or donating substituents either directly located at the atom involved in this coupling or, in the case of an aromatic compound, indirectly through the π system. The coupling constants generally increase with an increase of the s character of the hybrid orbitals involved or when electron withdrawing substituents are present.

The methyl $^{13}C\text{-}^{1}H$ coupling constants in $MeAc_4rF$ are only slightly larger than those in *o*-xylene (126 Hz) [26], indicating a somewhat lower π electron density in the benzene subnucleus of $MeAc_4rF$ compared to *o*-xylene. It is interesting to note that the coupling constants in free and protein-bound FMN become non-equivalent and steadily increase for the $CH_3(7)$ and decrease for the $CH_3(8)$ group on going from free to protein-bound FMN. The increase in the coupling constant for $CH_3(7)$ most probably reflects the increase of the partial positive charge on C(7) due to the increase of the sp^2 hybridization of the N(10) atom. This interpretation is in agreement with the observed downfield shifts of the carbon atoms in question (Table 1). The trend of the $^{13}C\text{-}^{1}H$ coupling constants of the $CH_3(8)$ is reversed on increasing the polarization of the flavin molecule in the direction of the C(8), C(6), N(5), C(10a) and C(2) atoms. The coupling constants of free and protein-bound flavin suggest that the $CH_3(8)$ group of the

Table 3. *Direct ^{13}C-^{13}C coupling constants in free and protein-bound [4,4a,10a-$^{13}C_3$] flavins in the oxidized and reduced state*
The accuracy of the coupling constants is ± 0.5 Hz. *N*(3)-Methyl-tetraacetylriboflavin was measured in C^2HCl_3; FMN and *D. vulgaris* flavodoxin were measured in 100 mM potassium pyrophosphate, pH 8.0

Redox state	Compound	Coupling constants	
		$^1J[^{13}C(4)$-$^{13}C(4a)]$	$^1J[^{13}C(4a)$-$^{13}C(10a)]$
		Hz	
Oxidized	$MeAc_4rF$	75.5	53.3
	FMN	75.4	55.9
	D. vulgaris flavodoxin	76.9	57.3
Reduced	$MeAc_4rFH_2$	79.1	84.5
	$FMNH_2$(pH = 4.8)	82.0	81.6
	$FMNH^-$ (pH = 8.0)	84.0	74.4
	D. vulgaris flavodoxin	86.7	72.8

flavin molecule is involved in hyperconjugation, the degree of hyperconjugation paralleling the degree of polarization of the molecule.

The ^{13}C-^1H coupling constants of the C(6)-H and C(9)-H bonds in $MeAc_4rF$, free and protein-bound FMN are somewhat larger than those in benzene (159 Hz) [26]. The larger coupling constant for C(6)-H as compared to that for C(9)-H is in accordance with the corresponding ^{13}C chemical shifts which indicate a decreased π electron density at C(6).

It is generally accepted that the magnitude of the one-bond ^{13}C-^{13}C coupling constants reflects approximately the s character of the orbitals making up the bond [26]. The coupling constant of the C(4)-C(4a) bond shows little variation among $MeAc_4rF$, FMN and *D. vulgaris* flavodoxin (Table 3). The corresponding coupling constant in *M. elsdenii* flavodoxin is almost identical with that in *D. vulgaris* flavodoxin. A detailed interpretation of this coupling constant has been given previously [4]. The $^1J[^{13}C(4a)$-$^{13}C(10a)]$ increases slightly in going from $MeAc_4rF$, FMN to *D. vulgaris* flavodoxin. As previously explained [4] the results indicate that the s character of C(10a) increases in going from $MeAc_4rF$ to *D. vulgaris* flavodoxin. This interpretation is in agreement with the ^{13}C chemical shifts of this atom.

The $^1J[^{15}N(3)$-$^1H]$ coupling constant in *D. vulgaris* flavodoxin (Fig. 4) is somewhat smaller than that in Ac_4rF which indicates as decreased sp^2 hybridization of the N(3) atom in the protein as compared to that of free flavin. The corresponding coupling constant of FMN in aqueous solution could not be determined because of the fast proton exchange reaction.

The hydrogen bond pattern derived from the NMR results is remarkably similar to that derived from the crystallographic data on *D. vulgaris* flavodoxin. There are, however, some differences between the two sets of data. The presence of a weak hydrogen bond towards the N(5) atom of protein-bound FMN was not obvious in the crystal structure [19, 25]. The rather strong hydrogen bond to the N(1) atom, as revealed by ^{15}N NMR, was also not observed in the X-ray structure. This could be due to crystal packing forces leading to a crystal structure differing slightly from that in solution.

Studies on the reduced state

^{13}C and ^{15}N NMR spectra of two-electron-reduced *D. vulgaris* flavodoxin are shown in Figs 5 and 6. Reduction of the protein by two electrons leads to an upfield shift of the ^{13}C and ^{15}N resonances, except for C(10a), which is shifted downfield as compared to the oxidized molecule (Table 1, Fig. 3). The largest upfield shifts are exhibited, in decreasing order, by the resonances due to C(4a), C(8), C(6), C(7) and C(4). The order for the ^{15}N chemical shifts is N(5), N(10), N(3) and N(1). Comparing the ^{13}C chemical shifts of the two-electron-reduced free and protein-bound FMN it is interesting to note that those due to the benzene subnucleus of the prosthetic group of flavodoxin are shifted further upfield than the corresponding ones in free flavin.

The chemical shifts due to C(7) and C(9a) are very similar. In order to ensure a correct assignment the spectra were run under proton decoupling and in the absence of decoupling conditions. Under the latter condition the signal at 130.7 ppm showed significantly more broadening, both in free and bound $FMNH^-$, due to the coupling of the C(7) with the protons of the $CH_3(7\alpha)$ group.

The results strongly indicate that, on two-electron reduction, the π electron density in the benzene subnucleus of FMN increases more in the protein-bound state than in the free state. A comparison of the chemical shifts due to C(10a) and C(2) in Ac_4rFH_2, $FMNH^-$ and flavodoxin shows unambiguously that the protein-bound FMN is ionized. This is further supported by the ^{15}N chemical shift of the N(1) atom (Table 1) and the absence of a one-bond coupling between N(1) and a proton. In protein-bound FMN the relevant ^{13}C and ^{15}N chemical shifts are pH-independent in the range 6.0 – 8.5. This means that the pK_a value of the N(1) atom in protein-bound flavin is decreased compared to that of free flavin. This observation has already been made with *M. elsdenii* flavodoxin [4, 8] and has been suggested from data obtained by the light absorption technique [12]. In this context we would like to stress the following point. Model studies [2] indicated that the molar absorption coefficient at 450 nm reflects the planarity of the flavin molecule in the reduced state. At that time a consensus existed among the flavin chemists that ionized reduced flavin possessed a bent structure and flavin enzymologists usually extrapolated this idea to flavoproteins. This often led to the conclusion that the prosthetic group in two-electron-reduced flavodoxins is coplanar and in the neutral state. While the planar structure of reduced flavodoxin has been proven by X-ray data [12], the ionization state of the protein-bound flavin could not be revealed by this technique. In a previous paper [4] we have discussed the relationship between the molar absorption coefficient and the planarity of the flavin ring. The main point is that this

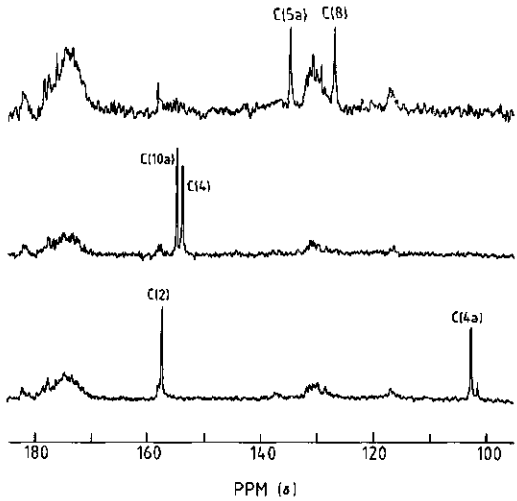

Fig. 5. *^{13}C NMR spectra of* D. vulgaris *apoflavodoxin reconstituted with [4,10a-$^{13}C_2$]FMN, (5840 acquisitions), [2,4a-$^{13}C_2$]FMN (13020 acquisitions) and [5a,8-$^{13}C_2$]FMN (155000 acquisitions) in the reduced state in 100 mM potassium pyrophosphate, pH = 8.0.* The small peak at high field of the C(4a) signal is due to the C(4a) atom of free $FMNH^-$. The corresponding C(2) signal at low field is not observed owing to overlap with natural-abundance ^{13}C signals due to protein groups

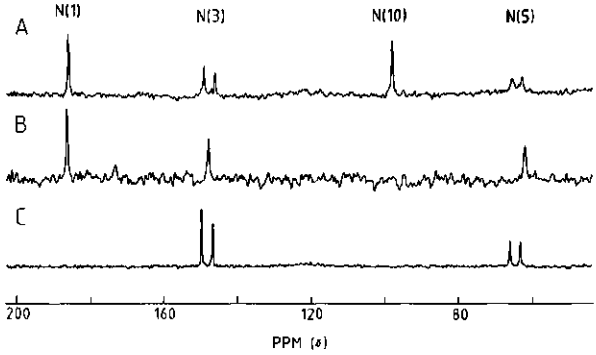

Fig. 6. *^{15}N NMR spectra of* D. vulgaris *apoflavodoxin reconstituted with [1,3,5,10-$^{15}N_4$]7-methyl-10-ribityl-isoalloxazine 5'-phosphate (A) (70310 acquisitions) and [1,3,5-$^{15}N_3$]FMN (B,C) in the reduced state in 100 mM potassium pyrophosphate, pH = 8.0.* Spectrum B (89110 acquisitions) was taken under broad-band proton decoupling and spectrum C (123960 acquisitions) was obtained applying the DEPT pulse sequence

relationship is still valid, although in a refined manner [9], but it is not useful as a diagnostic tool for the ionization state of reduced protein-bound flavin. On the other hand, the NMR technique reveals both the degree of planarity (see below) and ionization state of protein-bound flavin. The results also indicate that the N(1) atom in reduced flavodoxin-bound FMN is not at all accessible to protons from solvent water. This feature seems to hold for other flavoproteins as well [27, 28].

The ^{13}C and ^{15}N chemical shifts due to C(2), C(4), N(3) and N(5) indicate hydrogen bonds to O(2α) and N(3)H, whose strength is somewhat less than those in free flavin in aqueous solution. N(5)H forms a stronger hydrogen bond with the apoprotein than the corresponding atom in free FMN in water. The chemical shift of C(4) indicates that no hydrogen bond is formed with O(4α), i.e. the microenvironment is probably hydrophobic.

The order of the $^1J(^{13}C\text{-}^1H)$ coupling constants of C(6)H and C(9)H follows the corresponding ^{13}C chemical shifts and therefore reflects the π electron density at these centers, as expected from theory [26]. The same trend is also observed between the ^{13}C chemical shifts of C(7) and C(8) and the methyl coupling constants of the corresponding groups. In contrast to the corresponding methyl coupling constants in the oxidized molecules, the coupling constants remain equivalent for reduced Ac_4rF and FMN.

The fact that the $^1J[^{15}N(5)\text{-}^1H]$ and $^1J[^{15}N(3)\text{-}^1H]$ can be observed in reduced *D. vulgaris* flavodoxin shows that these centers are not accessible to bulk solvent. If these protons still exchange with the protons of the solvent water the exchange

rate has to be very slow i.e. $\tau \gg J^{-1}$, $\tau \gg 10$ ms. The almost complete absence of a nuclear Overhauser effect on the $^{15}N(3)$ and $^{15}N(5)$ resonances on decoupling of the protons indicates that the isoalloxazine is tightly bound to the apoprotein. As shown in Fig. 6, these coupling constants can be determined rather easily by enhancement of the ^{15}N signals by polarization transfer using the DEPT pulse-sequence [17]. The coupling constants given in Table 2 show that the N(3) atom of flavin bound to the apoflavodoxin and that in Ac_4rFH_2 are both highly sp^2-hybridized, the sp^2 hybridization is in fact increased as compared to those of the oxidized molecules. The $^1J[^{15}N(5)\text{-}^1H]$ of flavodoxin shows about the same value as that of Ac_4rFH_2 (Table 2) but it is considerably smaller than that found in *M. elsdenii* flavodoxin [8]. The larger value observed in the latter protein demonstrates that the N(5) atom in *M. elsdenii* flavodoxin is almost completely sp^2-hybridized whereas that in *D. vulgaris* flavodoxin is apparently considerably sp^3-hybridized. However, the strong interaction between the N(5)H group with the apoprotein leads to an increase of the N-H bond distance, influencing the value of the coupling constant, i.e. a decrease. Therefore only the combination of the coupling constant and the ^{15}N chemical shift of the N(5)H group leads to the conclusion that the N(5) atom of the flavin in *D. vulgaris* flavodoxin possesses a high degree of sp^2 hybridization, i.e. is in the molecular plain. This interpretation is in accordance with the published molar absorption coefficient at 450 nm for reduced *D. vulgaris* flavodoxin [29].

Our interpretation supports the 'first model' proposed for the hydrogen bond interaction between the apoflavodoxin and FMN as deduced from X-ray data on the semiquinone form of *D. vulgaris* flavodoxin [25]. In the comparison of these data with our NMR results it is, however, assumed that the structure of the two-electron-reduced state is very similar with that of the one-electron-reduced state. This is a reasonable assumption considering the crystallographic data on *Clostridium MP* flavodoxin [12]. It is suggested that this delicate structural difference between the flavodoxins from *M. elsdenii* and *D. vulgaris* is related to the fact that the electron exchange reaction between the semiquinone and hydroquinone forms is much faster in *M. elsdenii* flavodoxin [30] than in *D. vulgaris* flavodoxin (J. Vervoort, unpublished results).

The direct $^{13}C\text{-}^{13}C$ coupling constants of the C(4a)-C(10a) and the C(4)-C(4a) bonds indicate that the s character of the former bond decreases and that of the latter bond increases on going from Ac_4rFH_2, $FMNH_2$, $FMNH^-$ to *D. vulgaris* flavodoxin (Table 3). There seems to be a contradiction between this decrease in the $^{13}C\text{-}^{13}C$ coupling constant of the C(4a)-C(10a) bond and the increased chemical shift observed for the C(10a) atom on going from $FMNH_2$ to $FMNH^-$. One should however, remember that the electrical field of the negative charge at N(1) in $FMNH^-$ influences considerably the ^{13}C chemical shift of the C(10a). This makes it difficult to evaluate precisely the influence of the chemical shift of the C(10a) atom on the coupling constant. But from the comparison of $^1J[^{13}C(10a)\text{-}^{13}C(4a)]$ and the chemical shift of the C(4a) atom it can be concluded that the π electron density increases at the C(4a)-C(10a) bond and decreases at the C(4)-C(4a) bond in $FMNH^-$ as compared to $FMNH_2$. In *D. vulgaris* flavodoxin one observes a small decrease of the coupling constant of the C(4a)-C(10a) bond and a small increase for the C(4a)-C(4) bond as compared to free $FMNH^-$. Accordingly the C(4a)-C(10a) bond has a slightly higher and the C(4)-C(4a) bond has a slightly lower π electron density. The coupling constants in the flavodoxin were, in fact, used to ensure the assignment of the C(4) and the C(10a) resonances.

On consideration of the chemical shifts of the isoalloxazine ring system, one notices clearly the strong upfield shift of several carbon atoms of the isoalloxazine ring in *D. vulgaris* flavodoxin as compared to free $FMNH^-$. This increase in π electron density at the carbon atoms is most likely due to the electron-donating effect of the N(5) and N(10) atoms. The downfield shift observed for these two nitrogen atoms in *D. vulgaris* flavodoxin indicates an increase in sp^2 character and hence π electron density is reallocated at other centers [9], i.e. preferentially in the benzene subnucleus. The N(1) atom in *D. vulgaris* flavodoxin is shifted even more downfield than in $FMNH^-$. This indicates that, although it carries a negative charge, this charge is not compensated by a positive charge of the protein. It is interesting to see that in *M. elsdenii* flavodoxin the negative charge at N(1) is compensated to a small extent.

Our theory that charge-charge interactions are the main factor which determine the redox potential of the flavodoxins [11], is supported by these differences observed for the ^{15}N chemical shifts due to N(1) in *D. vulgaris* flavodoxin and in *M. elsdenii* flavodoxin. We hope to present in the near future further experimental data in support of this new theory.

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Chapter 3. A COMPARATIVE CARBON-13, NITROGEN-15 AND PHOSPHORUS-31 NUCLEAR MAGNETIC RESONANCE STUDY ON THE FLAVODOXINS FROM CLOSTRIDIUM MP, MEGASPHAERA ELSDENII AND AZOTOBACTER VINELANDII[+]

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ABSTRACT

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

INTRODUCTION

Flavodoxins are a group of relatively small flavoproteins (14000-23000 D) consisting of one polypeptide chain and containing a single molecule of non-covalently-bound riboflavin 5'-phosphate (FMN) (Mayhew & Ludwig, 1975).

The flavin coenzyme can exist in three redox states, i.e. oxidized, one-electron reduced or semiquinone and two-electron reduced or hydroquinone. During in vivo redox reactions the flavodoxins probably function only as one-electron carriers, shuttling between the hydroquinone and the semiquinone states. In vitro the flavin molecule shuttles between the oxidized, semiquinone and hydroquinone states. The redox potential of the different redox states is strongly altered as compared with free FMN. The

redox potential for the semiquinone-reduced couple is profoundly modified i.e. from -124 mV in free FMN to about -400 mV or even -500 mV in the flavodoxins (Mayhew & Ludwig, 1975; Anderson, 1983).

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

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

that there exist marked differences in electronic structure between free and bound flavin.

^{31}P NMR studies have been done on the flavodoxins from Megasphaera elsdenii (Moonen & Müller, 1982), Azotobacter vinelandii (Edmondson & James, 1979) and Desulfovibrio vulgaris (Favaudon et. al., 1980). These studies revealed information not only on the phosphate binding site of FMN (Moonen and Müller, 1982) but also on the rate of electron transfer between the semiquinone and reduced state in M.elsdenii flavodoxin (Moonen and Müller, 1984d) and on a covalently bound disubstituted monophosphate in A.vinelandii flavodoxin (Edmondson & James, 1979). In order to complement these studies and to unravel the protein active site a two-dimensional ^{1}H NMR project has been started and it has been shown that in spite of the relatively large molecular mass of M.elsdenii flavodoxin the desired information can be obtained (Moonen & Müller, 1984e; Moonen et. al., 1984f).

This paper shows that subtle differences in interaction between the apoflavodoxins from Megasphaera elsdenii, Clostridium MP and Azotobacter vinelandii and FMN reveal themselves by different ^{13}C, ^{15}N and ^{31}P NMR chemical shifts. All carbon and nitrogen atoms of the isoalloxazine moiety of protein-bound FMN have been investigated and assigned. The results obtained with these three flavodoxins are compared with the results obtained on Desulfovibrio vulgaris flavodoxin (Vervoort et al., 1985) and a further FMN-containing protein, Old Yellow Enzyme from yeast (Beinert et al., 1985a, 1985b).

MATERIALS AND METHODS

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

The flavodoxins were isolated and purified according to published procedures by Mayhew & Massey (1969) (Clostridium MP and Megasphaera elsdenii flavodoxin) and Hinkson & Bulen, (1967, Azotobacter vinelandii OP

flavodoxin). The apoproteins were prepared by the trichloroacetic acid precipitation method (Wassink & Mayhew, 1975). The reconstitution of the apoflavodoxins with ^{13}C or ^{15}N labeled prosthetic groups was carried out in neutral buffered solutions at 4°C. Excess of flavin was removed on a Biogel P-6DG column. The protein-FMN complex was then concentrated by either ultrafiltration (Amicon) or by means of lyophilization.

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

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

Pure [^{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 shifts are reported relative to liquid NH_3 at 25°C (δCH_3NO_2-δNH_3 = 381.9 ppm for the magnetic field parallel to the sample tube (Witanowski et al., 1981)). Values are reported as true shieldings i.e. corrected for bulk volume susceptibilities. ^{31}P chemical shifts were determined relative to an external standard of 85% phosphoric acid. The accuracy of the reported values is about 0.1 ppm for ^{13}C and ^{31}P NMR and about 0.3 ppm for ^{15}N NMR chemical shift values.

Quadrature phase detection was used. The instrumental settings were: 30° pulse, repetition time 1 s, 8 K data points. For determination of the nuclear Overhauser effects (NOE) a repetition time of 10 s was used. The temperature of the samples was 26±2°C for M.elsdenii and Clostridium MP

flavodoxin, and 20°±2°C for Azotobacter vinelandii flavodoxin. Reduction of the samples was conducted by the addition of the desired amount of a dithionite solution to the anaerobic solution. Anaerobiosis was achieved by carefully flushing the solution in the NMR tube with argon for about 10 min. The NMR tube was sealed with a rubber cap (suba seal).

RESULTS AND DISCUSSION

^{31}P NMR studies in the oxidized and reduced state.

Table 1 shows the effect of redox state on the ^{31}P chemical shifts of flavodoxins. The chemical shifts reported in this study appear to be different from those reported previously (Moonen & Müller, 1982; Favaudon et al., 1980; Edmondson & James, 1979).

Table 1 ^{31}P chemical shifts (in ppm) of the various flavodoxins in the oxidized and reduced state in 100 mM Tris-HCl, pH 8.0.[a)]

	Redox state	
Flavodoxin	Oxidized δ	Reduced δ
free FMN	5.1	5.1
C.MP	5.7	5.8
M.elsdenii	5.3; 4.8[b)]	5.4; 4.9[b)]
D.vulgaris	5.4; 5.0[c)]	5.5; 5.0[c)]
A.vinelandii	6.3, 0.9; 5.6[d)], 0.8[d)]	6.4, 1.0; 5.4[d)], 0.4[d)]

a) Chemical shifts are relative to 85% H_3PO_4. Positive chemical shifts are downfield from 85% H_3PO_4.

b) From Moonen and Müller (1982)

c) From Favaudon et al. (1980)

d) From Edmondson and James (1979)

However, the spectra measured previously were recorded on a NMR instrument, equipped with a conventional electromagnet where the magnetic field is perpendicular to the sample tube. We performed our measurements on an NMR apparatus equipped with a superconducting magnet where the external magnetic field is parallel to the sample tube. When the correction for the differences in magnetic susceptibility is applied (Gadian, 1982), some differences remain for the chemical shift of the phosphate resonances reported previously and those given in Table 1, but the differences are very small. Yet, the chemical shift of the covalently bound phosphorus residue in A.vinelandii flavodoxin still differs by about 0.6 ppm from that reported previously (Edmondson & James, 1979), after correction for differences in magnetic susceptibility. The chemical shifts reported here indicate that in all flavodoxins the phosphate group of FMN is bound in the dianonic form. This is deduced from the fact that the ^{31}P resonance of free FMN is found at 5.1 ppm in the dianionic form (pH= 9.0). All ^{31}P resonances in the flavodoxins are downfield shifted from that of free FMN. These downfield shifts can be interpreted as (small) distortions of the O-P-O bond angles (Gorenstein, 1975). The largest distortion is observed in A.vinelandii flavodoxin.

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

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

^{13}C and ^{15}N NMR studies in the oxidized state

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

A typical ^{15}N NMR spectrum of flavodoxins in the oxidized state is shown in Figure 1A for clostridial flavodoxin. The ^{15}N chemical shifts of the flavodoxins are collected in Table 2 and for convenience presented diagrammatically in Figure 2. From these results it can be concluded that M.elsdenii and C.MP apoflavodoxin do not form a hydrogen bond with the N(5) atom of FMN. This conclusion follows from the fact that the ^{15}N chemical shifts due to the N(5) atoms of FMN in these two flavodoxins appear even at lower field than that of TARF in an apolar solvent ($CHCl_3$) (Table 2, Figure 2). The shifts for the N(5) atom of FMN in D.vulgaris (Vervoort et al., 1985) and A.vinelandii flavodoxin appear at an intermediate field between that of TARF and FMN, indicating the formation of a weak hydrogen bond between the apoflavodoxins and the N(5) atom of protein-bound FMN. The fact that the ^{15}N chemical shifts of N(5) in the former flavodoxins are downfield from that of free FMN also suggests that the π electron density at the N(5) atom of protein-bound FMN is lower than that in free oxidized flavin.

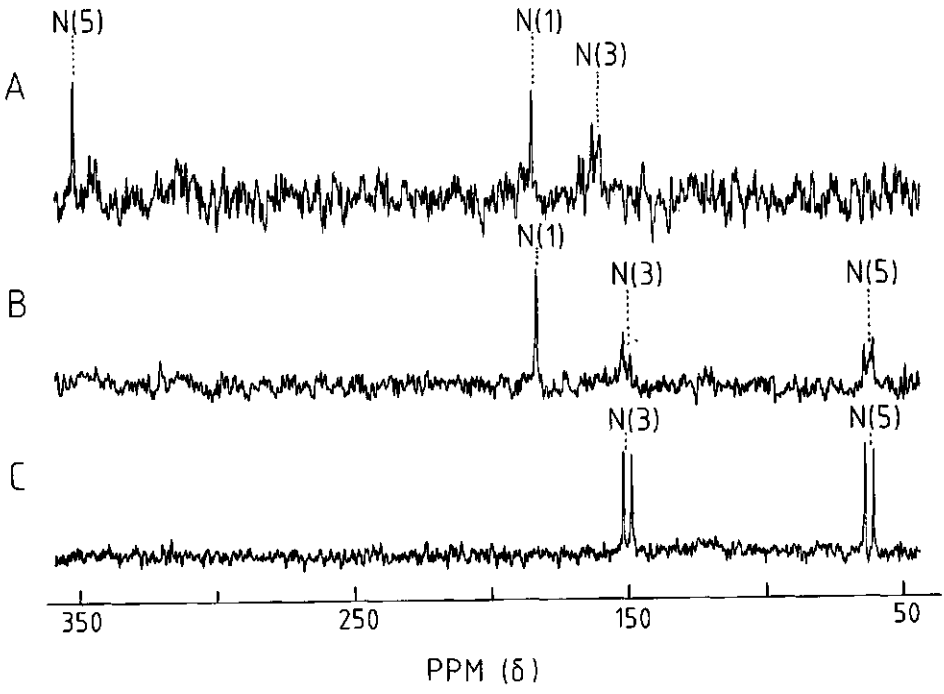

Figure 1. ^{15}N NMR spectra op C.MP apoflavodoxin reconstituted with [1,3,5 - $^{15}N_3$]FMN in the oxidized state (A) (55800 acquisitions) and in the reduced state (B,C) in 100 mM potassium pyrophosphate, pH = 8.0. Spectrum B (119800 acquisitions was taken under proton-coupled conditions and spectrum C (54300 acquisitions) was obtained applying the DEPT puls sequence.

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

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

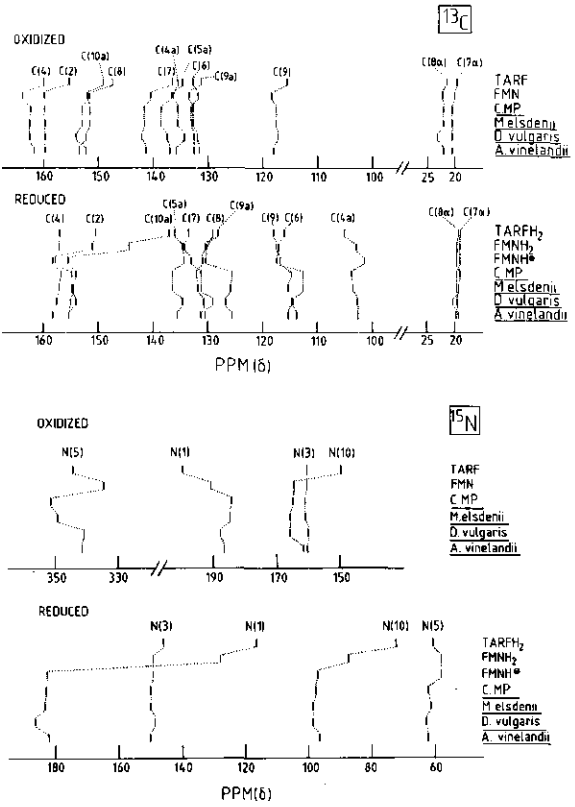

Figure 2. Correlation diagram of ^{13}C and ^{15}N chemical shifts of free FMN and bound to C.MP, M.elsdenii, D.vulgaris and A.vinelandii apoflavodoxin in the oxidized and in the reduced state.

As previously described (Moonen et al., 1984b) the N(10) atom in free oxidized flavin shows an unexpected large downfield shift on going from apolar to polar solvents. Since this pyrrole-like nitrogen atom cannot form a hydrogen bond the downfield shift of its resonance had to be explained as an increase in sp^2 hybridization. This increase in hybridization only occurs when the polarization of the isoalloxazine ring is stabilized by hydrogen bond formation with C(2α) and C(4α). In keeping with this interpretation, which is also supported by ^{13}C data (see below), M.elsdenii and D.vulgaris flavodoxin show an increased degree of sp^2 hybridization as

Table 2 ^{13}C and ^{15}N chemical shifts (in ppm) of various selectively-enriched free and protein-bound FMN derivatives in 100 mM potassium pyrophosphate, pH 8.0. ^{13}C chemical shifts are relative to Me_4Si. ^{15}N chemical shifts are relative to liquid NH_3. Values for Old Yellow enzyme are taken from Beinert et al. (1985). Values for TARF and $TARFH_2$ were determined in $CHCl_3$.

Atom	^{13}C and ^{15}N chemical shifts in						
Oxidized	A.v.	D.v.[a]	M.e.	C.MP.	OYE[b]	FMN[a]	TARF[c]
C(2)	159.6	159.7	159.8	159.8	160.6	159.8	155.2
C(4)	161.7	162.4	162.4	162.3	164.2	163.7	159.8
C(4a)	135.7	134.3	135.6	135.5	137.1	136.2	135.6
C(5a)	136.9	137.4	138.4	138.5	135.2	136.4	134.6
C(6)	132.6	132.5	133.0	133.1	-	131.8	132.8
C(7)	141.2	142.0	141.4	141.4	141.2	140.4	136.6
C(7α)	20.4	20.5	20.6	20.5	19.3	19.9	19.4
C(8)	152.2	154.0	153.0	153.0	151.8	151.7	147.5
C(8α)	21.9	23.3	22.1	22.0	21.9	22.2	21.4
C(9)	117.9	117.2	117.5	117.4	117.7	118.3	115.5
C(9a)	131.5	131.9	132.9	132.9	131.7	133.5	131.2
C(10a)	153.5	152.3	151.5	151.4	152.9	152.1	149.1
N(1)	186.4	188.0	185.0	184.5	194.3	190.8	199.9
N(3)	160.2	159.9	160.9	161.1	164.1	160.5	159.8
N(5)	341.4	341.1	349.3	351.5	319.4	334.7	344.3
N(10)[d]	161.5	165.6	165.6	164.8	161.5	164.6	150.2
Reduced	A.v.	D.v.[a]	M.e.	C.MP.	OYE[b]	$FMNH^-$[a]	$FMNH_2$[c]	$TARFH_2$[c]
C(2)	158.3	157.5	156.9	156.6	159.4	158.2	151.1	150.6
C(4)	155.2	154.0	154.8	154.8	163.0	157.7	157.2	157.0
C(4a)	102.6	102.7	103.5	103.7	95.3	101.4	103.1	105.2
C(5a)	135.5	134.6	136.3	136.5	133.9	134.2	134.4	136.0
C(6)	113.8	114.5	112.4	112.4	-	117.3	117.1	116.1
C(7)	130.4	130.7	131.1	131.3	131.8	133.0	134.3	133.6
C(7α)	19.8	19.4	19.6	19.6	18.3	19.0	19.1	19.1
C(8)	125.5	126.7	125.9	125.6	128.5	130.3	130.4	129.0
C(8α)	19.3	20.3	19.2	19.1	19.5	19.4	19.4	19.3
C(9)	115.2	114.7	115.3	114.8	117.0	116.8	117.4	118.0
C(9a)	131.2	129.1	131.8	132.1	131.8	130.9	130.4	128.2
C(10a)	155.2	155.0	154.5	154.1	157.7	155.5	144.3	137.1
N(1)	182.0	186.6	183.4	182.8	187.4	182.6	128.0	116.7
N(3)	150.0	148.3	149.7	150.1	153.2	149.3	149.7	145.8
N(5)	61.7	62.1	61.3	61.9	48.6	57.7	58.0	60.4
N(10)[d]	96.7	98.4	98.3	97.7	97.6	97.2	87.3	72.2

a) From Vervoort et al., 1985
b) From Beinert et al., 1985a,b
c) From Moonen et al., 1984b
d) [1,3,5,10-$^{15}N_4$]7-Methyl-10-ribityl-isoalloxazine-5'-phosphate

compared with that of free FMN and C.MP flavodoxin, whereas that of A.vinelandii flavodoxin is considerably decreased with respect to that of free FMN. Therefore the isoalloxazine ring of protein-bound FMN is somewhat less planar in A.vinelandii flavodoxin with respect to the N(10) atom than in the other three flavodoxins studied.

The ^{1}J ($^{15}N(3)$-^{1}H) coupling constants of the protein-bound FMN have been determined using the DEPT pulse sequence of Doddrell et al. (1982), optimized for a coupling constant of 90 Hz (Franken et al., 1984). This technique yields theoretically a ten-fold increase in the signal to noise ratio in the ^{15}N spectra (see also below) (Fig. 1 C). This allowed us to determine the coupling constants rather easily. The data are given in Table 3 and compared with published values for D.vulgaris and M.elsdenii flavodoxin, and TARF. The $^{1}J(^{15}N(3)-^{1}H)$ coupling constant is a few Hz smaller in the flavodoxins than in TARF. The fact that the coupling constant can be observed in these proteins indicates that the exchange of the N(3)-H protons is slow on the NMR time scale. Apparently the solvent has no access to this position in the proteins i.e. the lifetime of the N(3)-H group in the proteins >> 10 ms.

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

The nuclear Overhauser effect (NOE) of e.g. N(H) or C(H) groups

Table 3 ^{15}N-^{1}H coupling constants (in Hz) of free and protein-bound flavins in the oxidized and reduced state. The accuracy of the coupling constants is ± 1.2 Hz. Tetraacetylriboflavin (TARF) was measured in $CHCl_3$. The flavodoxins were measured in 100 mM potassium pyrophosphate, pH 8.0.

Flavodoxin	Coupling constant	
	$^{1}J[^{15}N(3)-^{1}H]$	$^{1}J[^{15}N(5)-^{1}H]$
Oxidized state		
C.MP flavodoxin	90.3	-
M.e. flavodoxin	88.2[a]	-
D.v. flavodoxin	90.6[b]	-
A.v. flavodoxin	90.6	-
TARF	92.7[a]	-
Reduced state		
C.MP flavodoxin	91.6	94.0
M.e. flavodoxin	93.1[a]	92.1[a]
D.v. flavodoxin	89.8[b]	86.2[b]
A.v. flavodoxin	89.7	90.3
$TARFH_2$	93.1[a]	87.5[a]

a) From Franken et al. (1984)

b) From Vervoort et al. (1985)

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

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

The ^{13}C chemical shifts of the flavodoxin from M.elsdenii, C.MP and A.vinelandii are given in Table 2. For comparison the ^{13}C chemical shifts of FMN (in H_2O), TARF (in $CHCl_3$) and of Old Yellow enzyme (Beinert et al., 1985b) are also given. Old Yellow enzyme is a FMN-containing protein. In contrast to the flavodoxins this enzyme is probably involved in the catalysis of oxidation or reduction of an organic molecule, although its precise biological function is not yet known. This makes a comparison with the electron-transfer flavoproteins interesting.

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

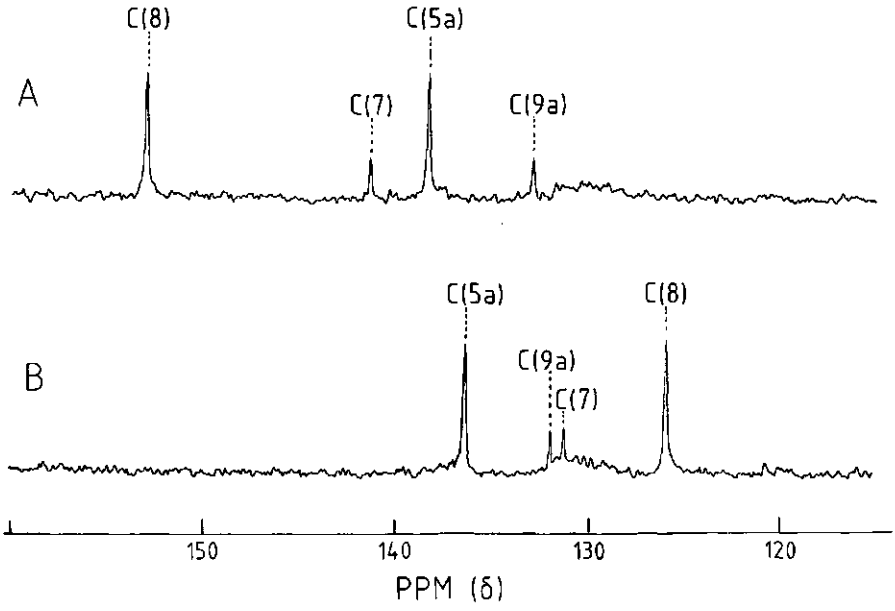

Figure 3. ^{13}C NMR spectra of M.elsdenii apoflavodoxin reconstituted with a mixture of ^{13}C enriched FMN containing 80% [8,5a-$^{13}C_2$]FMN and 20% [7,9a-$^{13}C_2$]FMN in the oxidized state (A) (105000 acquisitions) and in the reduced state (B) (109000 acquisitions) in 100 mM potassium pyrophosphate, pH = 8.0.

The ^{13}C chemical shifts due to C(2) of FMN in all four flavodoxins (Table 2, Figure 2) are very similar and resemble that of FMN in aqueous solution, but differ from that of TARF in $CHCl_3$. This indicates that a rather strong hydrogen bond exist between C(2α) and the apoflavodoxins. These hydrogen bondings seem to be of similar strength in all four flavodoxins as deduced from the ^{13}C chemical shifts. It must be noticed however, that the strength of the hydrogen bond formed between the N(1) atom of FMN and the apoprotein counteracts the ^{13}C chemical shifts of the neighboring carbon atoms, i.e. leading to an upfield shift of the resonance due to C(2)

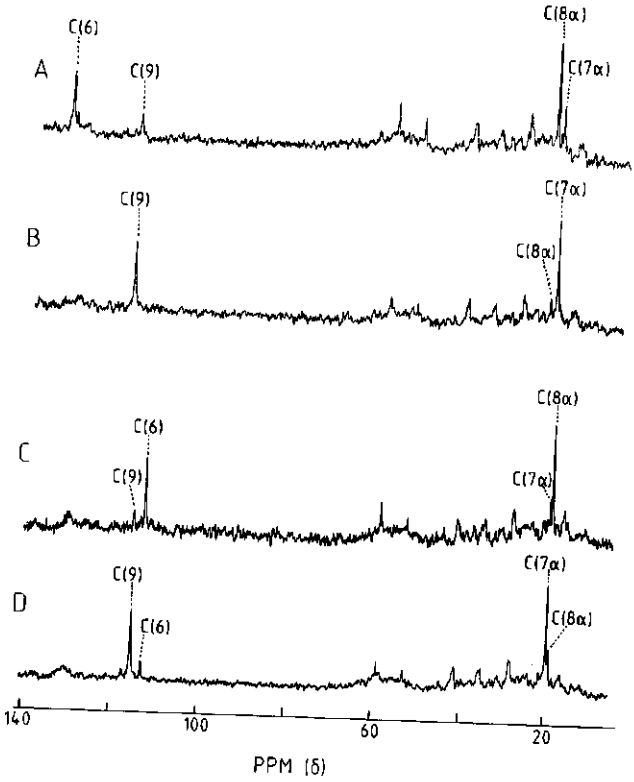

Figure 4. ^{13}C NMR spectra of C.MP apoflavodoxin reconstituted with various mixtures of ^{13}C enriched FMN in the oxidized state (A, B) and in the reduced state (C, D) in 100 mM potassium pyrophosphate, pH = 8.0.

A: 80% [6,8α - $^{13}C_2$] and 20% [7α,9 - $^{13}C_2$]FMN (11200 acquisitions)

B: 80% [7α,9 - $^{13}C_2$] and 20% [6,8α - $^{13}C_2$]FMN (5800 acquisitions)

C: 80% [6,8α - $^{13}C_2$] and 20% [7α,9 - $^{13}C_2$]FMN (5200 acquisitions)

D: 80% [7α,9 - $^{13}C_2$] and 20% [6,8α - $^{13}C_2$]FMN (17000 acquisitions)

and C(10a). Therefore it must be concluded that the hydrogen bond with C(2α) is stronger in C.MP and M.elsdenii flavodoxins than in A.vinelandii and D.vulgaris flavodoxin but that in all four flavodoxins the hydrogen bonds are stronger than that of C(2α) of FMN with the solvent water. These results also demonstrate that the electronic structure of the flavin molecule is very complex and that an environmental influence at one particular atom also affects other atoms in the molecule as was already demonstrated for free flavin by the coherent anti-Stokes Raman spectroscopy technique (Müller et al., 1983).

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

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

edge of protein-bound flavin which is known to be accessible to solvent from crystallographic work (Smith et al., 1977, 1983; Watenpaugh et al., 1972). As a consequence it could also be concluded from our NMR results that of the four flavodoxins studied the FMN in A.vinelandii flavodoxin is the least accessible to bulk solvent and that in D.vulgaris flavodoxin is the most accessible.

The ^{13}C chemical shifts due to C(9a) and C(10a) of FMN in D.vulgaris and A.vinelandii flavodoxins deserve some further comments since they do not show the shifts expected on the basis of the results for free flavin (Moonen et al., 1984b). For D.vulgaris flavodoxin we have recently suggested (Vervoort et al., 1985) that the slight upfield shift for C(9a) instead of the expected downfield shift, is a result of ring current effects of Trp-60 (Watenpaugh et al., 1972; 1973; 1975). Yet, there is no equivalent aromatic amino acid residue in A.vinelandii flavodoxin (Dubourdieu and Fox, 1977), except maybe Tyr-102, unless the folding of the active site is completely different from the other flavodoxins which does not seem likely (Smith et al., 1977; 1983). Even if the N(10) atom, on the other hand, would be under the influence of ring current effects of an aromatic amino acid residue, the chemical shifts of the two atoms in question could not be explained satisfactorily. Therefore we propose that both atoms are shifted upfield by the partial positive charge (α-effect) developed by the increased sp^2 hybridization of N(10). This interpretation would explain fully the C(10a) shifts in both proteins, i.e. in D.vulgaris flavodoxin the hydrogen bond to N(1) is weaker than in the other flavodoxin, leading to a downfield shift, and in A.vinelandii flavodoxin the sp^2 hybridization of N(10) is relatively strongly decreased in comparison to the other flavodoxins yielding a larger downfield shift. Since the C(9a) atom is under the influence of the mentioned effect of N(10) and involved when the molecule is polarized the shift of C(9a) is more difficult to interpret in detail but seems consistent with the above given interpretation.

An increase in sp^2 hybridization of N(10) in free flavin leads to a downfield shift of the resonance due to C(9), C(7), C(7α) and C(5a) due to the delocalization of the partial positive charge on N(10) (Moonen et al., 1984b). This is also observed with the flavodoxins and the downfield shift indeed parallels that of N(10) (Figure 2, Table 2), except for C(9). The relative upfield shift of C(5a) in D.vulgaris and in A.vinelandii flavo-

doxin compared to M.elsdenii and C.Mp flavodoxin reflects probably the hydrogen bonding to N(5) in the former two which strength is less than in FMN (Figure 2).

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

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

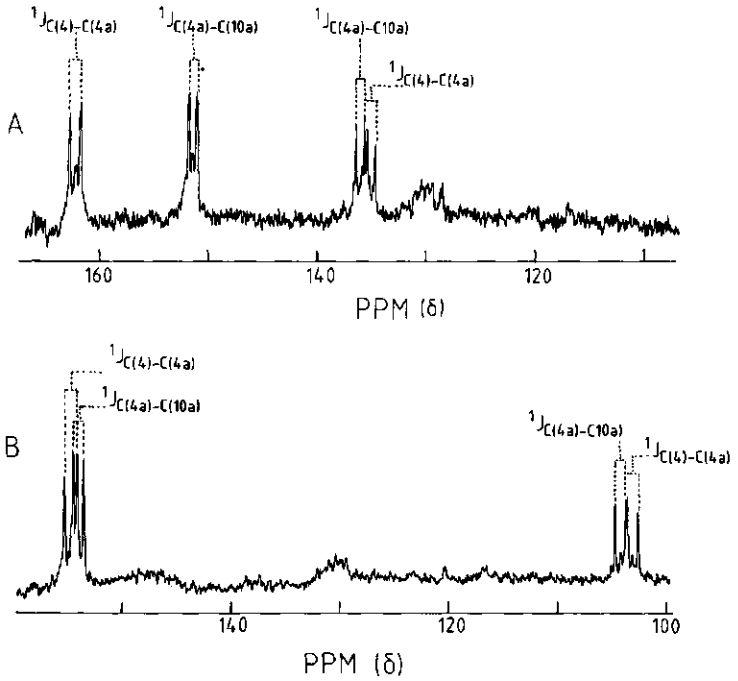

Figure 5. ^{13}C NMR spectra of C.MP apoflavodoxin reconstituted with [4,4a,10a - $^{13}C_3$]FMN in the oxidized (A) (35400 acquisitions) and in the reduced state (B) (54100 acquisitions) in 100 mM potassium pyrophosphate, pH = 8.0.

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

Table 4 Direct ^{13}C-^{13}C coupling constants of free and protein-bound [4,4a,10a - $^{13}C_3$]flavin in the oxidized and reduced state. The accuracy of the coupling constants is ± 0.5 Hz.

Redox state	Compound	Coupling and coupling constants (in Hz)	
		$^1J[^{13}C(4)-^{13}C(4a)]$	$^1J[^{13}C(4a)-^{13}C(10a)]$
Oxidized	TARF[a]	75.5	53.3
	FMN[a]	75.4	55.9
	C.MP flavodoxin	76.3	56.2
	M.e. flavodoxin	76.5	56.5
	D.v. flavodoxin[a]	76.9	57.3
	A.v. flavodoxin	76.3	56.5
Reduced	$TARFH_2$[a]	79.1	84.5
	$FMNH_2$[a]	82.0	81.6
	$FMNH^-$[a]	84.0	74.4
	C.MP flavodoxin	85.5	73.2
	M.e. flavodoxin	85.2	73.2
	D.v. flavodoxin[a]	86.7	72.8
	A.v. flavodoxin[b]	87.9	72.0

a) From Vervoort et al., 1985

b) The accuracy of the coupling constants is 1.0 Hz

^{13}C and ^{15}N NMR studies in the reduced state

A detailed interpretation of the chemical shifts observed for the reduced compounds will not be given here because the fundamental concept is the same as described above for the oxidized compounds. The discussion will be confined to the most useful findings.

It is most convenient to discuss again first the ^{15}N chemical shifts. All nitrogen atoms in the reduced state of flavin are of the pyrrole-type. The chemical shifts of N(1) of FMN bound to all of the apoflavodoxins are very similar to that of $FMNH^-$ (Figure 2, Table 2). This already indicates that the prosthetic group in reduced flavodoxins is ionized, information that cannot be obtained by other physical techniques.The ionization of $FMNH_2$ in the flavodoxins is also supported by the fact that no N(1)-H coupling is observed. The ionization of N(1) is also reflected by the large downfield shift of the resonance due to the neighboring carbon atoms C(2) and C(10a) (Figure 2, Table 2). These downfield shifts are caused by the negative charge (field effect) on N(1) (Van Schagen and Müller, 1981). The chemical shifts of N(1) of $FMNH^-$ in the flavodoxins is independent of pH in the range from 6.0 to 8.5. This implies that the pK_a value of the N(1) atom of flavin in flavodoxins is decreased compared with that of $FMNH_2$.

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

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

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

Flavin in the reduced state is an electron-rich molecule. On two-electron reduction the ^{13}C chemical shifts of apoflavodoxin-bound flavin are different from those of $FMNH^-$. An upfield shift is observed for the resonances of all sp^2 carbon atoms, except those of C(4a), C(5a) and C(9a).

The downfield shift of these carbon atoms indicates that both N(10) and N(5) nitrogen atoms possess an increased sp^2 hybridization that delocalises the partial positive charge onto these carbon atoms and increasing the π electron density at C(8) and C(6) on the one side, and C(7) and C(9) on the other. The fact that C(4) is also strongly upfield shifted even by comparision with that in $TARFH_2$ in an apolar solvent strongly suggests that some π electron density from N(5) is delocalized onto this atom. These facts make it difficult to derive from the ^{13}C chemical shifts of C(2) and C(4) whether or not these two atoms are involved in hydrogen bonding, because counteracting effects mask the influence of hydrogen bonding. In D.vulgaris flavodoxin the further upfield shift of the resonance of C(4), which is accompanied by a downfield shift of resonance due to C(6) and C(8), and an upfield shift of those of C(5a) and C(9a), suggests that π electron density is redistributed from C(8) and C(6) onto C(4) and C(9a) (from N(5)) and from N(10) onto C(5a) and C(4a) due to a further increase of the sp^2 hybridization of N(10) (Figure 2).

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

The ^{13}C-^{13}C coupling constants for the $^{13}C(4a)$-$^{13}C(4)$ and $^{13}C(4a)$-$^{13}C(10a)$ bonds are given in Table 4 (Figure 5). From these results it follows that the s character (Stothers, 1972) of the C(4)-C(4a) bond increases on going from free to protein-bound flavin and that the s character of the C(4a)-C(10a) bond is decreasing. These coupling constants were also used to assign unambiguously the resonance of the C(4) and C(10a) atoms.

The magnitude of the NOE for N(3)H and N(5)H in D.vulgaris and M.elsdenii flavodoxins were determined to be -0.40 and -0.57, respectively. Moreover the NOE for C(6)H and C(9)H were the same as those found for the oxidized proteins. These results strongly suggest that $FMNH^-$ bound to

apoflavodoxins is rigidly bound, as found for the oxidized proteins. It should be noted however, that we had to use rather high concentration of proteins to obtain these results and that the results should not be extrapolated to very dilute solutions. On the other hand, the flavodoxin concentrations found in the corresponding bacteria is rather high, approaching the concentrations used in the NMR measurements. Therefore the above statement should be of biological relevance.

The above results demonstrate that the electronic structure of flavin is altered on binding to apoflavoproteins. The electronic structures of the flavin in M.elsdenii and C.MP flavodoxin are very similar, but they differ from those in A.vinelandii and D.vulgaris flavodoxin. Even the latter two flavodoxins show distinct differences. On the other hand it is also obvious that there is a greater similarity among the flavodoxins than between the flavodoxins and Old Yellow enzyme (Table 2). This shows the power of the NMR technique especially in the combination of different nuclei and using labeled prosthetic groups allowing the detection of all atoms. Although it is obvious that the specific interactions between the prosthetic group and the apoprotein contributes considerably, if it is not the major part to the alteration of the electronic structure of protein-bound flavin, which ultimately prepares it for the catalysis of a certain biological reaction, more data on other flavoproteins are needed to unravel the complex electronic structure of protein-bound flavin. Such a program is now under active investigation. The higher complexity of the electronic structure of protein-bound flavin by comparison with free flavin also makes it very difficult to deduce the endocyclic angles of N(10) and N(5) from the NMR data, as has been done with free flavins (Moonen et al., 1984b). However, in the protein-bound state this information can be obtained from the ^{15}N-^{1}H coupling constant; this coupling cannot be observed in free flavin in aqueous solution. It has been suggested, based mainly on circular dichroism and UV spectra, that flavodoxins can be divided into a pasteurianum type (M.elsdenii, C.Mp) and a rubrum type (A.vinelandii, D.vulgaris) flavodoxin (D'Anna and Tollin, 1972). Our results indicate caution should be used in classifying the flavodoxin from A.vinelandii and D.vulgaris together (D'Anna and Tollin, 1972) because their NMR properties show considerable variation. Their circular dichroism spectra are similar, but such spectra cannot reveal directly the electronic structure of flavin in great detail.

The three-dimensional structures of C.MP (Burnett et al., 1974; Ludwig et al., 1976; Smith et al., 1977; 1983) and D.vulgaris flavodoxins have been determined. The flavin in D.vulgaris flavodoxin is flanked by Tyr-98 on one side and by Trp-60 on the other side. Tyr-98 is nearly coplanar with the flavin ring and located above the pyrazine moiety (central ring) of flavin. Trp-60 overlaps primarly with the benzene subnucleus of FMN, covering the C(8α), C(8), C(9) and C(9a) atoms of flavin but it is not coplanar with the flavin. There is an angle of 45° between the normals of the two planes. C.MP flavodoxin has a methionine in place of tryptophan on the one side of the flavin and tryptophan in place of tyrosine on the opposite site. The tryptophan residues overlaps with the benzene subnucleus of flavin. The role of these amino acid residues in the particular function of the flavodoxins is not yet known. Considering the differences in the electronic structure of the two flavodoxins (Figure 2), which are rather large in the reduced state, it is suggested that these residues play a role in the particular π electron density distribution in the two flavodoxins and in the stabilization of the resulting mesomeric structures.

The hydrogen bonding between FMN and apoflavodoxin from D.vulgaris as revealed by NMR results are in agreement with the "first model" proposed from X-ray data (Watenpaugh et al., 1971). The hydrogen bonding pattern of the four flavodoxins studied, as deduced from our NMR data, are presented schematically in Figure 6. It is interesting to note that C.MP and M.elsdenii flavodoxins do not form a hydrogen bond with the N(5) atom in the oxidized state. In the reduced state a strong hydrogen bond is formed. These results are in agreement with crystallographic studies on C.MP flavodoxin (Smith et al., 1977) showing that the peptide backbone at Glu-17 undergoes a conformational change on reduction that facilitates the interaction of the carbonyl group of Glu-17 with the proton at N(5) of flavin. A similar situation exists in M.elsdenii flavodoxin as deduced from the NMR results. Another common feature seems to be the absence of hydrogen bonding with C(4α) of flavin in all four flavodoxins. However, it should be kept in mind that the complexity of the electronic structure of flavin in reduced flavodoxins did not allow us to deduce with certainty the absolute absence of any hydrogen bonding interactions. Nevertheless it has been

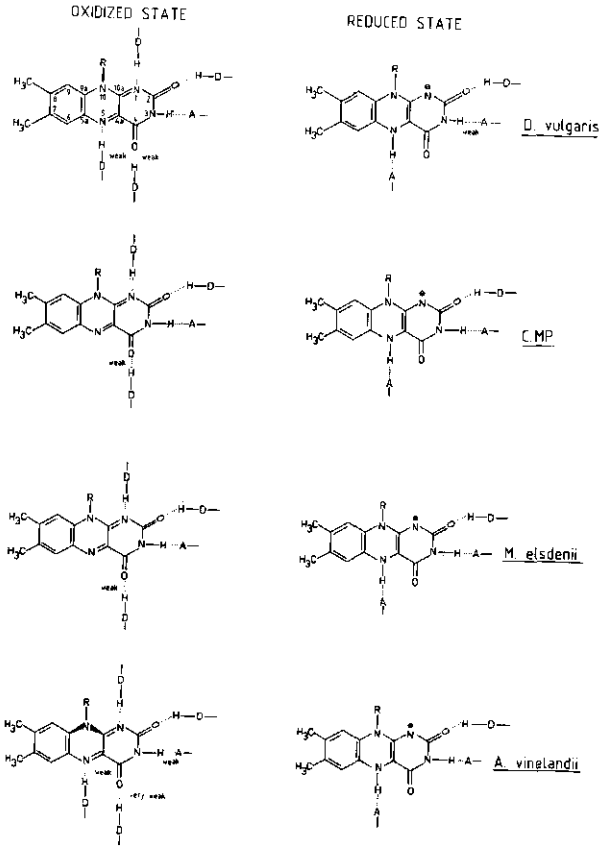

Figure 6. The hydrogen bond pattern between apoflavodoxin and FMN in the oxidized and reduced state as deduced from the ^{13}C and ^{15}N chemical shifts.

found in C.MP flavodoxin (Smith et al., 1977) that O(17) from Glu (17) is very close to the C(4α) atom of FMN. It has been suggested that this configuration must be associated with an unfavorable Coulombic interaction, i.e. repulsive. This may have some chemical consequences for the electron redistribution in reduced flavodoxins leading to the destabilization of certain mesomeric structures observed with free reduced flavin (Moonen et al., 1984b). Therefore, we believe that the unusual chemical shifts due to C(4) in the flavodoxins are also reflecting this effect.

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Chapter 4. PROPERTIES OF THE COMPLEX OF RIBOFLAVIN 3',5'-BISPHOSPHATE AND MEGASPHAERA ELSDENII APOFLAVODOXIN

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SUMMARY

Megasphaera elsdenii apoflavodoxin has been reconstituted with riboflavin 3',5'-bisphosphate. Several biochemical and biophysical properties of the complex have been investigated and the results are compared with the properties of the native protein. The dissociation constant of the modified complex is increased by a factor of about 23 by comparison with that of the native protein. The rate constant for the formation of the complex is about 70 times lower than that for the native protein. The stability of the complex is governed by the dissociation rate constant. The redox potential of the transition between the oxidized and semiquinone state is similar to that of the native protein. On the other hand the redox potential of the semiquinone-hydroquinone transition is about 20 mV more negative than that of the native protein. Absorbance and circular dichroism studies reveal no substantial differences between the protein-bound artificial prosthetic group and the protein-bound natural prosthetic group in all three redox states. Both prosthetic groups were specifically enriched in the isoalloxazine ring with ^{13}C and ^{15}N isotopes. In both the oxidized and in the fully reduced state only minor differences in interaction between the isoalloxazine ring and the apoprotein for the two flavin derivatives are found. ^{31}P NMR studies show that the 5'-phosphate group of the two flavin derivatives is bound in the same way and that it is dianionic in the complex. In contrast, the 3'-phosphate group in riboflavin 3',5'-bisphosphate is monoanionic or even neutral when bound to the protein. The 3'-phosphate group is also close to or on the surface of the protein. The results are discussed in the light of the recently proposed theory that various kinds of charge-charge interactions are mainly responsible for the regulation of the redox potential in flavoproteins.

INTRODUCTION

Flavodoxins are small flavoproteins (15,000 - 21,000 Da) which contain one molecule of FMN as prosthetic group and which function as electron carriers of low redox potential in many biological reactions [1]. They fall into two classes according to certain biophysical properties and the flavin-binding specificity of their apoproteins [1-3]. The proteins in one class (e.g. Megasphaera elsdenii, Clostridium MP) bind only riboflavin derivatives which contain a free phosphate group at the 5'-position; those in the second class (e.g. Desulfovibrio vulgaris, Azotobacter vinelandii) are much less specific. In contrast, both kinds of apoflavodoxin bind flavins which contain modifications in the isoalloxazine part of the molecule.

M.elsdenii apoflavodoxin has been used to assay for FMN, to analyze FMN for its riboflavin 5'-monophosphate content by fluorimetric techniques [5], and to prepare pure riboflavin 5'-monophosphate from crude FMN by affinity chromatography [8]. However, it has been shown recently that the specificity of this apoflavodoxin is not quite as high as was originally thought. It also binds riboflavin 3',5'-bisphosphate (Fig. 1), a flavin discovered in small amounts in commercial preparations of FMN [4], and FMN preparations synthesised in the laboratory, and occurring together with two other riboflavin bisphosphate and two riboflavin monophosphate contaminants [4-7]. The stability of the complex was somewhat less than that of native flavodoxin, but the complex was fully active as an electron carrier in an assay involving the oxidation of molecular hydrogen and the reduction of metronidazole [4]. These observations implied that the additional phosphate group on the flavin has a minimal effect on the properties of the complex.

A further investigation of the complex has therefore been carried out, the results of which are reported in this paper. It seemed possible that this flavodoxin derivative might help to assess a theory proposed recently that charge-charge interactions are a major factor in the regulation of the redox potential of flavoproteins [9].

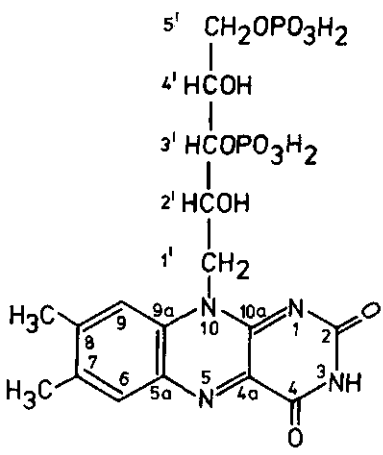

Fig. 1. Structure of riboflavin 3',5'-bisphosphate.

MATERIALS AND METHODS

Riboflavin 3',5'-bisphosphate was purified from FMN as described previously [4]. The synthesis of ^{15}N enriched flavins has been described elsewhere [10]. The selectively ^{13}C and ^{15}N enriched phosphorylated riboflavin derivatives were purified on a preparative scale by stepwise elution on a conventional DEAE-cellulose column as described previously [11].

Freeze dried samples (40-80 mg based on flavin absorbance at 450 nm) of the 100 mM ammonium carbonate fraction, containing the bisphosphorylated flavin analogues [11], were rechromatographed on a DEAE Sepharose 6B Fast Flow (Pharmacia) column (10x2,5 cm) by gradient elution at room temperature in the dark.

The anion exchanger was pre-equilibrated with 20 mM ammonium acetate, pH 5.9, the sample loaded on top of the column and the column washed with one column-volume of the acetate buffer. Riboflavin 3',5'-bisphosphate was eluted from the column with a 0-1 M ammonium acetate linear gradient (pH 5.9). The flow rate was kept constant at 40 ml/h with an LKB microperpex S peristaltic pump and 6.0 ml fractions were collected with an LKB Redirac fraction collector. Yellow fractions were tested for purity by analytical ion exchange chromatography on a MONO Q HR 5/5 column using the FPLC system of Pharmacia and by fluorescence titrations with apoflavodoxin from Megasphaera elsdenii [5]. Before recording the ^{31}P-NMR spectra the flavin analogues were eluted over Chelex-100 (Biorad) to remove traces of metal ions.

The flavodoxin from Megasphaera elsdenii was isolated as described previously [12]. The apoprotein was prepared by the trichloroacetic acid precipitation method [5]. The reconstitution of the apoflavodoxin with the FMN derivatives was conducted in neutral buffered solutions at 4°C. Excess of FMN or riboflavin 3',5'-bisphosphate was removed on a Biogel P6DG column. The samples were concentrated by means of ultrafiltration (AMICON).

The redox potentials of the flavodoxin complexes were determined as described previously [13,14]. Light absorption spectra were taken on a Varian Cary 16 and Perkin-Elmer 550B spectrophotometers, fluorescence experiments were done on Aminco SPF-500 spectrofluorometer and circular dichroism spectra on a Jobin Yvon auto-dichrograph Mark V. All spectrometers were equipped with thermostatted cell holders. The experiments were done at 22°C.

Wilmad 10 mm precision NMR tubes were used for ^{13}C and ^{31}P NMR. Wilmad 15 mm precision NMR tubes were used for ^{15}N NMR. The samples contained 1-5 mM flavodoxin in 100 mM potassium pyrophosphate buffer, pH 8.0, for ^{13}C and ^{15}N NMR and 100 mM Tris-HCl, pH 8.0, for ^{31}P NMR, all in the presence of 10% $^{2}H_2O$. The sample volume was 1.6 ml for ^{13}C and ^{31}P NMR and 4 ml for ^{15}N NMR. All measurements were performed on a Bruker CXP 300 spectrometer operating at 120.5 MHz for ^{31}P NMR, 75.6 MHz for ^{13}C NMR and 30.4 MHz for ^{15}N NMR measurements. Proton decoupling was achieved with the Waltz-16 pulse sequence [15] at -16 dB from 20 W. All spectra were recorded using 30° pulses and a repetition time of 1.0 s. The temperature was kept at 17°C, unless indicated otherwise.

Reduction of the samples was achieved by the addition of the desired amount of a dithionite solution to the anaerobic solution of flavodoxin. Anaerobiosis was achieved by carefully flushing the solution of the NMR tube with argon for about 10 min.

RESULTS AND DISCUSSION

Analytical anion exchange chromatography

Chemical phosphorylation of riboflavin yields three different types of monophosphorylated as well as three bisphosphorylated forms of riboflavin. These compounds can be separated on a semi-preparative scale by reversed phase HPLC [4]. For our purpose it was sufficient to purify the riboflavin derivatives at the mono- and bisphosphorylated level because apoflavodoxin is very specific for monophosphorylated riboflavin with the phosphate at the 5' position [4,5] and for bisphosphorylated riboflavin with the phosphates at the 3' and 5' position [4]. Therefore the enriched riboflavin 3',5'-bisphosphorylated compounds were purified on a preparative scale using conventional ion-exchange chromatography as described under Materials and Methods. Fig. 2 shows the elution pattern of the preparative purification of [4-10a $^{13}C_2$]riboflavin 3',5'-bisphosphate. In Fig. 3 the analytical FPLC pattern of the pooled preparative fraction of [4-10a $^{13}C_2$]riboflavin 3',5'-bisphosphate (Fig. 3A) is compared with those of commercial FMN (Fig. 3B) and riboflavin 3',5'-bisphosphate, purified by reversed phase HPLC (Fig. 3C). The purity of the various riboflavin 3',5'-bisphosphate pools after preparative anion exchange chromatography obtained from separate syntheses was further checked by fluorescence titrations with apoflavodoxin. When a two-fold excess of apoflavodoxin was added to the sample shown in Fig. 3A the residual fluorescence was 17%, indicating that the compound has a purity of about 80%, a value in good agreement with the FPLC pattern. Similar tests on other samples, showed that their purity varied from 60-80% but that they did not contain more than 1% of FMN as judged by analytical anion exchange chromatography on the FPLC system. The procedure described above is gives high yields and it is sufficient to separate from a mixture the monophosphorylated from the bisphosphorylated compounds that bind to apoflavodoxin.

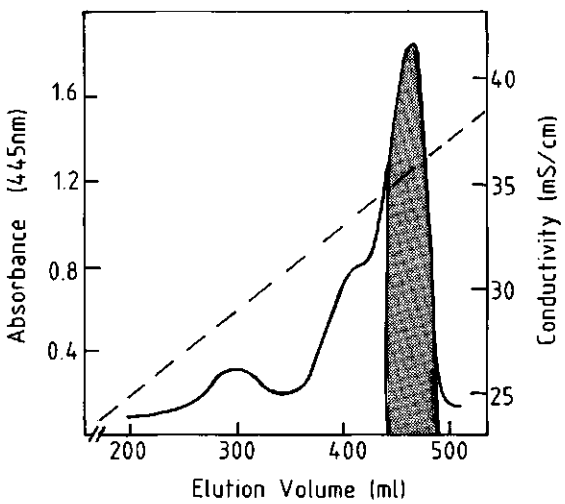

Fig. 2. Preparative Anion Exchange Chromatography of 4-10a ^{13}C Enriched Riboflavin 3',5'-bisphosphate on DEAE-Sepharose 6B Fast Flow. 100 µmol of partially purified [4-10a $^{13}C_2$]riboflavin 3',5'-bisphosphate [11] were bound on top of the column in 20 mM ammonium acetate, pH 5.9 (start buffer). After washing the column with one volume start buffer the flavin analogues were eluted using a linear gradient of 0-1 M ammonium acetate, pH 5.9, in the start buffer. The flow rate was 40 ml/h. 6 ml fractions were collected and pooled as indicated (shadowed area). Absorbance at 445 nm was measured by dilution of a 100 µl aliquot of the different fractions.

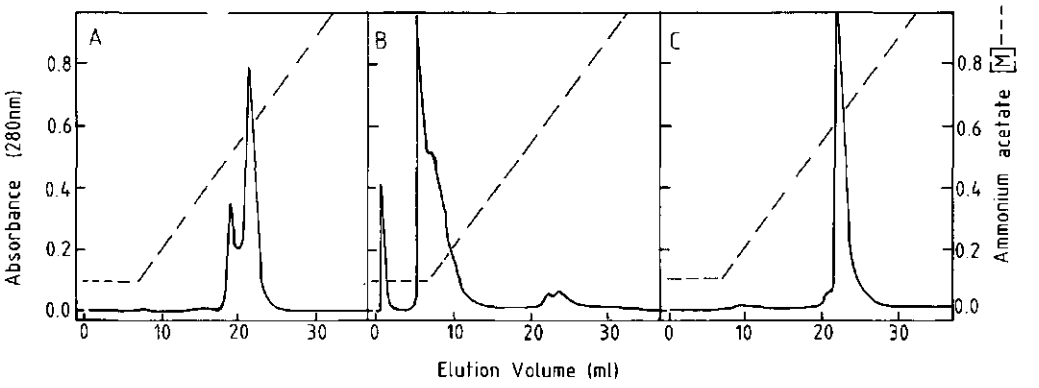

Fig. 3. Analytical Anion Exchange Chromatography of various riboflavin derivatives.

A: Pooled fraction of [4-10a $^{13}C_2$]riboflavin 3',5'-bisphosphate after preparative anion exchange chromatography.
For the purification of riboflavin 3',5'-bisphosphate a 50 µl aliquot of the pooled fractions in Fig. 1, containing about 100 nmol flavin, was analyzed.
Gradient composition: Buffer A: 20 mM ammonium acetate pH 6.0.
Buffer B: 1 M ammonium acetate pH 6.0.
Flow rate 1,5 ml/min.
The gradient was made as indicated by the dashed line. After each run the column was washed with 2 ml 1 M ammonium acetate and pre-equilibrated again with 5 ml 20 mM ammonium acetate.

B: Commercial FMN.

C: Reversed phase HPLC purified riboflavin 3',5'-bisphosphate [4].

Kinetic and thermodynamic results

The dissociation constant of the M.elsdenii apoflavodoxin riboflavin 3',5'-bisphosphate complex has been determined by fluorometric titration experiments [5] at pH 7 and 22°C, using the highly purified flavin derivative [4] (see Fig. 2C). The dissociation constant was found to be 10 nM. This value is the same as that determined independently in a previous study [4]. Although riboflavin 3',5'-bisphosphate binds tightly to the apoprotein, its dissociation constant is about a factor of 23 larger than that of the native protein [16]. The value for the native protein was however obtained in 25 mM sodium acetate, pH 6.55, and at 20°C. In a similar but kinetic experiment at pH 7, using a two-fold excess of apoflavodoxin over the riboflavin 3',5'-bisphosphate, the second-order association rate constant for the formation of the complex was determined to be 1.8×10^3 $M^{-1}s^{-1}$. This value is about 70 times smaller than the value determined for the native protein at pH 4.4 and 0.5°C [16] (Table 1). Although the results of the two kinetic measurements cannot be compared directly, the previously determined ionic strength-independent and pH-independent association rate constant for the native protein was found to be 3.5×10^5 $M^{-1}s^{-1}$ [17], a value close to that given in Table 1 and therefore making the comparison reasonable. From the appropriate dissociation constants and the association rate constants the dissociation rate constants have been calculated (Table 1). Since the reciprocal value of the dissociation rate constant represents the lifetime of the complex it is obvious that the stability of the riboflavin 3',5'-bisphosphate complex is governed by the dissociation rate constant. The kinetic constants shown in Table 1 deserve some further comments. The association rate constant for the native enzyme might have been expected to be diffusion controlled. The fact that the association rate constant for riboflavin 3',5'-bisphosphate is decreased by two orders of magnitude strongly suggests that formation of the complexes is accompanied by dehydration of the highly solvated phosphate group(s) of flavin.

The complex of apoflavodoxin and riboflavin 3/,5'-bisphosphate has been shown to be slightly more active than native flavodoxin in catalysing electron transfer from hydrogenase to metronidazole [4]. It was therefore of interest to determine the redox potentials of the complex for comparison with those of the native protein. The potential of the oxidized-semiquinone

Table 1

Comparison of Some Physico-Chemical Properties of Native Flavodoxin from M.elsdenii and the Complex between its Apoprotein and Riboflavin 3',5'-bisphosphate.

The proteins were dissolved in 50 mM potassium phosphate, pH 7.0, unless otherwise stated. The temperature was 22°C.

Parameter	pH	Prosthetic group	
		FMN	FB-3',5'
k_{on}	7.0	$2.59 \times 10^{5} M^{-1}s^{-1}$ [a]	$1.82 \times 10^{3} M^{-1}s^{-1}$
k_{off} [b]	7.0	$1.1 \times 10^{-4} s^{-1}$	$1.8 \times 10^{-5} s^{-1}$
K_D	7.0	0.43 nM [a]	9.7 nM [c], 10 nM
E_m(ox./semiquinone) [e]	7.0	-115 mV [d]	-111 mV, -118 mV
E_m(semiquinone/two-electron reduced) [e]	6.1	--	-376 mV
	6.56	-375 mV	-390 mV
	7.05	-390 mV, -373 mV [d]	-410 mV
	7.51	-393 mV	-419 mV
ε(molar absorption coefficient)			
oxidized state at 445 nm		10200 $M^{-1}cm^{-1}$ [d]	10200 $M^{-1}cm^{-1}$
semiquinone state at 580 nm		4500 $M^{-1}cm^{-1}$ [d]	4000 $M^{-1}cm^{-1}$

a) Taken from ref. 16.

b) Calculated from the corresponding values of k_{on} and K_D.

c) Taken from ref. 4.

d) Taken from ref. 13.

e) Measured at 25°C.

couple was measured with indigodisulphonate as indicator and using photochemical reduction with EDTA and deazaflavin to introduce reducing equivalents into the system [14,23]. Two equilibrium points were obtained, and these gave values of -111 mV and -118 mV for the midpoint redox potential of the complex. The values are very similar to the value reported for the native protein [13,14,24] (Table 1). The redox potential for the semiquinone-hydroquinone couple was measured by equilibration with H_2 using hydrogenase as catalyst [13,14]. The experimental points at four pH values (Table 1) fit a straight line rather than the curve observed with the native protein [13,14 24], and the value at pH 7.05 (-410 mV) is 20 mV more negative than the value determined for the native protein in a parallel experiment (Table 1). The values determined for the native protein are more negative than several published values determined by a variety of methods [13,14,24] but similar to a value obtained recently by pulse polarography [18]. The reason for these different values for the native protein is not known. The strain of M.elsdenii used as the source of flavodoxin was the same for all of the measurements, but we cannot yet exclude the possibility of a recent mutation in the strain.

It is surprising that the phosphate group at the 3'-position in riboflavin 3',5'-bisphosphate has such minor effects on the redox potentials of the complex of flavin and apoflavodoxin. The observation that the potentials of the complex are so similar to those of native flavodoxin indicate that the dissociation constants for the bound flavin change with the redox state of the flavin in a way similar to that deduced for the native protein [1]. The two redox potentials for protein-free riboflavin 3',5'-bisphosphate have not been measured. However, it is assumed that they are the same as those of riboflavin 5'-monophosphate (-124 mV and -314 mV for the semiquinone/hydroquinone and oxidized/semiquinone couples respectively [25]), values for the dissociation constants for the complexes of apoflavodoxin and the semiquinone and hydroquinone can be calculated from the equation [1,13,26]:

$$E_m(\text{bound flavin}) = E_m(\text{free flavin}) + 0.059 \log \frac{K_{D,ox}}{K_{D,red}} \qquad (1)$$

where, $K_{D,ox}$ refers to the oxidized and semiquinone forms of flavodoxin for the couples oxidized/semiquinone and semiquinone/hydroquinone respectively; $K_{D,red}$ referes to the semiquinone and hydroquinone forms of flavodoxin for

the couples oxidized/semiquinone and semiquinone/hydroquinone respectively.

The values calculated fo the dissociation constants are 4.2 pM and 0.3 μM for the semiquinone and hydroquinone respectively. Nevertheless, they show that the ratios of the dissociation constants for the oxidized and semiquinone complexes on the one hand, and for the semiquione and hydroquinone complexes on the other are very similar for the modified and native proteins [1]. The much weaker binding of the hydroquinone indicated by this calculation is supported by NMR measurements (see below).

The weaker binding of the hydroquinone has been ascribed to a difference in the conformation of the free and bound flavin; the crystal structure of fully reduced Clostridium MP flavodoxin was found to be coplanar, while the free flavin was thought to have a bent conformation, thus making the interaction with the apoprotein thermodynamically less favourable [1]. Recent ^{13}C and ^{15}N NMR measurements have indicated that the isoalloxazine structure of fully reduced flavins is also coplanar in the protein-free state [19]. It is unlikely therefore that a conformational change in the flavin of the kind originally proposed can explain the decreased stability of the reduced complex.

An alternative explanation for the negative shift of the redox potential for the semiquinone/hydroquinone couple of FMN in flavodoxin has been proposed recently [9]. In this theory, the charge-charge interaction between the negative charges of the phosphate group of FMN and the negative charge on N(1) of the isoalloxazine moiety of FMN hydroquinone [10,11,20] and the monopole-monopole interaction between the latter and the negative charges on the protein account for the shift in redox potential and could also account for the decreased stability of the hydroquinone complex. The more negative potential of the complex with riboflavin 3',5'-bisphosphate is consistent with this theory. The fact that the potential is not shifted even more might indicate that the 3'-phosphate group in the complex carries less than two negative charges; support for this charge composition is given below. The theory is not adequate to account for the positive shift in potential for the oxidized/semiquinone couple because the isoalloxazine systems for both redox species is neutral. However, the theory does predict that the additional phosphate group should not affect the potential of the oxidized semiquinone couple, as was observed.

Absorbance and circular dichroism studies

The shape of the absorption spectra of the native and modified flavodoxin complexes are identical or almost identical in all three redox states in the 320-700 nm region. Although the molar absorption coefficients seem to be identical in the oxidized state they differ by about 10% in the semiquinone state (Table 1).
The circular dichroism spectrum of the modified flavodoxin exhibits only very small differences in the 320-700 nm region in the three redox states as compared with native flavodoxin. The spectra are virtually identical with published spectra [3]. The intensity in the visible region of the circular dichroism bands of the modified protein is slightly decreased as compared with those of the native protein [3].

Nuclear magnetic resonance studies

^{31}P NMR

The ^{31}P NMR spectrum of free riboflavin 3',5'-bisphosphate, purified by HPLC [4], is shown in Fig. 4A. Two resonances are observed under proton-decoupling conditions. The low-field signal is assigned to the 5'-phosphate group resonating at the same position as the phosphate group of FMN. The 3'phosphate group resonates at higher field (Table 2). These two lines are further split into a triplet and a doublet, respectively, when the spectrum is taken in the absence of proton-decoupling conditions (Fig. 4B). The two $^{3}J(^{31}P\text{-}^{1}H)$ couplings arise by interaction with the 5'-CH_2 group yielding a triplet and the 3'-CH group giving a doublet. The coupling constants are: 5.1 Hz for the former case and 9.3 Hz for the latter case. Fig. 4C shows the ^{31}P NMR spectrum of [4a, 10a-$^{13}C_2$]riboflavin 3',5'-bisphosphate which was obtained as a byproduct by the phosphorylation of the riboflavin derivative. It was purified according to the procedure described in Materials and Methods. The spectrum demonstrates that the compound is at least 80% pure, as also deduced by HPLC techniques. The spectrum is slightly upfield shifted due to the lower pH value of the solution as compared to those of the spectra shown in Fig. 4A and B.

Table 2

^{31}P, ^{13}C and ^{15}N chemical shifts (in ppm) of selectively enriched protein-bound riboflavin 3',5'-bisphosphate and FMN bound to apoflavodoxin from M.elsdenii.

^{31}P chemical shifts were determined in 100 mM Tris, pH = 8.0. ^{13}C and ^{15}N chemical shifts were determined in 100 mM potassium pyrophosphate, pH = 8.0.

^{13}C chemical shifts are relative to Me_4Si, the ^{15}N chemical shifts relative to liquid NH_3 and the ^{31}P chemical shifts relative to H_3PO_4.

Atom	Prosthetic Group			
	3',5'-FB	FMN	3',5'-FB	FMN
	OXIDIZED		REDUCED	
C(2)	159.7	159.8	156.7	156.9
C(4)	162.4	162.4	154.9	154.8
C(4a)	135.6	135.6	103.2	103.5
C(10a)	151.6	151.5	154.5	154.5
N(1)	185.0	185.0	183.6	183.4
N(3)	160.6	160.9	149.5	149.7
N(5)	349.3	349.3	61.6	61.3
N(10)	164.8	165.3	97.5	98.2
5'-phosphate	5.15; 5.10[a)]		5.25	
3'-phosphate	2.00; 4.90[a)]		1.65	

a) ^{31}P chemical shifts of the free prosthetic group.

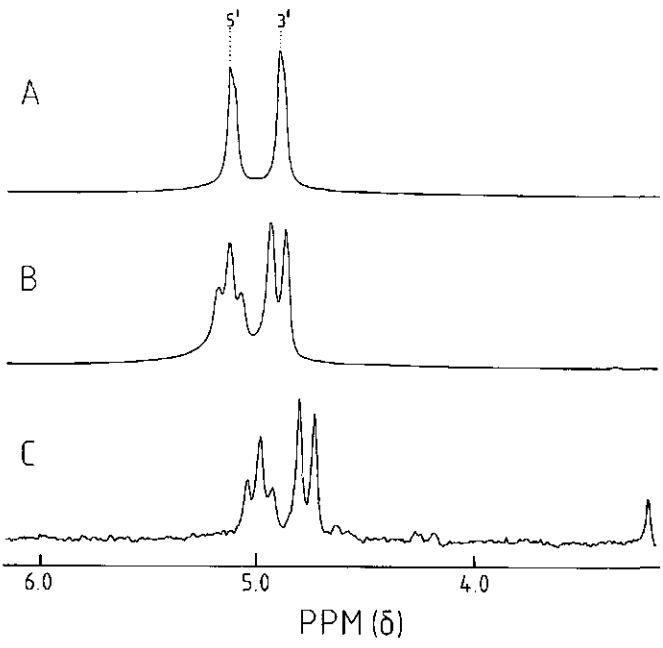

Fig. 4. ^{31}P NMR spectra of free riboflavin 3',5'-bisphosphate.
A: Proton-decoupled, pH = 9.2 (717 transients).
B: Proton-coupled, pH = 9.2 (4750 transients).
C: [4,10a-$^{13}C_2$] derivative, proton coupled, pH = 8.2 (18000 transients).

On binding of riboflavin 3',5'-bisphosphate to the apoprotein from M.elsdenii the ^{31}P NMR spectrum changes drastically (Fig. 5A). The high-field line appears now about 3 ppm at higher field than in the free state, whereas the low-field line shifts only slightly downfield. The latter line resonates at the same position as the 5'-phosphate group of FMN in the native protein [7]. The proton-coupled spectrum of the modified protein shows a doublet for the high-field peak, $^3J(^{31}P-^1H)$ = 8.4 Hz (data not shown). The low-field line exhibits no splitting under these conditions, but the linewidth is slightly increased as compared with that shown in Fig. 5A (for an explanation see [7]). The spectrum shown in Fig. 5A contains also some additional, much less intense lines at about 4.6 ppm. In contrast

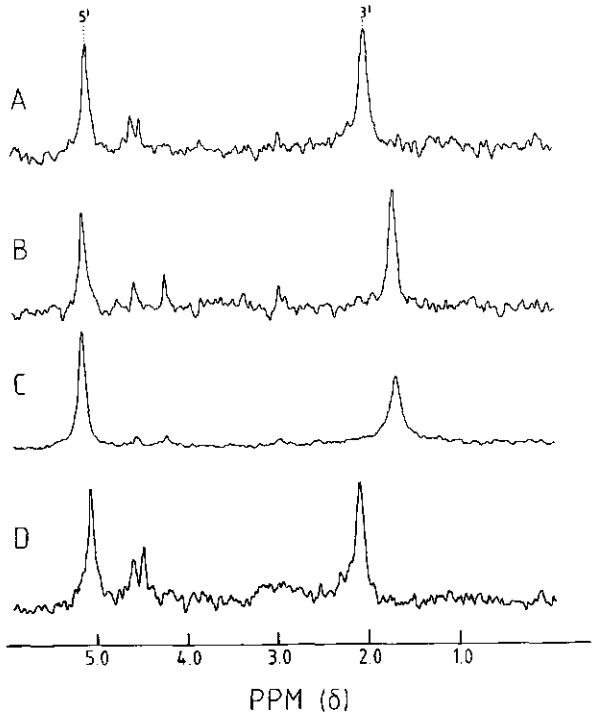

Fig. 5. ^{31}P NMR Spectra of *apoflavodoxin* (3 mM) recombined with *riboflavin* 3',5'-bisphosphate in 100 mM Tris/HCl, pH = 8.0.

A: Oxidized state (line broadening 3 Hz, 1200 transients)
B: Reduced state (line broadening 3 Hz, 1200 transients)
C: Reduced state, 0.3 mM in the presence of Mn^{2+} (line broadening 3 Hz, 43190 transients)
D: [4,10a-$^{13}C_2$]-enriched, flavin in the oxidized state (line broadening 3Hz, 1400 transients).

to the two main peaks, the position of the minor peaks is pH-dependent and these lines are completely broadened on addition of Mn^{2+} as a paramagnetic probe [21], one tenth the concentration of the protein. These results show that the minor peaks are due to a small amount of free riboflavin 3',5'-bisphosphate.

Upon two-electron reduction of the modified protein the high-field line shifts further upfield by 0.3 ppm, the low-field line undergoes a slight downfield shift (Fig. 5B), as was also observed for the native protein [7].

Addition of a substoichiometric amount of Mn^{2+} (see above) to the modified protein leads to a substantial broadening of the high-field peak, both in the oxidized and reduced state (Fig. 5C). Increasing the concentration of Mn^{2+} to 40% that of flavodoxin causes the high-field line to broaden beyond detection .The low-field line is not affected by Mn^{2+} (Fig. 5C).

In Fig. 5D the ^{31}P NMR spectrum of the complex between apoflavodoxin and [4,10a-$^{13}C_2$]riboflavin 3',5'-bisphosphate is shown in the oxidized state. The spectrum differs only slightly from that shown in Fig. 5A.

The ^{31}P NMR spectra indicate that the 5'-phosphate group in modified flavodoxin is in the dianionic form, irrespective of the redox state of the protein, as was also found for native protein [7]. The 5'-phosphate group is buried in the protein and therefore not accessible for Mn^{2+}. The large upfield shift of the resonance line due to the 3'-phosphate group upon binding to the apoflavodoxin suggests that this group is in the monoanionic or even in the neutral state. This interpretation is in agreement with the observation that monoprotonation of dianionic phosphate always leads to a large upfield shift and that further protonation causes only an additional small upfield shift [22]. It should however be noted that these upfield shifts depend on the nature of the phosphorus compounds, i.e. dependent on the degree of esterification. Furthermore, the accessibility of the 3'-phosphate group to Mn^{2+} in the modified flavodoxin strongly suggests that this group is located close to the surface of the protein. The fact that the 3'-phosphate group in flavodoxin is at most in the monoanionic state in the oxidized state and probably even in the neutral state in the two-electron reduced state, as indicated by the further upfield shift, suggests that the protein is preventing further anionization of the phosphate group either by providing a lipophilic environment or by strong electrostatic interactions affecting the angle of the O-P-O bonds in such a way that the resonance occurs at high field [22]. Apparently the conformation of the 3'-phosphate binding site is somewhat altered upon two-electron reduction of the protein as suggested by the further upfield shift of the corresponding resonance line.

The temperature-dependences of the ^{31}P NMR spectra of the modified flavodoxin have been measured in both the oxidized and reduced state (results not shown). The protein was studied at 3°C, 17°C and 31°C. In the oxidized state the amount of free flavin increased slightly going from 3°C to 31°C. In addition, the resonance line due to the 5'-phosphate group underwent a small downfield shift of 0.15 ppm, whereas the peak due to the 3'-phosphate group is shifted upfield by 0.23 ppm upon increasing the temperature from 3°C to 31°C. In the reduced state the corresponding shifts amount to 0.14 ppm and 0.16 ppm, respectively. No increase of free flavin could be observed on raising the temperature indicating that the complex in the reduced state is rather thermostable.

^{13}C NMR

The ^{13}C NMR spectra of protein-bound [2,4a-$^{13}C_2$]riboflavin 3',5'-bisphosphate in the oxidized and reduced state are shown in Fig. 6.

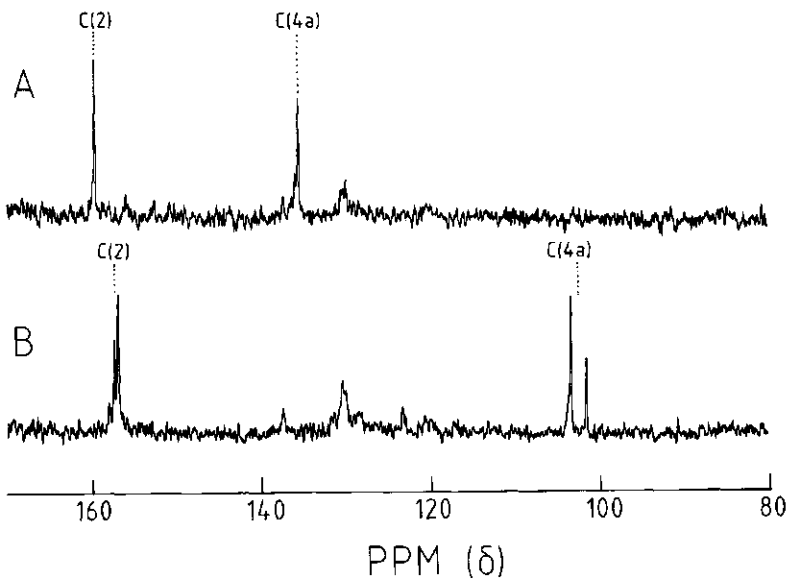

Fig. 6. ^{13}C NMR spectra of apoflavodoxin (3 mM) recombined with [2,4a-$^{13}C_2$] riboflavin 3',5'-bisphosphate in the oxidized state (1000 transients) (A) and in the reduced state (1000 transients) (B) in 100 mM potassium pyrophosphate, pH = 8.0.

The ^{13}C chemical shifts are presented in Table 2 and compared with those of the native protein. The ^{13}C chemical shifts for both proteins are the same indicating that there is no perturbation of the interactions between the isoalloxazine moiety of the prosthetic group and the apoprotein. In the oxidized state both peaks (Fig. 6) are accompanied by a small shoulder indicating the presence of a small amount of free flavin. Upon reduction two signals are observed for both resonance lines. The increase in free flavin in the reduced state as compared with the oxidized state shows that the complex is less stable in the reduced state. This explains also our observation that, although unbound flavin was removed by means of a Biogel P-6DG column prior to the NMR experiments, we sometimes observed in the course of the measurements the appearance of free flavin.

^{15}N NMR

The ^{15}N NMR spectra of apoflavodoxin reconstituted with [1,3,5,10-$^{15}N_4$]riboflavin 3',5'-bisphosphate are shown in Fig. 7 for the oxidized and the reduced state. The ^{15}N chemical shifts are collected in Table 2. Except for the chemical shift of the N(10) atom, which is shifted slightly upfield in both redox states as compared with that for native protein, the chemical shifts are identical. The small upfield shift suggests a slightly higher sp^3 hybridization of N(10) in modified than in native protein (see [19]). The splitting of the N(3)H resonance line is not so evident in the oxidized state (Fig. 7A), although proton-decoupling gives a sharp singlet (data not shown). In the reduced state the two doublets of the N(3)H and N(5)H groups are well resolved (Fig. 7B). The coupling constants for the two groups are: $^1J(^{15}N(3)-^1H) = 91 \pm 1.5$ Hz and $^1J(^{15}N(5)-^1H) = 93 \pm 1.5$ Hz. These coupling constants are identical with those of native protein and indicate that both nitrogen atoms are highly sp^2 hybridized and that the protons on these atoms are not freely accessible for bulk solvent.

^{31}P NMR spectra of the apoflavodoxin recombined with [1,3,5,10-$^{15}N_4$] riboflavin 3',5'-bisphosphate are virtually identical to those of Fig. 4A and Fig. 4B.

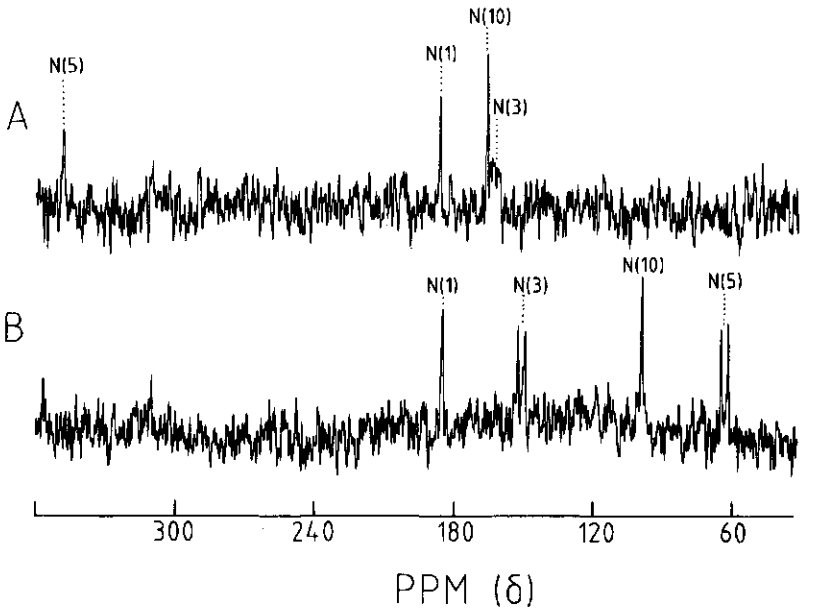

Fig. 7. ^{15}N NMR spectra of apoflavodoxin (5 mM) recombined with [1,3,5,10-$^{15}N_4$]riboflavin 3',5'-bisphosphate in the oxidized state (A) (17022 transients) and in the reduced state (B) (35965 transients) in 100 mM potassium pyrophosphate, pH = 8.0. No proton-decoupling was applied.

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Chapter 5.

Bacterial Luciferase: A Carbon-13 and Nitrogen-15 Nuclear Magnetic Resonance Investigation[+]

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ABSTRACT

The ^{13}C and ^{15}N NMR spectra of specifically ^{13}C and ^{15}N enriched FMN were measured in the presence of bacterial luciferase from Vibrio harveyi. In the oxidized state hydrogen bonds to both carbonyl groups are found, albeit weaker than those of FMN in water. In contrast, the N(1) and N(5) atoms both have stronger hydrogen bonds than FMN in water. The C(8) and C(7) resonances indicate that the isoalloxazine moiety of luciferase-bound FMN is not as strongly polarized as free FMN in aqueous medium, and much less than flavodoxin-bound FMN.

On reduction of the bound FMN, all ^{13}C resonances, except that due to the C(10a) atom, shift upfield, indicating increased π electron density at these carbon centers. The isoalloxazine ring carries a negative charge at the N(1) atom and its negative charge possibly interacts with a positively charged group on the protein. The results further indicate that the N(3)H group probably forms a hydrogen bond with the protein, whereas the N(5)H group does not. The N(5) atom of luciferase-bound $FMNH^-$ is highly sp^2 hybridized indicating an almost planar structure of the reduced prosthetic group, except that the N(10) atom is somewhat placed out of the molecular plane. Using highly active luciferase only one oxidized flavin molecule per dimeric luciferase is bound strongly. We have however observed that excess reduced flavin is bound to apoluciferase, probably in an aspecific manner, indicating that luciferase has two different binding sites for reduced flavin, the filling of the stronger specific site depending on the specific activity of the luciferase preparation.

INTRODUCTION

Bacterial luciferase is a flavoprotein containing riboflavin 5'-phosphate (FMN)[1] as prosthetic group. It catalyzes the oxidation of long chain aliphatic aldehydes with the emission of light with a spectral maximum around 490 nm. Hastings and Gibson (1963) showed that bacterial luciferase can form a long lived intermediate after the attack of molecular oxygen on the protein-bound reduced flavin molecule. In the absence of the aldehyde, the intermediate breaks down, forming H_2O_2 and oxidized FMN. Numerous studies have been performed on the nature of this intermediate and on the reaction scheme in general (Ziegler and Baldwin, 1981, Lee, 1985). Unfortunately, relatively few studies have been performed on the physical and chemical properties of the protein. The binding between FMN and apoluciferase has been studied by conventional methods in both the oxidized and reduced state (Ziegler and Baldwin, 1981). It was shown that a negative charge on the isoalloxazine side chain at about 0.9 nm away from the N(10) atom is required for good reactivity (Meighen and MacKenzie, 1973). Nielsen et al. (1983) showed that of all phosphorylated riboflavin analogs only the 5'-phosphate analog (FMN) gives a high bioluminescence activity. A remarkable aspect of the equilibrium binding studies is that FMN hardly binds in the oxidized state, K_d = 0.12mM, 3°C, for Vibrio harveyi luciferase (Baldwin et al., 1975), while $FMNH_2$ binds about 100 times more tightly, $K_d \approx 1\mu M$, 6°C (Becvar and Hastings,

1975). These dissociation constants are much higher than those of most other flavoproteins.

Various $FMNH_2$ analogs have been used to investigate the flavin reaction site. Meighen and MacKenzie (1973) showed that the N(3)H group of flavin is essential for reaction, alkylation of this group abolishes the bioluminescence activity. Watanabe et al. (1980), and Chen and Baldwin (1984) showed that 8 α-substituted $FMNH_2$ derivatives produce bioluminescence with Photobacterium phosphoreum luciferase. They suggested that the N(5)H group of $FMNH_2$ is involved in the binding to the luciferase molecule. Chen and Baldwin (1984) recently showed that 8 α substituted $FMNH_2$ derivatives also produce bioluminescence with V. harveyi luciferase and suggested that the methyl group at the 8 position of flavin is exposed to solvent in the complex.

There is some uncertainty about the number of flavins bound to the luciferase molecule and/or involved in the catalytic mechanism. Ziegler and Baldwin (1981) concluded that only one flavin reacts (binds) per luciferase dimer from the first-order behavior of the bioluminescence kinetics and the luciferase:flavin quantum yield ratios. Matheson and Lee (1983) however, showed that the kinetics are more complex and that the major bioluminescence pathway is via a bimolecular reaction.

NMR spectroscopy is being used extensively in the study of enzyme ligand interactions, yielding insight into specific interactions of the ligand with the binding site. In our case information about the electronic perturbation of flavin upon

binding to an apoflavoprotein and the conformation of protein-bound reduced flavin can also be obtained (Vervoort et al., 1985). Although bacterial luciferase is a relatively large biomolecule possessing a molecular mass of ∿80,000Da it lends itself to NMR study as it is a highly soluble protein produced in quantity by the bioluminescent bacteria. It is also relatively easy to purify (O'Kane et al., 1986). For these reasons we decided to study luciferase by NMR techniques. In this paper we report on the interaction of FMN with apoluciferase using ^{13}C and ^{15}N enriched FMN derivatives. Since all atoms of the isoalloxazine moiety of FMN have been labeled a rather detailed insight into the interactions of FMN with apoluciferase is obtained.

MATERIALS AND METHODS

FMN selectively enriched with ^{13}C and ^{15}N isotopes was prepared as described previously (Van Schagen and Müller, 1981; Müller et al., 1983; Franken et al., 1984). The synthesis of FMN derivatives enriched with ^{13}C in the benzene subnucleus will be published elsewhere.

[1,3,5,10-$^{15}N_4$] riboflavin was synthesized in the following way. A mixture of 16g conc. H_2SO_4 and 11.2g 14M [^{15}N]HNO_3 were slowly added under vigorous stirring to 12g 1,2-dimethylbenzene at 0-5°C. 11.2g 14M [^{15}N]HNO_3 was prepared from 14.4ml 9M [^{15}N]HNO_3 (Amersham, UK) by distillation to a constant boiling point of 121.7°C. The reaction mixture was kept for 3 h at 0-5°C and then for 1 h at 5-10°C. Then ice was added and the reaction mixture neutralized with solid Na_2CO_3. The mixture was extracted three times each with 50 ml CH_2Cl_2, the organic phase dried over Na_2SO_4 and evaporated to dryness. The yield of nitration products was 13.0g. The product was taken up in petroleum ether (fp. 40-60°C) and applied to a silia gel 60 (70-230 mesh, product of Merck AG., FRG) column (2.5 x 30 cm). Washing the column with petroleum ether eluted a pale yellow product which was identified as 2,3-dimethylnitrobenzene by ^{13}C NMR (yield 5.0g). An intense yellow band was eluted using petroleum ether containing 1% ether. The product was shown to be 3,4-dimethylnitrobenzene by ^{13}C NMR (yield 6.2g). Using petroleum ether containing a much higher percentage of ether (up to 30%) relatively small amounts of dinitroxylene and other

nitration products were eluted. The purity of the two mononitro compounds can be monitored by thin layer chromatography using plastic sheets impregnated with a fluorescent indicator (Merck, FRG) and a mixture of isopropylether and petroleum ether (1:1.5, by volume) as mobile phase. The desired product, 3,4-dimethyl-nitrobenzene was then dissolved in methanol and reduced catalytically with H_2 and Pd/C as catalyst at atmospheric pressure. The aniline derivative thus obtained was crystallized from n-hexane after removing the catalyst from the solution by filtration and evaporating the solvent. All other steps were identical with those described for the synthesis of 7-methyl-10-ribityl-isoalloxazine 5'-phosphate (Müller et al., 1983; Franken et al., 1984).

Bacterial luciferase was from an aldehyde requiring dark mutant of V. harveyi strain 392 (MAV) and was purified to homogeneity (O'Kane et al., 1986). Luciferase was assayed for bioluminescence specific activity (photons $s^{-1}A_{280}^{-1}$) at room temperature as described (Lee, 1982), using decanal. Photometer calibration was made by reference to the NBS standard lamp and NBS absolute photodiode photometer via the luminol chemiluminescence reactions (Lee & Seliger, 1965; Matheson et al., 1984).

The NMR samples contained luciferase, which was at least 80% of the maximum obtainable specific activity unless indicated otherwise. For reconstitution experiments ^{13}C and ^{15}N enriched FMN was added on a molar ratio with respect to

luciferase protein, unless indicated otherwise. The protein concentration was determined by absorbance (ε_{280} = 85,000 M^{-1} cm^{-1}; O'Kane et al., 1986). All ^{13}C and ^{15}N NMR measurements were done in 50 mM potassium phosphate buffer, 0.5 mM EDTA, 10 mM β-mercaptoethanol, pH=7.0, unless indicated otherwise. ^{13}P NMR measurements were done in 200 mM Tris, 0.5 mM EDTA, 10 mM β-mercaptoethanol, pH = 7.5. The temperature was kept at 5°C during the measurements.

Wilmad 10-mm precision NMR tubes were used for ^{13}C and ^{31}P NMR experiments and Wilmad 15-mm precision NMR tubes were used for ^{15}N NMR experiments. The sample volume was 1.6 ml for ^{13}C and ^{31}P NMR, and 4.0 ml for ^{15}N NMR measurements. The samples contained 10% $^{2}H_2O$ to lock the magnetic field. Broadband decoupling of 0.5 W was used for ^{13}C NMR experiments except for the C(6) and C(9) atoms where 2.0 W was used. All spectra were recorded using 30° pulses and a repetition time of 1.0 s. The measurements were done on a Bruker CXP 300 NMR spectrometer operating at 30.4 MHz for ^{15}N NMR, at 75.6 MHz for ^{13}C NMR, and at 120.5 MHz for ^{31}P NMR spectra.

Dioxane (3 μl) served as an internal standard for ^{13}C NMR measurements. Chemical shift values are reported relative to TMS (δ dioxane - δ TMS = 67.84 ppm). Neat [^{15}N]CH_3NO_2 was used as an external reference for ^{15}N NMR measurements 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 (δ CH_3NO_2 - δ NH_3 = 381.9 ppm for the magnetic field parallel to the sample tube). For ^{31}P NMR

measurements 85% H_3PO_4 was used as an external reference. Broadband decoupling of 0.5 W was used for ^{31}P NMR experiments. Values are reported as true shieldings, i.e. corrected for bulk volume susceptibilities. The accuracy of the reported values is about 0.2 ppm for ^{13}C NMR, 0.4 ppm for ^{15}N NMR and 0.1 ppm for ^{31}P NMR chemical shift values.

Reduction of the sample was made by the addition of the desired amount of a dithionite solution to the anaerobic solution of luciferase and flavin. Anaerobiosis was achieved by carefully flushing the solution in the NMR tube with argon for about 10 min. The NMR tube was sealed with a serum cap.

RESULTS AND DISCUSSION

In previous papers (Franken et al., 1984); Vervoort et al., 1985; Beinert et al., 1985a) we have used [1,3,5,10-$^{15}N_4$]7-methyl-10-ribityl-isoalloxazine-5'-phosphate as a prosthetic group to study the ^{15}N NMR characteristics of FMN containing flavoproteins. This labeled prosthetic group was used instead of the corresponding FMN derivative because of the easy availability of the staRting material [^{15}N]p-nitrotoluene. In the case of old yellow enzyme (Beinert et al., 1985a) it was noticed that the activity of the reconstituted enzyme containing the labeled prosthetic group was only one third of that when the apoenzyme was recombined with FMN. These results indicate that a small modification of the prosthetic group can drastically influence the enzymatic properties of a flavoprotein while the affinity of the apoprotein for the modified flavin is not affected as compared to that for FMN. To avoid possible ambiguities in our studies we decided to synthesize [1,3,5,10-$^{15}N_4$]FMN, as described in Materials and Methods. Under our experimental conditions a relatively high amount of mononitroxylene (65% yield) is obtained on nitration of xylene. The two isomeric nitro compounds, 1-nitro-2,3-dimethylbenzene and 1-nitro-3,4-dimethylbenzene, can be quantitatively separated using column chromatography, yielding 45% of the former and 55% of the latter compound relative to the total amount of the mononitro compounds. It should be noted that the nitration mixture must be added slowly and the temperature maintained at 0-5^{o}C at the

initial stage of the synthesis, otherwise a higher amount of dinitro xylene is formed at the expense of the desired product. The purified mononitro isomers have been unambigueously identified by ^{13}C NMR (Table I). The carbon atoms carrying the nitro function are doublets in the ^{13}C NMR spectra due to coupling with the ^{15}N mucleus. It is obvious that only 1-nitro-3,4-dimethylbenzene is the appropriate product to be used to synthesize flavin, which was achieved by catalytic reduction of the nitro compound to the corresponding aNiline derivative and condensation of the product with D-ribose. This and the subsequent steps of the synthesis are identical to those described previously for [1,3,5,10-$^{15}N_4$]7-methyl-10-ribityl-isoalloxazine-5'-phosphate (Müller et al., 1983; Franken et al., 1984).

^{31}P NMR of luciferase

The ^{31}P NMR chemical shift values of the phosphate group of FMN bound to V. harveyi luciferase are 5.2 ppm in the oxidized and 5.6 ppm in the reduced state. Both resonances are downfield shifted as compared to that of free FMN in the dianionic state (δ= 5.1 ppm, pH = 9.0). The ^{31}P chemical shifts for luciferase therefore indicate that the phosphate group of FMN in luciferase is bound in the dianionic state. The small downfield shift observed in luciferase as compared to free FMN can be interpreted as a small distortion of the O-P-O angle in luciferase bound FMN (Gorenstein, 1975). The small downfield shift of 0.4 ppm in reduced luciferase as compared to oxidized luciferase probably reflects the higher binding of FMN in reduced luciferase (Becvar

Table I. Carbon-13 chemical shifts (in ppm) of [^{15}N]1-nitro-2,3-dimethylbenzene (1) and [^{15}N]1-nitro-3,4-dimethylbenzene (2) is chloroform. The chemical shifts are relative to TMS.

Compound	Chemical shifts							
	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	C(2α/3α)	C(3α/4α)
1	151.1[a]	139.4	130.2	125.9	121.3	133.5	20.0	15.3
2	146.1[a]	130.0	138.0	144.7	120.7	123.9	19.4	19.7

a) doublet

and Hastings, 1975). No covalently bound phosphate groups were observed in the ^{31}P NMR spectrum of luciferase, as observed in some flavoproteins (Edmondson and James, 1979).

^{13}C and ^{15}N NMR of Oxidized luciferase

Figure 1A shows the ^{15}N NMR spectrum of ^{15}N enriched FMN bound to luciferase from V. harveyi. Several natural abundance lines due to the protein are observed, but the resonances of the ^{15}N atoms of protein-bound FMN appear at fields where no natural abundance peaks are present enabling us to assign the resonances of the prosthetic group unambiguously. The natural abundance resonances at about 120 ppm and 140 ppm represent probably amide groups of the protein (Witanowski et al., 1981) and the low intensity resonances at about 75 ppm could be due to arginine residues (Lapidot and Irving, 1978). Another bulk of natural abundance resonances appear at about 310 ppm which remain unassigned.

The nitrogen shieldings of protein-bound FMN are summarized in Table II. According to common practice the nitrogen atoms in heterocycles are categorized as pyridine or β-type nitrogen and pyrrole or α-type nitrogen (Witanowski et al., 1981). Generally, the β-type nitrogens resonate at lower field than the α-type nitrogens. As can be seen from Figure 1A and Table II the nitrogen atoms of flavin fit well into this categorization. However, a more important characteristic of the two types of nitrogen atoms for our study is the fact that the chemical shifts of pyridine type nitrogen atoms are much more sensitive to hydrogen bond formation, leading to an increased shielding of the nitrogen atoms, than those of the pyrrole type nitrogen atoms

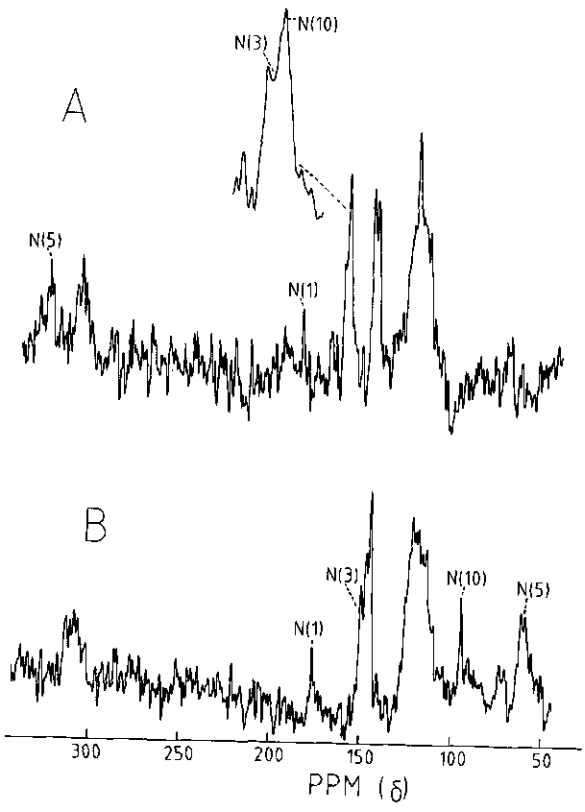

Figure 1. A. ^{15}N NMR spectrum of Vibrio harveyi luciferase (2.5 mM) reconstituted with [1,3,5,10-$^{15}N_4$]FMN in the oxidized state (90800 acquisitions). B. In the reduced state (140300 acquisitions). All samples were in a buffer of 50 mM potassium phosphate, 0.5 mM EDTA, 10 mM β-mercaptoethanol, pH = 7.0. The temperature was 5°C.

Table II. Carbon-13 and nitrogen-15 N chemical shifts (in ppm) of free FMN and bound to luciferase in the oxidized and in the reduced state. Luciferase and oxidized FMN were measured in 50mM potossium phosphate, pH = 7.0. TARF in the oxidized and reduced state was dissolved in $CHCl_3$. Reduced FMN in the neutral state was measured in 100 mM potassium phosphate, pH 5.0, and in the anionic state in 50 mM potassium pyrophosphate, pH 8.5. All measurements were done at 5°C. ^{13}C chemical shifts are relative to TMS. ^{15}N chemical shifts are relative to liquid NH_3.

	Chemical shifts in the						
Atom	oxidized state			reduced state			
	LUC	FMN[a]	TARF[b]	LUC	$FMNH^-$[a]	$FMNH_2$[b]	$TARFH_2$[b]
C(2)	158.5	159.8	155.2	157.9[c]	158.2	151.1	150.6
C(4)	162.6	163.7	159.8	157.2	157.7	158.3	157.0
C(4a)	137.4	136.2	135.6	103.5	101.4	102.8	105.2
C(5a)	135.7*	136.4	134.6	135.0*	134.2	134.4	136.0
C(6)	130.8	131.8	132.8	116.8	117.3	117.1	116.1
C(7)	139.0	140.4	136.6	132.7*	133.0	134.3	133.6
C(7)	20.2	19.9	19.4	19.4	19.0	19.0	18.9
C(8)	148.6	151.7	147.5	126.2	130.3	130.4	129.0
C(8)	21.9	22.2	21.4	19.7	19.4	19.2	18.9
C(9)	119.5	118.3	115.5	115.6	116.8	117.4	118.0
C(9a)	134.6*	133.5	131.2	130.7*	130.9	130.4	128.2
C(10a)	151.3	152.1	149.1	156.2[c]	155.5	144.0	137.1
N(1)	187.1	190.8[d]	200.1[d]	176.8[c]	181.3[d]	128.0[d]	121.1[d]
N(3)	162.3*	160.5[d]	159.6[d]	150.0[c]	150.0[d]	149.7[d]	150.3[d]
N(5)	325.8	334.7[d]	346.0[d]	59.9[c]	58.4[d]	58.0[d]	59.8[d]
N(10)	180.8*	163.5[d]	151.9[d]	94.6	96.5[d]	87.2[d]	78.0[d]

Chemical shifts marked with an asterisk are tentative assignments.

a) Taken from Vervoort et al. (1985).

b) Taken from Moonen et al. (1984a)

c) Independent of pH in the range 6.5 - 8.5.

d) This paper.

(Witanowski et al., 1981). In order to demonstrate these effects more clearly the nitrogen shieldings of free flavin in a polar (FMN in H_2O) and an apolar (TARF in $CHCl_3$) solvent are also given in Table II. For convenience the chemical shifts are presented schematically in Figure 2. The resonance signal due to the N(5) atom of luciferase-bound FMN (Figure 1A) appears at much higher field than that of both of FMN and TARF. This result indicates that this atom forms a rather strong hydrogen bond with the protein. A similar but weaker effect is observed with the N(1) atom of protein-bound FMN. In both cases the hydrogen bond is stronger than that formed with FMN in aqueous solution (Table II, Figure 2). The resonances of the N(3) and N(10) atom are observed at about 160 ppm (Table II). Owing to the lower resolution as compared to the spectra of flavodoxins (Franken et al., 1984; Vervoort et al., 1985) the assignment of the resonances is somewhat jeopardized. However, since the spectra have not been recorded under proton decoupling conditions the line due to N(10) should be more intense than that due to N(3), the last should be split into a doublet because it contains a covalently bound hydrogen. An expansion of the resonance lines at about 160 ppm (Figure 1A) suggests the presence of a doublet, partially overlapping with the peak at higher field (N(10) atom). Although the doublet nature of the low field lines cannot be unambiguously proven for reasons stated above we assign the low field part tentatively to the N(3) atom. Following this reasoning we estimated roughly the $^1J(^{15}N(3)-^1H)$ coupling constant to be about 90 ± 10Hz. The fact that the chemical shift value of N(3) of luciferase-bound FMN is further downfield

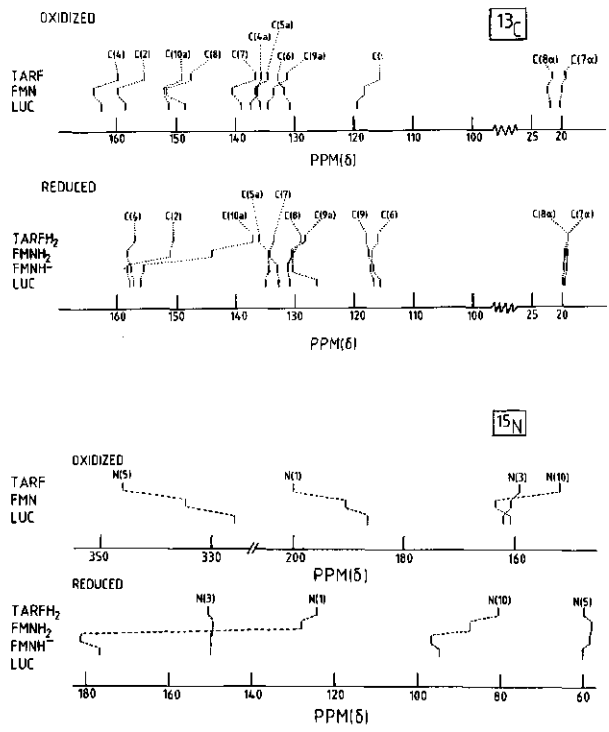

Figure 2. Correlation diagram of ^{13}C and ^{15}N NMR chemical shifts of free flavin and bound to Vibrio harveyi apoluciferase in the oxidized and in the reduced state.

shifted than that of FMN indicates that the N(3)H group of flavin probably forms a hydrogen bond with the protein and that this group is not accessible to bulk water in the protein. In fact this implies that the exchange rate for the proton of N(3) must be very slow, i.e. $\nu \leq$ 100Hz. On the other hand, if the exchange rate was very fast, one should observe a single sharp and a more intense line for N(3) than is the case, suggesting also coupling between $^{15}N(3)$ and its proton. These interpretations are supported and consistent with the finding that replacement of the proton at N(3) by a methyl group abolishes completely the bioluminescence activity of flavin and luciferase (Meighen and MacKenzie, 1973).

The resonance line of the N(10) atom is upfield from that of free FMN but considerably downfield from that of TARF (Table II, Figure 2). This indicates that the N(10) atom in luciferase-bound FMN possesses a high degree of sp^2 hybridization, somewhat less than that in free FMN (Moonen et al., 1984a). This means that the N(10) atom of protein-bound flavin is slightly out of the molecular plane.

A typical ^{13}C NMR spectrum of luciferase, recombined with [4,10a-$^{13}C_2$]FMN, is shown in Figure 3. Besides the two resonances due to the flavin, the usual spectral features expected for the natural abundance ^{13}C in a protein of this size are observed. The peptide carbonyl and carboxyl resonances appear between 170 ppm and 185 ppm. The arginine Cξ atoms are observed at 158.2 ppm. Aromatic carbon atoms of histidine, tyrosine, phenylalanine and tryptophan residues resonate between

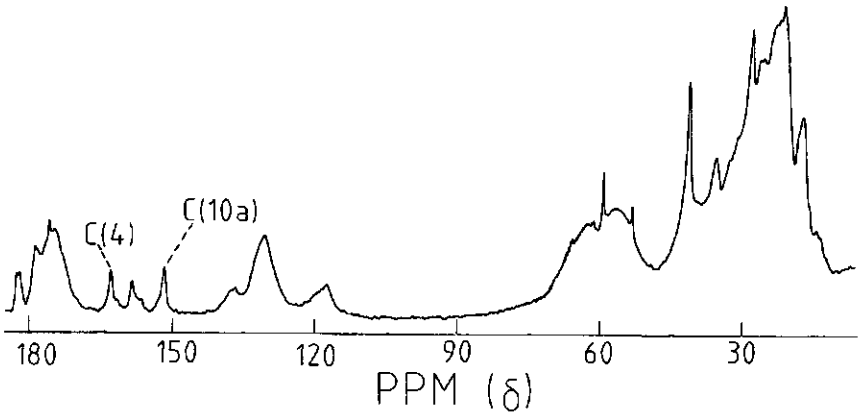

Figure 3. ^{13}C NMR spectrum of oxidized Vibrio harveyi luciferase (2mM) recombined with [4,10a-$^{13}C_2$]FMN in 50 mM potassium phosphate, 0.5 mM EDTA, 10 mM β-mercaptoethanol, pH = 7.0 (36000 acquisitions). The temperature was 5°C.

110 ppm and 140 ppm. No resonances appear in the spectral region from 70 ppm to 110 ppm. The Cα atoms resonate at about 60 ppm and all other aliphatic carbon atoms appear at higher field. It is obvious that the natural abundance resonance lines can interfer with the localization of the resonance lines due to certain atoms in protein-bound flavin, even when using highly enriched flavin derivatives. In order to arrive at a safe assignment, difference spectra have been recorded where necessary. Figure 4A shows for example the difficulty of observing directly the resonances of the protein-bound [6,8α-$^{13}C_2$]FMN. Usually we have recorded first the spectrum of the FMN-luciferase complex and then reduced it by an excess of dithionite in the same sample tube, followed by recording the spectrum under identical instrumental settings to avoid as much as possible spectral deviations. Figure 4B demonstrates the good quality of such difference spectra. Figure 4B shows also interesting features. In both redox states, the linewidth of the C(6) resonance is much larger than that of the C(8α) resonance. An identical observation (not shown) is made using [7α,9-$^{13}C_2$]FMN as a prosthetic group with respect to the C(9) resonance. This strongly indicates that the larger linewidths are not artifacts due to data handling. Tentatively, our interpretation of these observations is as follows. In general, the important relaxation mechanisms for ^{13}C resonances of proteins are the ^{13}C-^{1}H dipolar interactions and the chemical shift anisotropy (Wilbur et al., 1976). These relaxation sources determine not only the spin-lattice relaxation times (T_1) but also the linewidths (T_2) of the ^{13}C resonances. The

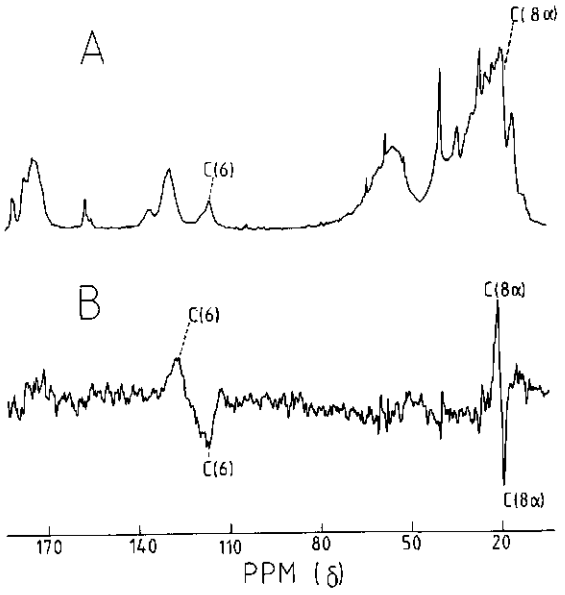

Figure 4. ^{13}C NMR spectra of Vibrio harveyi luciferase (2mM) recombined with [6,8α-$^{13}C_2$]FMN in the reduced state (A) (44100 acquisitions) in 50 mM potassium phosphate, 0.5 mM EDTA, 10 mM β-mercaptoethanol, pH = 7.0. B is a difference spectrum between the spectrum in the oxidized (26500 acquisitions) minus that in the reduced state of luciferase.

dipole-dipole relaxation is by far the dominant relaxation source at 7 Tesla (Norton et al., 1977) for aromatic and aliphatic carbon atoms bearing a covalently bound hydrogen atom, as C(6), C(9), C(7α) and C(8α) in FMN. The linewidth of the resonance lines of these atoms depends also on the rotational correlation time of the molecule under study (Norton et al., 1977). The calculated rotational correlation time for luciferase (M_r=80,000) is about 50 ns at 5^{o}C. Therefore, the two methyl groups of protein-bound flavin undergo fast internal rotations with a rotational correlation time much smaller than the isotropic overall tumbling of the protein, yielding sharp lines. On the other hand the larger linewidths of the resonances from C(6) and C(9) suggest that the isoalloxazine ring of FMN is rigidly associated with the protein with little independent motion in either redox state.

The ^{13}C chemical shifts of protein-bound FMN are collected in Table 2 and compared with those of free flavin in solvents of different polarity (see also Figure 2). The chemical shifts of the two carbonyl groups of protein-bound FMN appear at higher field than those of free FMN in aqueous solution indicating an apparent increase in π electron density on these C atoms suggesting that the two functional groups form weaker hydrogen bonds with the protein than FMN does with water, and much weaker than those of the prosthetic group in flavodoxins (Van Schagen and Müller, 1981; Moonen et al., 1984a; Vervoort et al., 1985). Since the N(1) atom forms a rather strong hydrogen bond with the protein, the chemical shift of the C(2) atom indicates an apparently very weak hydrogen bond, because a strong hydrogen

bond to N(1) masks a hydrogen bond to C(2)=O by counteracting its chemical shift. (The partial positive charge on N(1) leads to an upfield shift of the C atoms in α position.) The fact that C(8), C(8α), C(6) and C(10a) resonate at higher field than the corresponding atoms of free FMN in aqueous solution, but at lower field than those of TARF, indicates that the hydrogen bond to C(2)=O does not much affect these indirectly polarizable carbon atoms, i.e. there is only a little π electron delocalization from the benzene ring onto the C(2)=O group (Moonen et al., 1984a). As shown by model studies (Moonen et al., 1984a) polarization of the isoalloxazine ring of flavin requires the presence of a polar solvent possessing a high permittivity. It is interesting to note that FMN bound to apoflavodoxin is, by the interaction of C(2)=O with the protein highly polarized, in contrast to FMN bound to luciferase. Crystallographic studies on Clostridium MP flavodoxin showed (Burnett et al., 1974) that part of the benzene subnucleus of flavin is accessible to bulk solvent. In our opinion this contributes to the stabilization of the highly polarized prosthetic group (for tautomeric structure, see Moonen et al., 1984a). Therefore, it is suggested that the benzene ring in luciferase- bound FMN is in a hydrophobic microenvironment. This conclusion is in agreement with published circular dichroism and absorbance spectra (Baldwin et al., 1975).

The decreased sp^2 character of N(10) of protein-bound FMN as compared with that of N(10) of free FMN should lead to a downfield shift of C(9), C(7), C(7α) and C(4a) (Moonen et al., 1984a). This is found and is in accordance with the

interpretation of the N(10) chemical shift. However, the downfield shift for C(7) is less than expected, although $C(7_\alpha)$ follows the expected trend. This is attributed to a lower accuracy in the determination of the chemical shift of C(7) which leads to an apparent inconsistency of our interpretation.

In Figure 5A a schematic representation of the structure and hydrogen bond interaction of FMN with apoluciferase is given.

^{13}C and ^{15}N NMR of two-electron reduced luciferase

The ^{15}N NMR spectrum of protein-bound luciferase is shown in Figure 1B. The resonances due to N(1) and N(10) appear as sharp lines whereas those due to N(3) and N(5) are broader and less intense considering the fact that both nitrogen atoms carry a covalently bound proton. Again, as already outlined above, these last two resonances should show up as doublets if no fast proton exchange reaction is occurring. Owing to the lack of resolution of the resonance of N(3), which is obscured partially by natural abundance resonances, no definite statement can be made with respect to the presence or absence of a coupling, although the composite resonances at about 150 ppm do suggest it. At any rate the ^{15}N chemical shift of the N(3) atom suggests that the N(3)H group is involved in hydrogen bonding similar to that of $FMNH^-$ in aqueous solution (Table II, Figure 2). On the other hand the resonance line of N(5) is possibly split into a doublet with an estimated $^1J(^{15}N(5) - {}^1H)$ of about 85 ± 10 Hz, suggesting that no fast proton exchange reaction occurs. If a fast proton reaction would occur the resonance line of N(5) would be expected to be much sharper and more intense than observed. The N(5) chemical shift (Table II) strongly

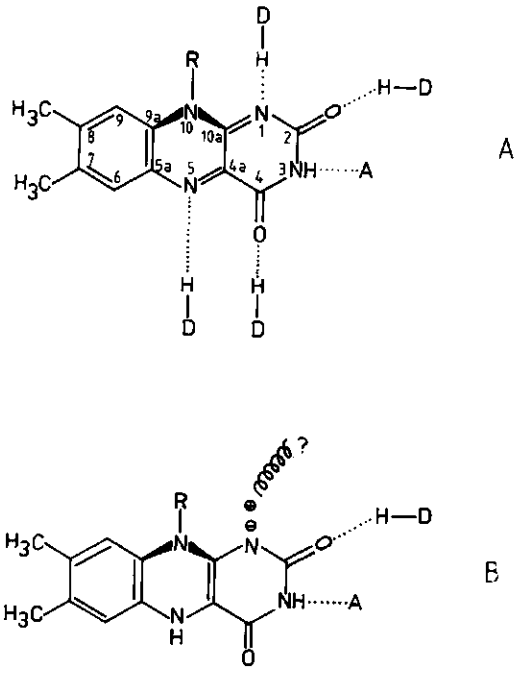

Figure 5. Structure and flavin-apoenzyme interactions of FMN bound to apoluciferase in the oxidized (A) and reduced (B) state as revealed by ^{13}C and ^{15}N NMR data. A = hydrogen bond acceptor; D = hydrogen bond donor.

indicates that the N(5)H group is not forming a hydrogen bond with the protein and that the N(5) atom possesses a high degree of sp^2 hybridization (Moonen et al., 1984a) (see also below). These results also indicate that the N(5)H group of luciferase-bound FMN experiences a rather hydrophobic microenvironment. The N(1) atom resonates at 176.8 ppm, a value very close to that of the corresponding atom in $FMNH^-$ (Table II, Figure 2). This proves that the N(1) atom in luciferase-bound reduced FMN is ionized. The chemical shift of N(1) is pH-independent in the range of 7.0 to 8.5. This indicates that the pK_a value of the ionization of the N(1)H group of $FMNH_2$ in luciferase is lower than that in free $FMNH_2$ (Van Schagen and Muller, 1981; Franken et al., 1984). This has also been observed for all flavodoxins studied (Van Schagen and Muller, 1981; Franken et al., 1984, Vervoort et al., 1985), for old yellow enzyme (Beinert et al., 1985 a,b), and for lipoamide dehydrogenase and glutathione reductase (Van den Berg et al., 1984). The only exception found up to now, in what seems to be a rule in flavoproteins, is riboflavin-binding protein (Moonen et al., 1984 b).

The resonance of the N(1) atom in luciferase appears about 5 ppm upfield from that of free $FMNH^-$ (Table II, Figure 2). This suggests that the negative charge on N(1) is either somewhat delocalized or it is counteracted by a nearby positively charged group, which could also be due to the positively charged pole of an α-helix. It has been found that two ionizable groups (pK_a

values of 6.2 and 6.8) are involved in the bioluminescence of $FMNH_2$ with V. harveyi luciferase (Nicoli et al., 1974). It was suggested that these pK_a values could be due either to $FMNH_2$ (phosphate and isoalloxazine moieties) or to cysteine (Nicoli et al., 1974) or histidine residues (Cousineau and Meighen, 1976). Our results indicate that the pK_a of 6.8 is probably due to $FMNH_2$ which must be ionized to bind.

Also the resonance due to the N(10) atom of luciferase-bound $FMNH^-$ is observed at higher field than that of free $FMNH^-$ (Table II, Figure 2). From this we conclude that the N(10) atom of protein-bound $FMNH^-$ possesses a slightly lower degree of sp^2 hybridization than that of free $FMNH^-$. Consequently, this atom is placed slightly out of the molecular plane of protein-bound $FMNH^-$ as compared with free $FMNH^-$ (Moonen et al., 1984a).

The ^{13}C chemical shifts of reduced flavin bound to luciferase are collected in Table 2 and diagramatically presented in Figure 2 (see also Figure 4). These chemical shifts support the interpretation of the ^{15}N chemical shifts and follow the reasoning discussed above for the oxidized luciferase-FMN complex. Therefore, only a few points will be considered here. The slight upfield shift of the resonance due to C(2), compared to $FMNH^-$, reflects the decreased charge on N(1), because the resonance of C(2) and C(10a) are very sensitive to the field from this negative charge. Therefore, despite the upfield shift of the C(2) resonance the result indicates that C(2) is still hydrogen bonding. The field effect does not apply to the C(4) atom and therefore the C(4)=O hydrogen bond is much weaker than

that on C(2)=O. The downfield shifts of the resonances due to C(4a) and C(5a) reflect the somewhat decreased sp^2 character of the N(10) atom as compared with that of free $FMNH^-$ (Moonen et al., 1984a). The strong upfield shift of C(8) and the lesser extent of an upfield shift of C(6) reflect the high sp^2 hybridization of N(5) and the π electron reallocation from the last atom onto the first two. In this context it should be noted that the sp^2 hybridization of N(5) as deduced from the ^{15}N and ^{13}C NMR results is in agreement with the fact that reduced luciferase exhibits an increased molar absorption coefficient at 450 nm as compared with free reduced flavin (Dudley et al., 1964). As discussed previously (Vervoort et al., 1985) both parameters reflect the high coplanarity of the flavin molecule around the N(5) atom, while the N(10) atom is somewhat out of the molecular plane (Figure 5B).

Luciferase reconstituted with [2,4a-$^{13}C_2$]FMN in the reduced state was also studied in the presence of tetradecanal and dodecanol in order to investigate possible influences of these compounds on the electronic system of the flavin. No influence of these compounds on the ^{13}C chemical shifts were found.

Luciferases are known as proteins binding the prosthetic group relatively weakly in the oxidized state (Ziegler and Baldwin, 1981) in contrast to most flavoproteins. In the reduced state the prosthetic group in luciferase is more tightly bound than in the oxidized state. For instance the dissociation constant for the V. harveyi luciferase-FMN complex in the oxidized state has been determined to be 0.12 mM at 3°C in 0.05

M Bis-Tris (2,2-bis (hydroxymethyl)-2',2',2'-nitriloethanol), pH 7.0, containing 0.2M NaCl (Baldwin et al., 1975), while the K_d for the reduced complex was found to be about 1 μM at 6^{o}C and in 0.1M Bis-Tris, pH 7.0, in the presence of 0.1 M NaCl and 0.2 mM dithiothreithol (Becvar and Hastings, 1975). Considering these facts and the fact that we used about 2 mM solutions of luciferase we should observe roughly 25% free flavin in the oxidized state and about 3% in the reduced state. The latter value is small enough not to be observed in the ^{13}C and ^{15}N NMR spectra. However, in the oxidized state we should observe a considerable contribution of free FMN in the ^{13}C spectra of luciferase, which is not observed. There are two arguments to explain this apparent discrepancy. Firstly, the luciferase preparations used possessed a high specific activity, in fact an activity exceeding those published before (O'Kane et al., 1986). Secondly, it has been found that maximal reconstitution and a tighter binding of FMN is obtained in 50mM phosphate buffer than in other buffer systems such as Tris and Bis-Tris (J. Lee, unpublished results). Therefore, it must be concluded that the affinity of luciferase for FMN is larger than published and it is tentatively proposed that the K_d should be about one order of magnitude smaller which would be consistent with our NMR results. Further support for these proposals will be presented below. In fact, the NMR results by Ghisla et al. (1978) also indicated a higher affinity of luciferase for FMN than expected on the ground of the published K_d (Baldwin et al., 1975).

It has been proposed that two flavin molecules are involved in the catalytic mechanism of luciferase (see e.g., Lee, 1985).

These findings are in contradiction to published work by others (see e.g., Ziegler and Baldwin, 1981). Therefore, this issue is worth considering in the light of the present results. In the course of this study we observed that ^{13}C resonances were split into doublets representing two flavin molecules in different environments. These doublets were observed in the reduced rather than the oxidized state of the protein. Figure 6 shows ^{13}C NMR difference spectra between the spectrum of oxidized minus that of reduced luciferase. The spectrum of Figure 6A was obtained by using a luciferase preparation which was only about 30% active (specific activity = 50 x 10^{12} photons $s^{-1}A_{280}{}^{-1}$), while the preparations used to obtain the results presented in Table 2 and Figures 1-4 had a specific activity of 160 x 10^{12} photons $s^{-1}A_{280}{}^{-1}$. Nevertheless the sample used in Figure 6A was reconstituted with [2,4a-$^{13}C_2$]FMN in a molar ratio of 1:1, flavin:protein. The spectrum of oxidized luciferase was then recorded and thereafter the protein reduced in the sample tube and the spectrum reregistered. The difference spectrum between the two redox states shows each one line for C(2) and C(4a) in the oxidized and two lines for C(4a) and one line for C(2) in the reduced state. The major peak appears at 101.5 ppm and the minor peak at 103.5 ppm. The ^{13}C chemical shifts correspond well with those of free and protein-bound $FMNH^-$ (Table II). The C(2) of the $FMNH^-$-luciferase complex appears as a singlet at about 158 ppm and is not split because of the very small difference in the chemical shift between free and protein-bound $FMNH^-$ at the pH value studied (Table II). On the other hand,

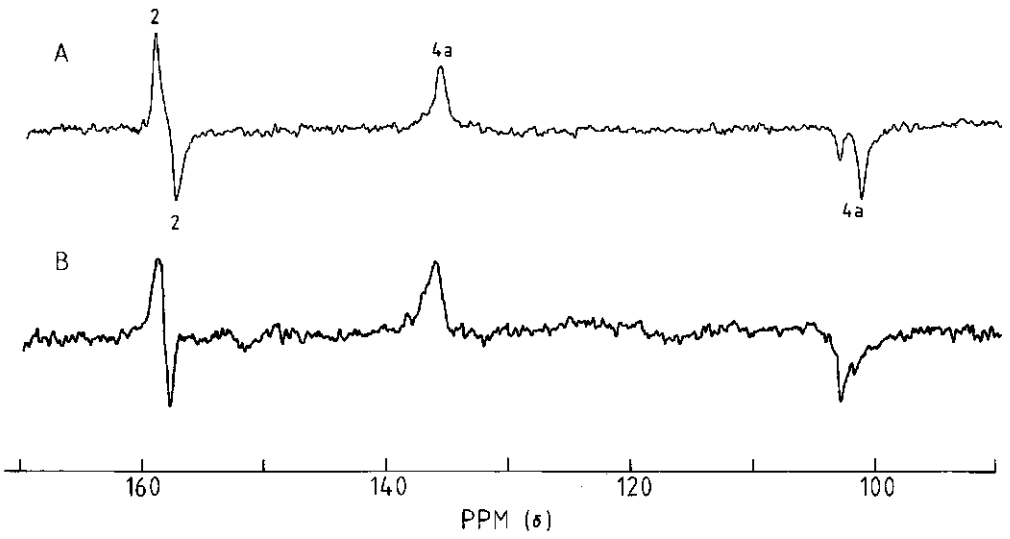

Figure 6. ^{13}C NMR spectra of Vibrio harveyi luciferase (3 mM) reconstituted with [2,4a-$^{13}C_2$]FMN in 200 mM potassium phosphate, 0.5 mM EDTA, 10 mM β-mercaptoethanol, pH = 8.5.

A: Specific activity of the enzyme was $50x10^{12}$ photons $s^{-1}A280^{1}$. Difference spectrum between the spectrum of oxidized (38500 acquisitions) minus that of reduced luciferase (42300 acquisitions).

B: Specific activity of the enzyme was $120x10^{12}$ photons $s^{-1}A_{280}^{-1}$. Difference spectrum between the spectrum of oxidized (39400 acquisitions) minus that of reduced luciferase (45100 acquisitions).

the difference in the chemical shifts between free and protein-bound flavin in the oxidized state is sufficient to be observed, if present (Table II).

Using an enzyme preparation showing a specific activity of 120 x 10^{12} photons $s^{-1}A_{280}^{-1}$ and following the same procedure as outlined above gave the difference spectrum shown in Figure 6B. The major peak appears now at 103.5 ppm and the minor one at 101.4 ppm. Furthermore, it was found that the line at 101.4 ppm is pH-dependent, whereas the line at 103.5 ppm is pH-independent (Table II). The pH-dependent line at 101.4 ppm follows exactly the pH titration curve of free $FMNH_2$ (Van Schagen and Muller, 1981). These results show that the ^{13}C spectra of reduced luciferase can be used to estimate the relative content of active luciferase molecules in preparations.

To check the possiblity that highly active luciferase can bind more than one molecule of $FMNH^-$, we added excess flavin stepwise to the solution of Figure 6B up to a molar ratio of 2 to 1 (results not shown). Upon addition of reduced flavin the intensity of the resonance line at 101.4 ppm (Figure 6B) increases in a concentration dependent fashion while remaining of the same broad width, and the intensity of the line at 103.5 ppm stays unchanged. The addition of a higher excess of $FMNH^-$ leads to the appearance of sharp resonance lines superimposed on those assigned to the aspecifically bound $FMNH^-$. These sharp lines are due to free $FMNH^-$. These results strongly suggest that luciferase can bind more than one reduced flavin. However, the binding interaction with the second molecule is much less specific than with the first molecule as can be deduced from the

chemical shifts. Since the linewidth of the resonances due to the weakly bound flavin is about 40Hz as compared with that of free flavin ($\Delta\nu_{1/2}$ 10Hz) there is no doubt that the second molecule interacts with luciferase, very much the same way as flavin binds to inactive luciferase. In fact, we have found that the interaction of excess flavin with luciferase resembles that of bovine serum albumin with flavin, although the latter possesses no specific flavin binding site. This result suggests that flavin interacts in both proteins, either with some hydrophobic sites or with charged groups via the phosphate moiety of $FMNH^-$. In both cases the flavin is accessible to bulk water, this in contrast with the flavin bound specifically by luciferase.

It can easily be demonstrated that the aspecifically bound $FMNH^-$ binds more weakly to luciferase than the other one. Figure 7A shows the ^{13}C NMR spectrum of luciferase reconstituted with [2,4a-$^{13}C_2$]$FMNH^-$. The specific activity of the sample was the same as that shown in Figure 6B, except the pH value of the solution was 7.0. This sample was poured over a Biogel P6-DG column in the presence of dithionite, the enzyme collected and measured again. The spectrum of Figure 7B shows that the aspecifically bound flavin has been removed completely. As evident from this spectrum only a very small amount of specifically bound flavin is possibly lost during the chromatographic procedure as revealed by the difference spectrum between the two spectra (Figure 7C). It should also be noted that in Figure 7A also the resonance of C(2) of the aspecifically

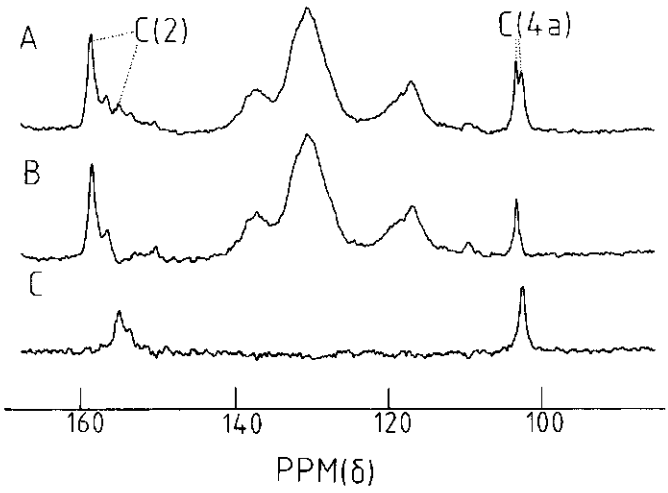

Figure 7. ^{13}C NMR spectra of Vibrio harveyi luciferase (specific activity $120x10^{12}$ photons $s^{-1}A_{280}^{-1}$) (3mM) reconstituted with [2,4a-$^{13}C_2$]FMN in the reduced state in 50 mM potassium phosphate, 0.5 mM EDTA, 10 mM β-mercaptoethanol, pH = 7.0.

A. The flavin/apoluciferase ratio was 2:1 (44100 acquisitions).

B. The sample of A was chromatographed under anaerobic conditions (see text) and the solution was measured again. (33400 acquisitions).

C. Difference spectrum between spectrum A minus that of B.

bound flavin can be seen due to the larger chemical shift difference between the two species at pH 7.0 (Table II).

The observation that the ^{13}C NMR spectra of luciferase recombined with oxidized flavin do not show different microenvironments for the strongly and more weakly bound flavin, in contrast to the spectra in the reduced state, indicates that a conformational change of the protein occurs on changing the redox state. Recently it was shown that such a conformational change indeed occurs (Aboukhair et al., 1985). This conformational change affects specifically the microenvironment of the flavin involved in the catalysis. Since the aspecifically bound flavin seems to experience the same microenvironment in both inactive and highly active luciferase its possible role in the catalysis needs further investigation.

We have also measured the ^{13}C NMR spectra of luciferase from V. fischeri and from Photobacterium phosphoreum in the presence of [2, 4a-$^{13}C_2$]FMNH$^-$. The ^{13}C chemical shifts are the same as those found in V. harveyi, so very likely the interaction between these luciferases and flavin are not very different from that observed in V. harveyi luciferase.

When comparing our results with those of Ghisla et al. (1978), who used [2,4a-$^{13}C_2$]FMN only, we notice some discrepancies in the interpretation of the ^{13}C NMR spectra. The ^{13}C NMR spectra of Ghisla et al. (1978) had a signal to noise ratio much lower than our spectra. Therefore, the minor differences can be attributed to this fact (see also Vervoort et al., 1986, accompanying paper).

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Chapter 6.

IDENTIFICATIONS OF THE TRUE CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTRUM OF THE STABLE INTERMEDIATE II IN BACTERIAL LUCIFERASE+

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ABSTRACT

Intermediate II in bacterial luciferase, formed in a reaction of luciferase with $FMNH^-$ and O_2, has been reinvestigated by ^{13}C NMR spectroscopy using ^{13}C enriched FMN derivatives. It is shown that the previously published spectrum of the intermediate by Ghisla et al. (Proc. Natl. Acad. Sci. USA 75, 5860-5863 (1978)) does not represent the intermediate, but is due to a contamination contained in commercially available ethylene glycol-d_6. Relaxation studies show that the resonance line due to the by-product of ethylene glycol-d_6 is easier to observe at low temperature than at room temperature explaining fully the spectral properties of the published artifact. The true C(4a)-resonance is found at 82.5 ppm and this is interpreted as due to considerable sp^2 hybridization at this position, indicating an almost planar configuration by comparison with a model compound.

INTRODUCTION

Bacterial luciferase is a flavoprotein utilizing riboflavin 5'-phosphate (FMN)[1] as a prosthetic group and catalyzing the oxidation of long chain aliphatic aldehydes. The reaction is accompanied by emission of light. In the course of the bioluminescence reaction several intermediates are formed and their involvement in the reaction is still under dispute (e.g. Lee, 1985; Ziegler and Baldwin, 1981). However, one of these is a species detected on reaction of O_2 and $FMNH^-$ with luciferase in the absence of aldehyde. This species is commonly termed intermediate II. From a comparison of the uv spectral properties of the intermediate with those of model compounds (Ghisla et al., 1973) it has been suggested that the compound is 4a,5-dihydro-4a-hydroperoxyflavin (Hastings et al., 1973). However, the light absorption technique is far from unambiguous in providing the chemical structure of a new compound, especially in the case of the flavin molecule possessing a complex electronic structure. The fact that intermediate II is rather stable in the protein-bound state makes it feasible to elucidate the structure of intermediate II by ^{13}C NMR, despite the low sensitivity of the technique (Allerhand, 1979), which can be overcome using ^{13}C enriched compounds. Ghisla et al. (1978) have pioneered such a study using [4a-^{13}C]FMN as a prosthetic group for luciferase and claimed to show proof for the substitution at C(4a) of flavin in the intermediate. Since the signal/noise ratio in the experimental spectra was rather low we suspected that the assignment of the resonance lines could be incorrect. When we repeated the experiments of Ghisla et al.

(1978) in the absence of ethylene glycol we could not observe the line at 74 ppm which they assigned to the intermediate. Since the data of Ghisla et al. (1978) are frequently cited as absolute proof for the existence of a C(4a) substituted flavin derivative in flavin-dependent hydroxylation reactions we considered it very important to reinvestigate the structure of the luciferase-bound intermediate (Lee, 1985). It is shown in this paper that the published spectrum for the intermediate is an artifact due to an impurity contained in deuterated ethylene glycol and that the intermediate is indeed a C(4a) substituted flavin which resonance appears at 82.5 ppm in the ^{13}C NMR spectrum.

MATERIALS AND METHODS

FMN selectively enriched with ^{13}C was prepared as described previously (Van Schagen and Müller, 1981). The isotopic enrichment was at least 92 atom %. Ethylene glycol-d_6 was purchased from Merck AG, Darmstadt, Germany. Bacterial luciferase was from an aldehyde requiring dark mutant of Vibrio harveyi strain 392 (MAV) and was purified to homogeneity (O'Kane et al., 1986). Luciferase was assayed for bioluminescence specific activity (photons s^{-1} A_{280}^{-1}) at room temperature as described (Lee, 1982), using decanal. Photometer calibration was made by reference to the NBS standard lamp and NBS absolute photodiode photometer via the luminol chemiluminescence reactions (Lee and Seliger, 1965; Matheson et al., 1984).

The NMR samples contained luciferase, which was at least 80% of the maximum obtainable specific activity, i.e. $120x10^{12}$ photons s^{-1} A_{280}^{-1}. For reconstitution experiments ^{13}C enriched FMN was added in a slight excess over apoluciferase. All measurements were done in 50 mM potassium phosphate buffer, pH=7, containing 10 mM β-mercaptoethanol. The intermediate II was prepared from reconstituted luciferase using the same buffer system mentioned above but saturated with dodecanol (Tu, 1979). The sample was then reduced and poured over a short Biogel P6DG column (2.7x7.5cm), previously equilibrated with the same buffer. After elution of the luciferase-bound intermediate the sample was concentrated to about 1.5 ml by ultrafiltration (Amicon). All procedures were done at about 0^oC.

The decay of the luciferase-bound intermediate was followed spectrophotometrically with time using an Aminco DW2A spectrophotometer, and by assays of the bioluminescence reaction. The temperature was kept at 0 ^{o}C.

Wilmad 10 mm precision NMR tubes were used for ^{13}C NMR measurements. The sample volume was 1.6 ml, containing 10% $^{2}H_2O$ to lock the magnetic field. Broadband decoupling of 0.5 W was used. All spectra were recorded using 30^{o} pulses and a repetition time of 1.0s. The temperature was 0$^{o}\pm$2^{o}C. Dioxan (3μl) served as an internal standard. The ^{13}C chemical shift values are reported relative to tetramethylsilane (δdioxane - δ TMS = 67.84ppm). The accuracy of the reported values is about 0.2 ppm. All measurements were done on a Bruker CXP300 NMR spectrometer operating at 75.6 MHz.

When luciferase recombined with FMN was studied in the reduced state reduction was done by addition of a dithionite solution to the anaerobic solution in the NMR tube. Anaerobiosis was achieved by carefully flushing the solution in the NMR tube with argon for about 10 min. The NMR tube was sealed with a serum cap.

RESULTS AND DISCUSSION

In contrast to Ghisla et al. (1978) we used doubly labeled ^{13}C enriched FMN derivatives throughout this study. This approach has a great advantage in the identification and assignments of the resonances in a complex ^{13}C NMR spectrum of a relatively large protein as luciferase. Since the ^{13}C chemical shifts in oxidized and reduced free flavin have been shown to be directly related to the electron density on the corresponding carbon atoms in the molecule (Van Schagen and Müller, 1980). the chemical shifts change in a predictable manner on reduction or modification of flavin. Therefore, a second label in the molecule is a reliable reference point for the other one in a ^{13}C NMR spectrum.

Figure 1A shows the ^{13}C NMR spectrum of luciferase-bound [2,4a-$^{13}C_2$] FMN in the two-electron reduced state. The spectrum shows one resonance line at 103.5 ppm for the C(4a) atom. The resonance line is shifted downfield by 2.1 ppm as compared with free $FMNH^-$(Moonen et al., 1984). This is in contradiction with the value reported by Ghisla et al. (1978) for the free flavin, i.e. 104 ppm. The resonance line due to C(2) (Table I) is hidden under the natural abundance lines of the C atom of arginine residues (Vervoort et al., 1986), the latter resonating at 158.2 ppm (Allerhand, 1979). However, a difference spectrum between the spectra of reduced and oxidized luciferase-bound FMN reveals that the C(2) atom resonates at 157.9 ppm in the reduced and at 158.5 ppm in the oxidized state (Vervoort et al., 1986) (Table I). These results indicate that luciferase-bound reduced FMN is ionized, i.e. carrying a negative

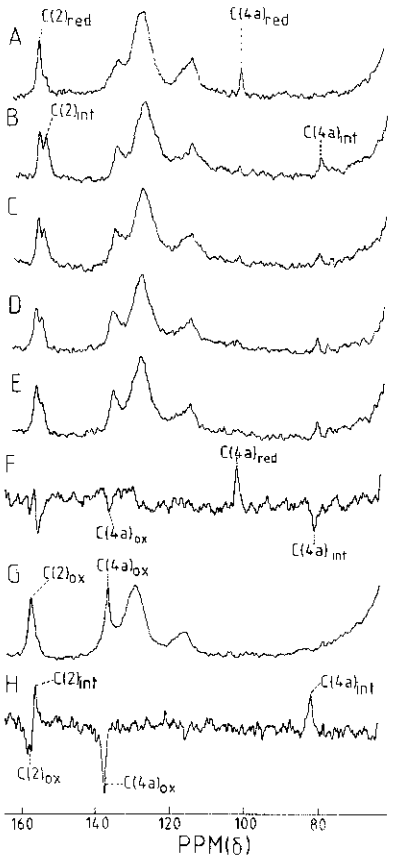

Figure 1. Carbon-13 NMR spectra of 3 mM luciferase in the presence of 2.4 mM [2,4a-$^{13}C_2$]FMN in 50 mM potassium phosphate, pH 7.0, containing 10 mM β-mercaptoethanol and a saturating concentration of dodecanol. The sample also contained 10% ^2H_2O to lock the magnetic field.

A. The anaerobic sample was reduced with a slight excess of dithionite and kept under argon. The NMR tube was sealed with a serum cap. 10,000 transients were accumulated.
B. Spectrum of intermediate II prepared by column chromatography and subsequent concentration by ultrafiltration (see Materials and Methods). Then the spectrum was recorded immediately. 7,200 transients were collected taking 120 min.
C. to E. Subsequent spectra of intermediate II collecting each 7,2000 transients.
F. Difference spectrum between that of A and that of B.
G. The sample of E was allowed to stand for 2 h at room temperature to achieve full reoxidation. After recooling the sample to 0°C, the spectrum was recorded (16,700 transients).
H. Difference spectrum between that of intermediate II (B to D) and that of the reoxidized sample (F).

All spectra were obtained at 0°C.

charge at N(1) (Van Schagen and Muller, 1981). This has also been proven by ^{15}N NMR data (Vervoort et al., 1986). The broad lines between about 120 ppm and 140 ppm are due to the natural abundance aromatic carbon atoms of tyrosine, tryptophan, phenylalanine and histidine residues (Allerhand, 1979).

Before continuing to the ^{13}C NMR spectra of the intermediate a few points deserve a brief discussion. In an accompanying paper (Vervoort et al., 1986) we will show that inactive luciferase also binds reduced FMN. In this case the C(4a) atom of $FMNH^-$ resonates at 101.4 ppm. Figure 1A shows that this resonance line is absent from the spectrum in agreement with the fact that only a slight excess of flavin over apoluciferase (see Materials and Methods) has been added in the reconstitution reaction in order to avoid further spectral complexity and side reactions during the preparation of the intermediate (see below). This means that the concentration of active reconstituted luciferase in the sample used to obtain Figure 1A is about 2.4 mM. Furthermore, we used a different buffer system and a different procedure to prepare the intermediate than Ghisla et al. (1978). We used 50 mM phosphate buffer (pH=7) in the presence of 10 mM β-mercaptoethanol and a saturating concentration of dodecanol instead of 300 mM phosphate buffer (pH=7.0) in the presence of 20% ethylene glycol-d_4(Ghisla et al., 1978). β-Mercaptoethanol is needed to preserve the activity of luciferase, especially in long lasting experiments (O'Kane et al., 1986). Dodecanol has been shown to stabilize intermediate II in luciferase (Tu, 1979). On the other hand high concentrations of phosphate buffer inhibit the

bioluminescence reaction, and possibly the interaction between luciferase and $FMNH^-$, competitively, K_i = 220 mM (Meighen and MacKenzie, 1973), leading possibly also to a decrease in the concentration of the intermediate. Furthermore, rather than preparing the intermediate directly in the NMR tube as done by Ghisla et al. (1978) we isolated the intermediate by column chromatography (Hastings et al., 1973; Becvar et al., 1978) at 0^oC and concentrated the sample subsequently by ultrafiltration. Although these steps take about 90 min, the loss of intermediate is small considering the very long lifetime of the intermediate at even 0^oC (see below). An advantage of the chromatographic step is that excess flavin, which was present in the sample used by Ghisla et al. (1978), is removed which if present, leads to the formation of a considerable amount of luciferase-bound flavosemiquinone (Kürfurst et al., 1982), also resulting in an additional decrease of the concentration of the intermediate. In order to be sure that we were measuring and following the decay of the intermediate we withdrew an aliquot of the prepared intermediate prior to the start of the NMR measurements and followed its decay spectrophotometrically and by its bioluminescence activity with time, parallel to the registration of the ^{13}C NMR spectra.

The first spectrum of the intermediate accumulated after the preparation is shown in Figure 1B. The initial concentration of the intermediate in the sample was about 2.2 mM as judged by uv spectrophotometry (Becvar et al., 1978). The accumulation of this spectrum took 2 h. Additional spectra of the same sample

were collected under identical instrumental settings (Figure 1,C-E). Each spectrum represents a 2 h accumulation time. The spectra of the intermediate exhibit lines at 82.5 ppm for the C(4a) atom and at 156.5 ppm for the C(2) atom, the latter overlapping slightly with the high field edge of the line due to C^{ζ} atom of arginine residues. The intensities of both lines decrease slowly with time. Concomitantly the resonance lines at 137.4 ppm and 158.5 ppm due to oxidized flavin increase in intensity. The changes observed in the ^{13}C NMR spectra parallel those of activity measurements and the uv spectra. The slow formation of oxidized luciferase-bound FMN is not accompanied by the formation of possible other species, at least not in concentrations high enough or lifetimes long enough to be observed in the ^{13}C NMR spectrum. The spectrophotometric experiments and the assay measurements indicate that the half life of the intermediate under our experimental conditions is 13 h at $0^{o}C$, a time long enough not to lead to a great loss of the intermediate during the chromatographic procedure and subsequent concentration of the sample (90 min). This is demonstrated in Figure 1 F showing the difference spectrum between that in Figure 1A and that in Figure 1 B. The low intensity of the C(4a) resonance of oxidized flavin indicates again the slow decomposition of the intermediate.

After registration of the spectrum shown in Figure 1E the sample was brought to room temperature and allowed to stand for 2 h at this temperature. Then the sample was cooled again to $0^{o}C$ and its spectrum registered (Figure 1G). The high field peak at 82.5 ppm has disappeared completely from the spectrum indicating

the full decomposition of the intermediate (Hastings et al., 1973; Becvar et al., 1978). This was also ascertained by spectrophotometry and assay measurements. Figure 1G clearly shows now the increased intensity of the lines due to oxidized flavin at 137.4 ppm and 158.5 ppm, and the decrease of intensity at about 156.5 ppm and at 82.5 ppm. Figure 1H shows the difference spectrum between that of the intermediate (Figure 1B) and that of the oxidized sample (Figure 1G). This difference spectrum shows the resonance lines of the intermediate very clearly and allows an unambiguous assignment of all resonance lines. The experiments described above have been repeated several times using freshly prepared luciferase. The results were identical.

The same type of experiments were done with luciferase reconstituted with [4,10a-$^{13}C_2$]FMN under identical conditions as described above. The results are collected in Table I. The resonance line of C(4) of the intermediate can be directly observed since at this resonance position no protein background is present. The C(10a) resonance of the intermediate on the other hand is strongly overlapping with the natural abundance lines of the C ζ atom of arginine residues at about 158 ppm and can only be identified by difference spectroscopy, which has been done.

The strong upfield shift by 21 ppm of the C(4a) atom in the intermediate as compared with that in the luciferase-FMNH$^-$ complex indicates that the C(4a) in the latter changes from a sp^2 hybridized to a more sp^3 hybridized one in the intermediate. In fact the ^{13}C chemical shift of C(4a) in the

Table 1. ^{13}C chemical shifts (in ppm) of FMN bound to luciferase from V. harveyi in the oxidized, reduced and the intermediate II state. All experiments were done in 50 mM potassium phosphate buffer, pH=7.0, in the presence of 10 mM β-mercaptoethanol. The temperature in all cases was 0°C. For comparison the relevant ^{13}C chemical shifts of 3-methyl-4a-propenyl-4a,5-dihydrolumiflavin (I) and 3-methyl-4a,5-epoxyethano-4a,5-dihydrolumiflavin (II) are also given. The ^{13}C chemical shifts are reported relative to TMS.

Carbon atom	Redox State of Luciferase				
	oxidized	reduced	intermediate II	I[a)]	II[a)]
C(2)	158.5	157.9	156.5	162.1	154.7
C(4)	162.6	157.2	166.9	167.7	164.9
C(4a)	137.4	103.5	82.5	63.0	83.5
C(10a)	151.3	156.2	157.7	160.0	156.4

a) From Lhoste et al. (1980).

intermediate is within 1 ppm of that of 3-methyl-4a, 5-epoxyethano-4a,5-dihydrolumiflavin (Lhoste et al., 1980) (Table I). The crystal structure of this compound is known (Bolognesi et al., 1978) and shows that the molecule is almost planar, forming an angle of 5.5^{0} between the two planes intersecting along the N(5)-N(10) axis of the molecule. The largest displacement was found for the C(4a) atom, indicating its increased sp^3 character. It is interesting to note that the C(4a) atom in C(4a) alkylated flavins resonate at about 60 ppm (Ghisla et al., 1978; Van Schagen and Müller, 1980) (Table I)indicating its fully sp^3 hybridized character. Substituting the C(4a) atom in flavin by a hydroxyl group as in 4a-hydroxy-5-alkyl-4a,5-dihydroflavins (pseudo base) shifts the resonance downfield by about 10 ppm (Ghisla et al., 1978) due to the electronegative character of the oxygen atom. The fact that the C(4a) atom in the intermediate resonates about a further 10 ppm downfield from that in the pseudo base (δ = 74.5ppm) is in agreement with the C(4a) hydroperoxide structure. The close resemblance of the chemical shifts of intermediate II with those of 3-methyl-4a,5-epoxyethano-4a,5-dihydrolumiflavin strongly suggests that the intermediate possesses a rather planar structure and that therefore C(4a) in the intermediate is more sp^2 hybridized than that in the pseudo base, as can be deduced from the fact that the chemical shifts of intermediate II are intermediate between those of compound I and compound II (Table I). The rather planar structure of the intermediate and consequently the decreased sp^3 hybridization of its C(4a) atom allows a certain conjugation between the residual π system of the

isoalloxazine ring and the C(4a) atom. This interpretation is in agreement with the fact that 5-ethyl-4a-hydroperoxy-4a,5-dihydrolumiflavin shows a pK_a of 9.2 for the hydroperoxy function and that the pK_a in 1,10-ethano-5-ethyl-4a-hydroperoxy-4a,5-dihydrolumiflavinium is lowered to about 7 (Eberlein and Bruice, 1983). Therefore it is postulated that the degree of planarily of the C(4a) hydroperoxy intermediate in the various flavoprotein hydroxylases determines its stability and consequently its hydroxylation power. This interpretation could offer an explanation for the observation that intermediate II is more stable in some flavoproteins and less stable in others.

The chemical shifts due to C(2) and C(10a) (Table I) indicate that the π electron density does not change much on formation of the intermediate, which also proves that oxygen is not attached to the C(10a) atom, which has also been proposed (Massey and Hemmerich, 1975).

The chemical shift of C(4) also indicates that oxygen is attached to C(4a) in the intermediate. On formation of the intermediate C(4) undergoes a relatively large downfield shift which can be explained as a change towards an electronically isolated sp^2 hybridized carbon atom with a vicinal nitrogen atom, resembling a peptide bond.

The linewidth of the resonance at 82.5 ppm ($\Delta\nu_{1/2}$= 50Hz) in the intermediate (Figure 1B) is slightly larger than that of the line at 103.5 ppm (Figure 1A) or that of the line at 137.4 ppm (Figure 1H) ($\Delta\nu_{1/2}$ = 40Hz). Furthermore, it is obvious that the intensity of the line of C(4a) in the intermediate is slightly less that of the lines of C(4a) in the oxidized and

reduced state, a result observed in all experiments. This reflects the slow conversion of the intermediate to the oxidized state during the experiment, but differences in T_1 of the C(4a) atom in the different states probably also contribute to these effects. It is not unlikely that the spin-lattice relaxation time (T_1) of the C(4a) atom in the intermediate is larger than in the oxidized and reduced state. This would mean that the chemical shift anisotropy (CSA) contributes only slightly, if at all, to the spin lattice relaxation of C(4a) in the intermediate as compared with that in the oxidized and reduced state, i.e. the shielding tensors of the C(4a) atom in the intermediate become less anisotropic and will therefore decrease the contribution of the CSA to the spin lattice relaxation time. This interpretation is in agreement with the fact that the CSA is a major relaxation source for the spin lattice relaxation of quaternary aromatic carbon atoms (Allerhand, 1979; Moonen and Müller, 1983).

It is obvious that our results are in contradiction with those of Ghisla et al. (1978). We have shown in this paper that the C(4a) atom in the intermediate II resonates at 82.5 ppm and not at 74 ppm. Although we have used different conditions, it is difficult to imagine such a large chemical shift difference for the same protein-bound intermediate. Moreover, a direct comparison between our ^{13}C NMR spectra of the intermediate and that published by Ghisla et al. (1978) reveals a very striking difference, i.e. in our spectra several natural abundance resonances of the protein can be observed in addition to the resonances of the ^{13}C enriched flavin carbons, whereas no

intensity at all was observed for the protein resonances in the published spectrum of Ghisla et al. (1978). This can only be explained by a drastic difference in T_1 and/or T_2 values for the C(4a) of the intermediate in the different experiments. Therefore, we will discuss these aspects in detail in the following.

For a tight protein-flavin complex we are dealing with the situation where $\omega\tau_r > 1$ for the field strength and viscosities used (Allerhand, 1979). Therefore, a linear relationship can be expected for T_2 (and thus linewith) and the rotational correlation time. Indeed, this is found in our study on increasing the viscosity by lowering the temperature. This has been demonstrated also by Ghisla et al. (1978). However, at -15°C the viscosity is about 15 cp for a mixture of 20% ethylene glycol and 80% water (Douzou, 1977). At 20°C a viscosity lower than 2 cp is found for this mixture. Therefore an increase in linewith by a factor of about 7 can be expected at -15°C for this mixture, thus lowering the signal/noise ratio for a single resonance by the same factor.

The viscosity change from 2 cp to 15 cp also has drastic effects on the T_1 values. T_1 values increase in a linear way upon an increase in the viscosity in the relevant region of the τ_r values. As T_1 values of about 2 s can be expected for the quarternary carbon atoms of the luciferase flavin complex at 1 cp, where we estimate the rotational correlation time to be 50 ns (Vervoort et al., 1986; Allerhand, 1979), we can expect much higher T_1 values (possibly up to 25 s) at -15°C in an ethylene glycol/water mixture. Using 90° pulses and repetition

times of 0.8s severe saturation can be expected for these quaternary carbon atoms. However, the majority of natural abundance carbon resonances are due to carbon atoms with a directly bound hydrogen atom, rendering the T_1 and T_2 relaxation much more effective for the latter carbon atoms than for the quaternary flavin carbon atoms. Therefore, at high viscosity compared with low viscosity and using acquisition parameters of Ghisla et al. (1978) one would expect that the natural abundance resonances of the protein appear with a relatively high intensity compared with the quaternary flavin carbon atoms. This is indeed observed in our spectra (data not shown). In the spectrum of the intermediate at -15^{o}C reported by Ghisla et al. (1978) no protein resonances can be observed at all. With the above given analyses, one would expect that the C(4a) atom of the flavin in the intermediate cannot be observed due to the increased linewith and increased saturation. However, the resonance at 74 ppm can be observed in their spectrum with a relatively good signal/noise ratio. This fact suggest strongly that the resonance at 74 ppm in their spectrum cannot originate from the C(4a) carbon atom of the intermediate. It should be noted, that the above given analysis is based mainly on dipole-dipole relaxation. Although this is correct for all protonated carbon atoms, it has been shown that the chemical shift anisotropy contributes to the relaxation values of the quaternary flavin atoms up to 50% (Moonen and Müller, 1983). If we take also the effect of the CSA into account, then we would expect an even larger intensity decrease for the C(4a) resonance of the intermediate, as the CSA for the sp^3 hybridized C(4a) atom of

the intermediate is undoubtedly decreased compared with the CSA of the C(4a) atom of the reduced complex.

It remains to be proven that the resonance line at 74 ppm, assigned to the C(4a) atom of flavin in the intermediate (Ghisla et al., 1978), originates from a compound other than flavin. Since we expected that this resonance is an artifact we repeated the experiment of Ghisla et al. (1978) in the absence of the protein, i.e. 0.3 M phosphate buffer containing 20% ethylene glycol-d_6 and 1 mM [4a-^{13}C]FMN at -15^{o}C. The spectrum is shown in Figure 2. This spectrum is identical with that published by Ghisla et al. (1978) claimed to represent the spectrum of the intermediate. The high field peak resonates at 73 ppm, 1 ppm upfield of that reported by Ghisla et al. (1978). This can be ascribed to the use of a different reference compound (Lhoste et al., 1980). The resonance of the C(4a) atom of oxidized flavin is found at 136 ppm in our spectrum (Figure 2), also 1 ppm upfield from that of C(4a) in the spectrum of Ghisla et al. (1978). During the deuteration process of ethylene glycol, diethylene glycol can be formed. In our case we found by gas chromatographic and mass spectral analysis that commercially available ethylene glycol-d_6 is contaminated with 2% of diethylene glycol-d_{10}. Due to the one-bond coupling between the deuterium and the C(2) atoms of diethylene glycol-d_{10} the resonance line at 73 ppm is, under the conditions used in Figure 2, a non-resolved quintet with $^{1}J(^{13}C-^{2}H)$= 22Hz (Kalinowski et al., 1984). This yields a resonance line with an apparent linewidth of about 50Hz, as also observed by Ghisla et al. (1978). The coupling constant is independent of the magnetic

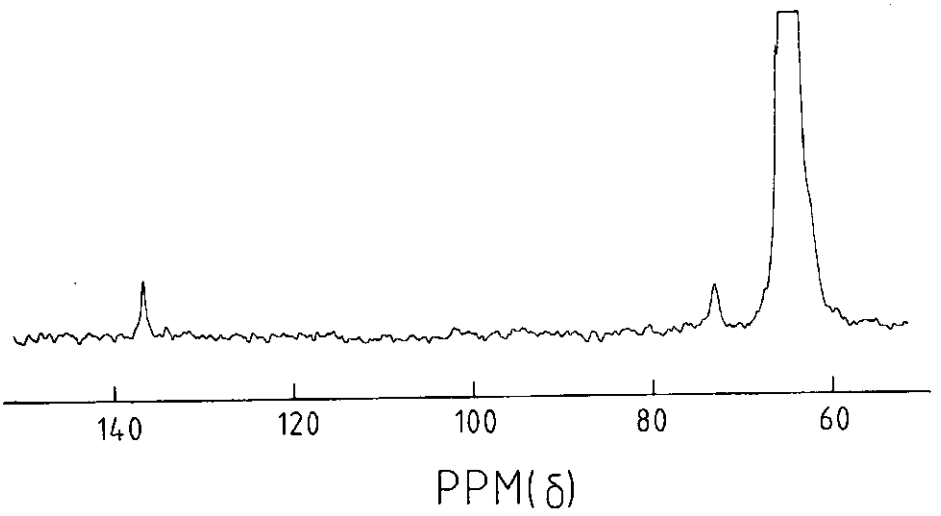

Figure 2 Carbon-13 NMR spectrum of 1 mM [4a-^{13}C]FMN in a mixture of 0.3 M potassium phosphate and ethylene glycol-d_6 (20%), pH = 7.0. The temperature was -15°C.

field strength. The linewidth of C(4a) of free flavin is 10Hz under these conditions. Ghisla et al. (1978) reported that the resonance at about 74 ppm disappeared from the spectrum on heating the sample to room temperature. In order to explain this result it is necessary to discuss the various relaxation effects in more detail.

From the size of diethylene glycol it can be calculated that the rotational correlation time (τ_r) is much smaller than 0.1 ns, so we are dealing with the "extreme narrowing limit", i.e. $\omega\tau_r << 1$(Allerhand, 1979; Kalinowski et al., 1984). Under these conditions the spin-lattice relaxation time (T_1) is dependent on τ_r of the molecule. We determined the T_1 of ethylene glycol-d_6 at 25°C and -15°C in a sample containing apoluciferase and 20% of ethylene glycol-d_6 in 0.3 M phosphate, pH=7.0. The T_1 was found to be about 25s at 25°C and about 7.5s at -15°C. It can be expected that the T_1 of the C(2) atoms of diethylene glycol-d_{10} is slightly different from that of ethylene glycol-d_6 owing to the small differences in the τ_r (Kalinowski et al., 1984). In both molecules however the ^{13}C nuclei relax primarily through dipole-dipole (protonated and deuterated carbon atoms) interactions and the T_1 is therefore independent of the magnetic field strength. As a result, under the conditions used by Ghisla et al. (1978), i.e. 90° pulses and 0.8s repetition time, it is almost impossible to observe the resonance of diethylene glycol-d_{10} at 25°C, especially with the inferior signal/noise ratio reported by Ghisla et al. (1978). In fact a very close inspection of the published spectrum of Ghisla et al. (1978) at 25°C suggests the presence

of the resonance at about 74 ppm as a very broad line. On the other hand the resonance at 73 ppm is much easier to observe at $-15^{o}C$ due to the shorter T_1. The spin-lattice relaxation times of the protein, on the other hand, show an opposite behavior. These facts are illustrated by registering the spectra at various temperatures and comparing roughly the ratio of the intensity of the line at about 128 ppm due to the protein and that of the line at 73 ppm. Under our instrumental settings (30^{o} pulses and 1.0s repetition time) the following ratios were determined: 2.2 at $25^{o}C$, 1.0 at $0^{o}C$ and 0.4 at $-15^{o}C$. Using the conditions of Ghisla et al. (1978) a ratio of 5.6 was found at $25^{o}C$. These results definitely prove that the spectrum of the intermediate published by Ghisla et al. (1978) is an artifact due to a contamination in deuterated ethylene glycol, i.e. diethylene glycol-d_{10}.

This study also demonstrates that great caution must be excercised in the identification and assignments of NMR lines in complex spectra of biological material.

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Chapter 7. A ^{13}C, ^{15}N and ^{31}P NMR investigation of para-hydroxybenzoate hydroxylase

SUMMARY

Para-hydroxybenzoate hydroxylase from Pseudomonas fluorescens has been reconstituted with ^{13}C and ^{15}N enriched FAD. The protein preparations were investigated in the oxidized and in the two-electron reduced state by ^{13}C, ^{15}N and ^{31}P NMR techniques. The chemical shift values are compared with those of free flavin in water or chloroform. The substrate para-hydroxybenzoate has a marked effect on the ^{13}C and ^{15}N chemical shifts in both redox states. No effect of substrate on the ^{31}P NMR resonances was found. The results indicate that the isoalloxazine ring is fairly solvent accessible in the substrate free enzyme in the oxidized state. The results show that upon binding of substrate the isoalloxazine is shielded from solvent. It is also suggested that upon substrate binding a conformational change occurs as a result of which the positive pole of an α-helix dipole influences the ^{13}C and ^{15}N chemical shifts. In the reduced state the flavin is bound in the anionic form i.e. carrying a negative charge at N(1). The exchange of the N(3) and N(5) amide protons with bulk solvent is very slow (ν<<100 Hz) in the reduced state either in the presence or in the absence of substrate. The flavin is bound in a more planar configuration than free in solution. Upon binding of substrate the resonances of N(1), C(10a) and N(10) shift sharply upfield. It is suggested that these upfield shifts are either a result of a shift of the pK of the N(1) atom to higher pH values or a result of a similar conformational change as found in the oxidized state.

SUMMARY

Para-hydroxybenzoate hydroxylase from Pseudomonas fluorescens has been reconstituted with ^{13}C and ^{15}N enriched FAD. The protein preparations were investigated in the oxidized and in the two-electron reduced state by ^{13}C, ^{15}N and ^{31}P NMR techniques. The chemical shift values are compared with those of free flavin in water or chloroform. The substrate para-hydroxybenzoate has a marked effect on the ^{13}C and ^{15}N chemical shifts in both redox states. No effect of substrate on the ^{31}P NMR resonances was found. The results indicate that the isoalloxazine ring is fairly solvent accessible in the substrate free enzyme in the oxidized state. The results show that upon binding of substrate the isoalloxazine is shielded from solvent. It is also suggested that upon substrate binding a conformational change occurs as a result of which the positive pole of an α-helix dipole influences the ^{13}C and ^{15}N chemical shifts. In the reduced state the flavin is bound in the anionic form i.e. carrying a negative charge at N(1). The exchange of the N(3) and N(5) amide protons with bulk solvent is very slow ($\nu << 100$ Hz) in the reduced state either in the presence or in the absence of substrate. The flavin is bound in a more planar configuration than free in solution. Upon binding of substrate the resonances of N(1), C(10a) and N(10) shift sharply upfield. It is suggested that these upfield shifts are either a result of a shift of the pK of the N(1) atom to higher pH values or a result of a similar conformational change as found in the oxidized state.

INTRODUCTION

The enzyme p-hydroxybenzoate hydroxylase (pHBH) from Pseudomonas fluorescens is an external FAD containing monooxygenase catalyzing the hydroxylation of p-hydroxybenzoate (pHB) to 3,4-dihydroxybenzoate in the presence of molecular oxygen.

The reaction requires NADPH as an electron donor (Husain and Massey, 1979). It has been observed that the binding of substrate (pHB) to the oxidized enzyme causes large, characteristic spectral perturbations and fluorescence quenching (Visser et al., 1983). The reduction of the protein bound FAD by NADPH is accelerated enormously upon substrate binding (Hosokawa & Stainier, 1966). The crystal structure of the pHBH-pHB complex is known at 2.5 Å resolution (Wierenga et al., 1979). The primary sequence has also been determined (Weijer et al., 1982). Müller et al. (1979) showed that in solution the enzyme is present mainly as a dimer, and also as higher order structures.

The exact sequence of intermediates formed after the attack of molecular oxygen on the reduced pHBH-pHB complex is still unclear. It is generally accepted that the first intermediate observed is a C(4a)-hydroperoxide. However, because only absorbance data are available, its formation is not fully established yet. The possible structures of the other intermediates involved in the reaction are much more speculative (Wessiak et al., 1984; Schopfer et al., 1984, Visser, 1984).

The occurence of a C(4a)-hydroperoxide intermediate has been shown recently in the bacterial luciferase reaction (Vervoort et al., 1986b). In the latter enzyme also a C(4a) hydroxyflavin has been postulated as the emitter (Kurfürst et al. 1984). Interestingly both intermediates are also postulated to be present in the reaction catalyzed by pHBH and yet no light emission is ever observed under any circumstance. Certainly, some structural differences in the interaction between the flavin molecule and the apoprotein must be responsible for these different reaction mechanisms. It has been shown that nuclear magnetic resonance can yield information on the subtle differences in interaction between prosthetic group and apoenzyme (Moonen et al., 1984). Here we report on a ^{13}C, ^{15}N and ^{31}P NMR investigation of the interaction between FAD and the apoprotein of p-hydroxybenzoate hydroxylase.

MATERIALS AND METHODS

p-Hydroxybenzoate hydroxylase from Pseudomonas fluorescens was isolated and purified as described previously (Müller et al., 1979). Preparation of apoprotein and reconstitution was performed as described by Müller and Van Berkel (1982). The synthesis of flavin enriched in the isoalloxazine ring with the ^{13}C isotope was performed as described by Van Schagen and Müller (1980). The synthesis of flavin enriched in the isoalloxazine ring with the ^{15}N isotope was performed as described by Vervoort et al. (1986a). FAD was synthesized as described by Cramer and Neunhoeffer (1962), and purified by gradient elution on DEAE-Sepharose 6B.

Samples consisted of 0.6 mM pHBH in 50 mM potassium phosphate buffer (pH = 7.0) for ^{13}C NMR measurements and 0.6 mM pHBH in 50 mM Tris/Maleate (pH = 7.0) and 0.3 mM EDTA for ^{31}P NMR measurements. For ^{15}N NMR measurements 1 mM pHBH in 100 mM HEPES buffer containing 20% Me_2SO, 0.3 mM EDTA and 1 mM DTT, pH = 7.0 was used. Concentrations are expressed on FAD content. Wilmad 10 mm precision NMR tubes were used for ^{13}C NMR and ^{31}P NMR measurements and Wilmad 15 mm precision NMR tubes were used for ^{15}N NMR measurements.

The sample volume was 1.6 ml for ^{13}C NMR and ^{31}P NMR and 4.0 ml for ^{15}N NMR measurements. The samples contained 10% ^2H_2O. Broadband ^1H decoupling of 0.5 W was used for ^{13}C NMR and ^{31}P NMR measurements. No proton decoupling was applied for the ^{15}N NMR measurements. All spectra were recorded using 30° pulses and a repetition time of 1.0 s, except for ^{31}P NMR measurements where a repetition time of 1.1 s was used. The measurements were performed on a Bruker CXP 300 spectrometer operating at 30.4 MHz for ^{15}N NMR, at 75.6 MHz for ^{13}C NMR and at 120.5 MHz for ^{31}P NMR measurements. Dioxane (3 µl) served as an internal standard for ^{13}C NMR measurements. Chemical shift values are reported relative to Me_4Si. Neat [^{15}N] CH_3NO_2 was used as an external reference for ^{15}N NMR measurements. Chemical shift values are reported relative to liquid NH_3 at 25°C ($\delta_{CH_3NO_2} - \delta_{NH_3}$ = 381.9 ppm for the magnetic field parallel to the sample tube). Values are reported as true shieldings, i.e. corrected for bulk volume susceptibilities. For ^{31}P NMR measurements 85% H_3PO_4 was used as an external reference. The accuracy of the reported values is about 0.2 ppm for ^{13}C NMR, 0.4 ppm for ^{15}N NMR and 0.1 ppm for ^{31}P NMR measurements. The temperature of the samples was 20° for ^{13}C NMR measurements and 17°C for ^{15}N and ^{31}P NMR measurements.

Reduction of the sample was conducted by the addition of the desired amount of a dithionite solution to the anaerobic solution of para-hydroxybenzoate hydroxylase. Anaerobiosis was achieved by carefully flushing the NMR tube with argon for about 10 min. The NMR tube was sealed with a rubber cap (subaseal).

RESULTS AND DISCUSSION

^{31}P NMR

Figure 1 shows the ^{31}P NMR spectrum of pHBH. The pyrophosphate resonances of the protein-bound FAD molecule can be seen as broad lines at -9.0 ppm and at -9.6 ppm. Both resonances are upfield shifted compared to the

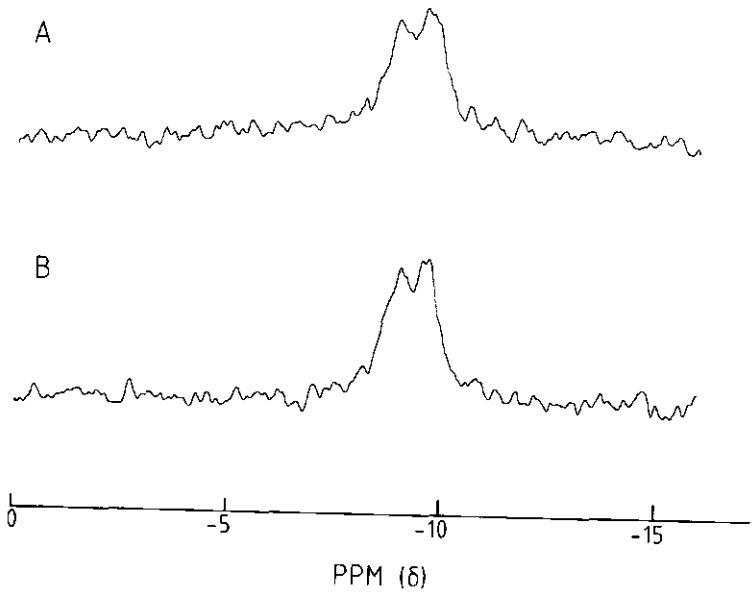

Figure 1: ^{31}P NMR spectra of para-hydroxybenzoate hydroxylase in the oxidized state in the presence (A) and absence of substrate (B).

ones found in free FAD in water i.e. at -9.9 ppm and at -10.55 ppm in free FAD. The resonance at the low field side of the pyrophosphate resonances of free FAD was assigned to that of the AMP moiety and the resonance at the high field side to that of the FMN moiety (Kainosho and Kyogoku, 1972). It

is unfortunately not possible to specifically assign the two resonances of the FAD bound to pHBH. Nevertheless, on comparison of the ^{31}P NMR spectrum of pHBH with those of e.g. glucose oxidase (James et al., 1981) or xanthine oxidase (Davis et al., 1984) one clearly notices large differences in the ^{31}P resonances of the pyrophosphate group of FAD. These differences in ^{31}P chemical shift must find their origins in differences in interaction between the apoprotein and the pyrophosphate group. It is known that ^{31}P NMR chemical shifts are very sensitive towards differences in the O-P-O bond angle (Gorenstein, 1975) and therefore it can be expected that small differences in interaction between the pyrophosphate group of FAD and the apoprotein lead to rather large differences in the ^{31}P NMR spectrum. Upon binding of substrate (Fig. 1B) the ^{31}P NMR spectrum is virtually the same. Therefore, although binding of substrate causes remarkable changes in the circular dichroism spectrum around 280 nm (Van Berkel, personal communication) and in the rate of reduction by NADPH, this conformational change is probably not extended to the pyrophosphate binding domain.

^{13}C and ^{15}N NMR. The oxidized state.

Figure 2 shows in a series of ^{13}C NMR spectra of pHBH, the dependence on the redox state, and on the absence or presence of substrate pHB. The resonances of the ^{13}C enriched carbon atoms C(2) and C(4a) are easily determined, either directly from the spectra for well resolved lines, or by using difference spectra spectra for overlapping lines. Similar experiments were performed with FAD enriched at the positions C(4) and C(10a) (data not shown). The data are collected in Table 1.

Figure 3 shows ^{15}N NMR spectra of pHBH in the oxidized state in the presence or absence of substrate. In the presence of the substrate, pHB, the N(1) and N(10) resonances are well resolved singlets (Fig. 3A). The N(3) and N(5) resonances, however, are not well resolved, and appear as broad lines with a linewidth of 110 Hz and 200 Hz respectively. This limits the accuracy of the determination of the chemical shifts (Table 1). In the

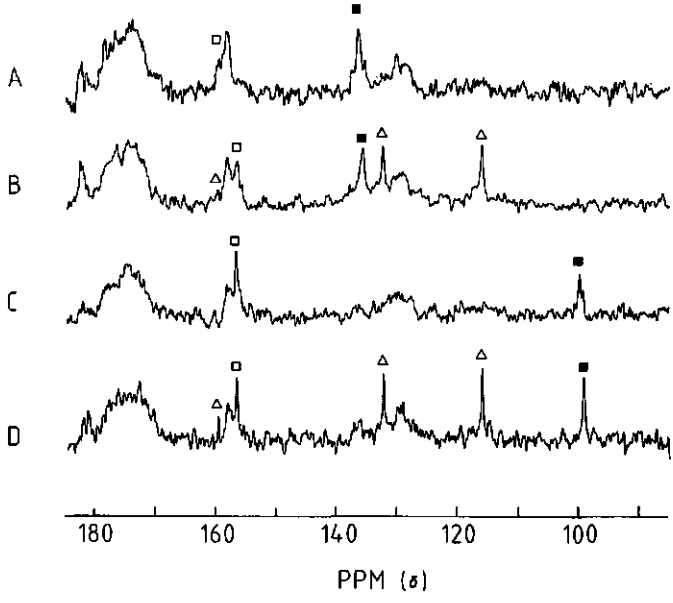

Figure 2: Low field part of the ^{13}C NMR spectra of parahydroxybenzoate hydroxylase recombined with [2,4a]$^{13}C_2$ FAD. A. Oxidized state. B. Oxidized state in the presence of 5 mM substrate. C. Reduced state. D. Reduced state in the presence of 5 mM substrate. Resonances marked with Δ represent natural abundance intensity of substrate. Resonances marked with □ and ■ represent the C(2) and C(4a) atom, respectively.

absence of substrate the signal to noise ratio of the spectrum (Fig. 3B) is less than when substrate is present (Fig. 3A), although the number of transients is doubled. The N(5) resonance is not resolved anymore, which may result from both a T_1 (spin-lattice relaxation) and T_2 (spin-spin relaxation) effect. The N(1) resonance has increased slightly in linewidth compared to Fig. 3A. The N(3) resonance is again very broad, linewidth 110 Hz. The resonances around 125 ppm arise from the natural abundance amide resonances of the protein. All ^{15}N chemical shifts are collected in Table 1.

Table 1. ^{13}C, ^{15}N and ^{31}P chemical shifts of parahydroxybenzoate hydroxylase in the oxidized or reduced state and in the absence (-) or presence (+) of substrate. The accuracy of the ^{13}C NMR data is ±0.2 ppm, of the ^{15}N NMR data ±0.3 ppm unless indicated otherwise and of the ^{31}P NMR data ±0.1 ppm.
^{13}C NMR spectra were measured in 50 mM potassium phosphate, pH = 7.0.
^{15}N NMR spectra were measured in 100 mM HEPES, Me_2SO (20% v/v), 0.3 mM EDTA, 1 mM DTT, pH = 7.
^{31}P NMR spectra were measured in 50 mM Tris-maleate + 3 mM EDTA, pH = 7.0

	Oxidized state				
Carbon atom	-substrate	+substrate	FMN		TARF
C(2)	159.5	156.6	159.8		155.2
C(4)	163.2	162.8	163.7		159.8
C(4a)	136.4	135.7	136.2		135.6
C(10a)	151.6	151.1	152.1		149.1
N(1)	191.6[a]	189.2[d]	190.9		199.9*
N(3)	159.8[b]	159.8[b]	160.4		159.8*
N(5)	?	348.0[e]	334.5		344.3*
N(10)	165.6[c]	165.6[c]	163.5		150.2*
	Reduced state				
	-substrate	+substrate	$FMNH^-$	$FMNH_2$	$TARFH_2$
C(2)	156.9	156.9	158.2	151.1	150.6
C(4)	157.6	158.1	157.7	158.3	157.0
C(4a)	99.9	99.3	101.4	102.8	105.2
C(10a)	155.6	153.3	155.5	144.0	137.0
N(1)	183.9	179.8	181.3	128.0	116.7*
N(3)	148.2[f]	149.0[f]	150.0	149.7	145.8*
N(5)	60.9[f]	61.5[f]	58.4	58.0	60.4*
N(10)	98.4	94.3	96.5	87.2	72.2*
	^{31}P chemical shifts				
	-substrate	+substrate			
	-9.0, -9.6	-9.0, -9.6			

*7-Methyl-10-tetraacetylribityl-isoalloxazine derivitive.

a) Linewidth ~ 50 Hz, precision of the chemical shift 0.5 ppm.
b) Linewidth ~110 Hz, precision of the chemical shift 1.0 ppm.
c) Linewidth ~ 25 Hz, precision of the chemical shift 0.3 ppm.
d) Linewidth ~ 30 Hz, precision of the chemical shift 0.4 ppm.
e) Linewidth ~200 Hz, precision of the chemical shift 2.0 ppm.
f) Linewidth ~120 Hz, precision of the chemical shift 0.5 ppm.

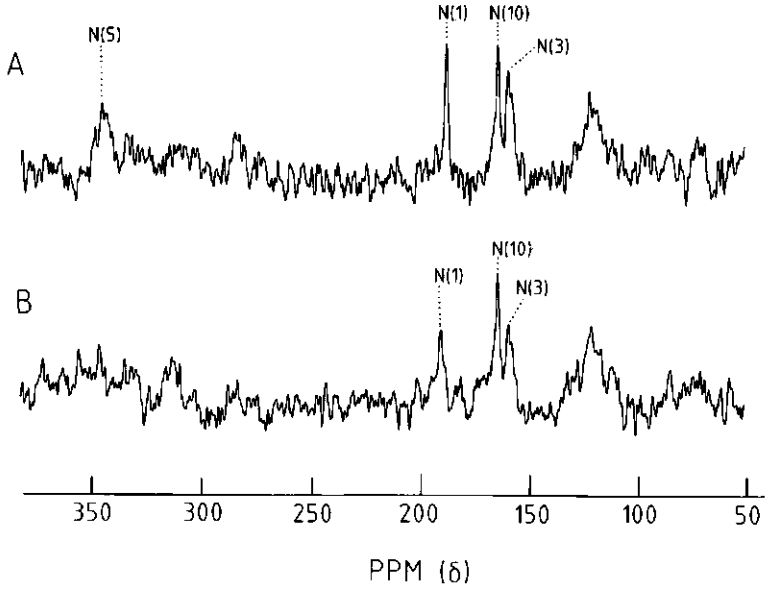

Figure 3: ^{15}N NMR spectra of para-hydroxybenzoate hydroxylase recombined with [1,3,5,10]$^{15}N_4$ FAD in the oxidized state. A, in the presence of 1.5 mM substrate; B, in the absence of substrate.

The ^{13}C and ^{15}N chemical shifts of the pHBH-bound FAD molecule are compared with those of free flavin in Table 1. The chemical shifts of free FMN are used because only the chemical shifts of the isoalloxazine ring are of interest. The results indicate that in the absence of substrate the isoalloxazine ring is polarized to about the same extent as in free flavin (FMN) in water. This suggests that the isoalloxazine ring, at least the pyrimidine part, is fairly exposed to solvent. The exposure to solvent would also be in agreement with the study of Claiborne et al. (1982). It is evident that in the absence of substrate the quality of the ^{15}N spectrum is rather low. This can partly be explained by the fact that the solubility of the protein is limited to about 700 μM. Müller et al. (1979) showed that the protein rather easily forms higher aggregates due to aspecific

hydrophobic association phenomena.

The N(10) resonance is downfield shifted by 2.1 ppm on binding of FAD to the protein. This downfield shift indicates that the N(10) atom has an increased sp^2 character, π electron donation to mainly O(2α) and O(4α) leads to the creation of a partial positive charge at N(10) (Moonen et al., 1984). The N(10) atom is fully sp^2 hybridized in the protein-bound FAD molecule.

Upon binding of the substrate pHB the solubility of the protein increases strongly. It is possible to achieve a concentration of 2 mM. The higher concentration used in the presence of substrate results in a better signal to noise ratio for the ^{15}N spectrum (Fig. 3A) compared to the spectrum where the substrate is absent (Fig. 3B).

The ^{13}C and ^{15}N chemical shifts show that several resonances shift upfield. The strongest upfield shifts are observed for the C(2) and the N(1) resonances. The C(2) resonates at 156.6 ppm which is 2.9 ppm upfield from the substrate free enzyme. This value of 156.6 ppm is very close to the value of TARF dissolved in chloroform (Table 1). This indicates that the π electron density at the C(2) carbonyl group is increased in the presence of substrate. The C(4) carbonyl group has a fairly strong hydrogen bond in the enzyme-substrate (ES) complex as indicated by the chemical shift. The C(4a) resonance is upfield shifted in the ES complex compared to the substrate free enzyme. In the substrate free enzyme it was already suggested that the isoalloxazine ring is fairly solvent accessible. As a result the N(5) would have a hydrogen bond, although its resonance is not resolved in the ^{15}N spectrum of the free enzyme. Upon binding of substrate the chemical shift of the N(5) resonance indicates that it forms no hydrogen bond with either water or the apoprotein (vide infra). This absence of a hydrogen bond would then lead to a small upfield shift of the C(4a) resonance, as observed in the ^{13}C NMR spectra.

The $^{15}N(5)$ chemical shift is downfield shifted from the one found in free flavin in chloroform (TARF). From this it can be concluded that the N(5) atom has no hydrogen bond and even that the polarity of the microenvironment is very low. It is evident from the spectrum in Fig. 3A that the N(5) resonance is very broad (linewidth 200 Hz). This broad line is probably caused by some dynamic process, e.g. a limited mobility of the substrate. The N(3) resonance is also very broad. A possible explanation

would be that it is split up into a non-resolved doublet due to the $^{1}J[^{15}N(3)-^{1}H]$ coupling. This splitting could exist if the proton exchange is very slow with bulk solvent (ν<<100 Hz). The chemical shift of N(3) and also of the N(10) do not change when substrate is bound and hence no change in π electron density occurs. In contrast, the N(1) is shifted by 2.4 ppm upfield which could be the result of a stronger hydrogen bond in the enzyme-substrate complex.

Hitherto cyrstallographic data are only available of the enzyme-substrate complex (Wierenga et al., 1979). It has been suggested from these data that hydrogen bonds exist to the N(1), O(2α), N(3) and O(4α) atoms of the isoalloxazine ring. From the ^{13}C and ^{15}N NMR data we indeed observe hydrogen bonds to these atoms. One might conclude that the C(2) carbonyl group has no or a very weak hydrogen bond in the ES complex as concluded from ^{13}C NMR data. However, from the crystallographic data it was shown that an α-helix points with its positive pole directly to N(1) in the enzyme-structure complex (Wierenga et al., 1979). This could explain the upfield shifts observed for the N(1) and for the C(2) and C(10a) atoms because these atoms can accommodate then a higher π electron density. The relative position of the α-helix must then be different in the latter case from the substrate free enzyme as suggested previously on the basis of ^{13}C data only (Moonen, 1983). Overall, it can be concluded that upon binding of the substrate the interaction between the prosthetic group and the apoprotein is affected. This is in agreement with changes observed in absorption and fluorescence spectra (Husain and Massey, 1979; Visser, 1983).
Upon binding of an effector, parafluorobenzoate (K_D = 150 μM, pH = 7), nearly the same chemical shifts are found within the accuracy of the measurements as when parahydroxybenzoate is bound (Table 2). This indicates that in the parafluorobenzoate complexed enzyme comparable conformational changes must occur.

Table 2: ^{13}C chemical shift of parahydroxybenzoate hydroxylase in the oxidized and in the reduced state in the presence of an effector, parafluorobenzoate [2.4 mM]. The buffer used was 50 mM potassium phosphate 0.3 mM EDTA, 1 mM DTT, pH = 7.0.

	Oxidized state
C(2)	156.8
C(4)	163.0
C(4a)	136.0
C(10a)	151.2

	Reduced state
C(2)	157.0
C(4)	158.3
C(4a)	99.6
C(10a)	153.6

The reduced state

Figure 4 shows ^{15}N NMR spectra of pHBH in the presence (Figure 4A) and in the absence (Figure 4B) of substrate. The N(1) and N(10) resonances can be seen as sharp singlets in both spectra. In contrast the N(3) and N(5) resonances can be seen as broad lines, perhaps even doublets, both in presence and absence of substrate. The ^{15}N chemical shifts are collected in Table 2. The ^{13}C and ^{15}N NMR chemical shifts clearly demonstrate that the reduced FAD is present in the anionic form ($FADH^-$). In the absence of substrate the C(10a) and the C(4) resonances are the same as the ones found in $FMNH^-$, whereas the C(2) and C(4a) resonances are shifted upfield by 1.3 and 1.5 ppm respectively. The upfield shift of the C(2) indicates that the hydrogen bond towards the carbonyl group is weaker than found in $FMNH^-$ in

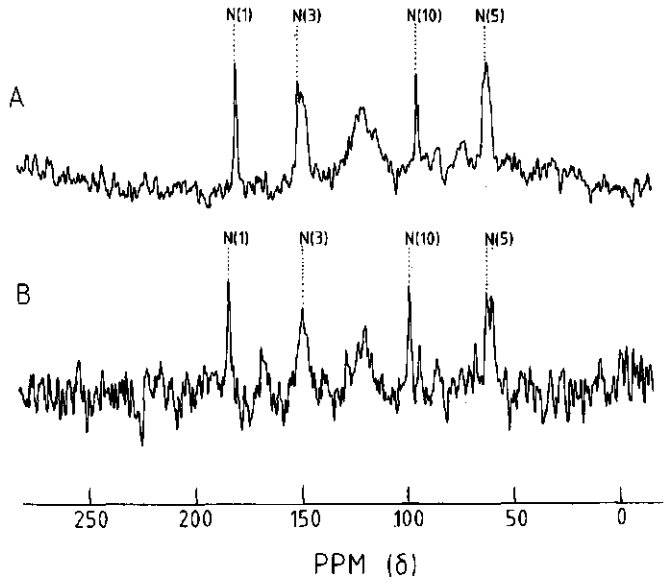

Figure 4: ^{15}N NMR spectra of para-hydroxybenzoate hydroxylase recombined with [1,3,5,10]$^{15}N_4$-FAD in the reduced state. A, in the presence of 1.5 mM substrate; B, in the absence of substrate.

water. The hydrogen bond towards the C(4) carbonyl group is a strong one as judged from the ^{13}C chemical shift. The rather weak hydrogen bond towards the O(2α) and the strong one towards the O(4α) indicate that the π-electron donation from the N(10) atom occurs mainly towards the C(4) carbonyl group (Moonen et al., 1984). The N(10) is a strong π electron donor in the protein-bound FAD because its chemical shift indicates an increased sp^2 character compared to free flavin (Moonen et al., 1984). The upfield shift observed for the C(4a) is also due to π electron donation from the N(10).

The ^{15}N chemical shifts clearly show that the protein-bound flavin has negative charge at the N(1) atom. This negative charge is very likely related to the oxygen reactivity of the reduced flavin. The anionic reduced flavin reacts much more rapidly with molecular oxygen than the neutral reduced flavin (Bruice et al., 1982). The flavin is more planar when bound to the protein than free in solution as judged from the N(10) and N(5) che-

mical shifts. Both nitrogens shift downfield on binding to the protein which can be related to the increased sp^2 character of both nitrogens (Moonen et al., 1984). The very broad resonances of the N(3) and perhaps even splitting of N(5) due to the $^1J[^{15}N(5) - {}^1H]$ coupling indicate that the amide protons at these groups do not exchange with water on the NMR timescale i.e. $\nu << 100$ Hz. This indicates that the reduced anionic flavin in substrate-free pHBH is not accessible to bulk water, more or less in contrast to the oxidized state.

Upon binding of the substrate (pHB) to the reduced enzyme resonances shift to a different extent, showing the specific influence of the substrate binding to reduced flavin. The C(10a) resonances shifts 2.3 ppm upfield. Also the N(1) and N(10) ^{15}N resonances shift upfield, both by 4.1 ppm. There are two possible explanations for these upfield shifts. First, binding of the substrate might cause a pK shift of the N(1) atom of the isoalloxazine ring system. All measurements were performed at pH = 7.0. The N(1) in free reduced flavin deprotonates with a pK of 6.6 (Dudley et al., 1964; Franken et al., 1983). A possible other explanation is that the binding of substrate causes a conformational change of the position of the α-helix dipole, as was postulated to exist in the oxidized state. The position of the α-helix is probably slightly different in the ES complex in the reduced state compared to the ES complex in the oxidized state. In the oxidized state the main effects are observed for the C(2) and N(1) atoms, while in the reduced state the N(1), C(10a) and N(10) are mainly affected. The small upfield shift observed for the C(4a) can also be explained by the presence of a positive pole of the α-helix. Further experiments will have to show which explanation is the most likely one.

The slight downfield shift observed for the C(4) atom indicates that the hydrogen bond towards the C(4) carbonyl group increases even in strength in the ES complex compared to the free enzyme. The N(5) chemical shift is slightly downfield in the ES complex compared to the substrate free enzyme. This points towards an increased sp^2 character of the N(5) (Moonen et al., 1984). The addition of an effector, para-fluorobenzoate, leads to the same shifts in the ES complex as compared with substrate, pHB. Therefore we can conclude that this effector is bound in the same way as the substrate is and that it has the same electronic effects on the flavin ring. It also can be expected that pHB is bound in the mono-anionic form as is para-fluorobenzoate.

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Chapter 8. MERCURIC REDUCTASE. A ^{13}C AND ^{31}P NMR INVESTIGATION

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Abstract

The interaction between FAD and the apoprotein of mercuric reductase from Pseudomonas aeruginosa was investigated using selectively ^{13}C enriched FAD. The reconstituted enzyme was studied in the oxidized (E), in the two-electron reduced (EH_2) and in the four-electron reduced (EH_4) state. The results are interpreted in terms of specific interactions between the apoprotein and the prosthetic group and the chemical structure of protein-bound FAD.

In the oxidized state the interactions between FAD and apoprotein are not much different from those found in free flavin in water. A downfield shift of the C(4a) resonance compared to free flavin indicates the absence of a hydrogen bond to N(5) in the protein.

The protein consists of an oxidized flavin and a reduced disulfide in the two-electron reduced state. The thiolate anion is located in the immediate neighbourhood of the C(4a) atom.

Binding of NADPH to the two-electron reduced enzyme leads to an increase of π electron density of the measured ^{13}C resonances, perhaps indicating charge transfer from the bound NADPH to the flavin molecule.

On four electron reduction the protein-bound flavin becomes anionic. The ^{13}C shifts indicate a bent flavin at the N(5) position. The results are discussed in relation to the published data of two related proteins, lipoamide dehydrogenase and glutathione reductase.

Introduction

Mercuric reductase (reduced NADP: mercuric ion oxidoreductase) is an indispensable enzyme in many bacteria in the reduction of toxic mercury ions to the much less toxic elemental mercury [1]. Mercuric reductase is a dimeric enzyme (M_r = 116000) and contains two active site electron acceptors (per subunit) in the oxidized state, i.e. FAD and a redox active disulfide [2]. In this respect it strongly resembles other FAD containing disulfide reductases. As pointed out by Fox and Walsh [2], especially glutathione reductase and lipoamide dehydrogenase show a strong resemblance to mercuric reductase.

A comparative NMR study was performed on the latter two enzymes using ^{13}C enriched FAD [3]. NMR is an excellent tool to study the interaction between FAD and apoprotein. Small differences in the microenvironment of the bound FAD reveal itself by different chemical shifts. The NMR study performed on glutathione reductase and lipoamide dehydrogenase showed that there are structural differences in the active site organization. These subtle differences are very likely responsible for the different catalytic mechanism of both enzymes. In this paper we will discuss the results obtained on mercuric reductase in relation to the two other enzymes mentioned above.

Materials and Methods

Mercuric reductase from Pseudomonas aeruginosa PAO95 ϕ1 (pVS1) was purified as previously described [4]. Preparation of the apoprotein was performed as follows. Purified enzyme (160 mg) was loaded on a phenyl-Sepharose column (Pharmacia) using a 50 mM potassium phosphate buffer which contained 30% ammonium sulphate, 1 M KBr and 1 mM EDTA (pH 6.5). Subsequently, FAD was removed from the protein by washing the column with 20 ml of 50 mM potassium phosphate buffer, containing 30% ammonium sulphate, 1 M KBr and 1 mM EDTA (pH 3.25). Then the column was washed with 15 ml of the same mixture omitting KBr. Recombination with ^{13}C labelled 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 (pH 6.5). The column was then washed with the same mixture, from which FAD was omitted.

The reconstituted protein was eluted from the column with a mixture of 10 mM potassium phosphate containing 1 mM EDTA and 50% ethylene-glycol (pH 6.5). The specific activity of the reconstituted protein was identical with that of the starting material. The reconstituted protein was concentrated by ultrafiltration and put onto a Biogel P6DG column to obtain the protein in the desired buffer. ^{13}C and ^{31}P NMR measurements were performed on a Bruker CXP300 spectrometer operating at 75.6 MHz for ^{13}C NMR and at 121.0 MHz for ^{31}P NMR measurements. Wilmad 10 mm precision NMR tubes were used. The samples of 1.6 ml solution contained 0.75 mM protein. 10% $^{2}H_2O$ was added to lock the spectrometer. Dioxane (3 µl) served as an internal standard for ^{13}C measurements. Phosphoric acid (85%) served as an external standard for ^{31}P measurements. 35^{o} pulses were used with a repetition time of 1s. The temperature of the samples was $25^{o}\pm2^{o}C$. Anaerobiosis was achieved by carefully flushing the sample with argon. Reduction of the samples was conducted by the addition of the desired amount of dithionite solution or of a borohydride solution to the anaerobic sample.

RESULTS AND DISCUSSION

It has been pointed out already by Fox and Walsh [2,5] that mercuric reductase belongs to the class of flavoproteins in which both flavin and oxido-reductive active disulfide groups are constituents of the active site. These flavoproteins show a long-wavelength absorbance band centered around 530 nm on two-electron reduction. The protein turns from yellow to red. This long wavelength absorbance band was interpreted as resulting from a charge transfer interaction between oxidized flavin and a reduced disulfide [6].

^{31}P NMR

Fig. 1 shows ^{31}P NMR spectra of mercuric reductase. Interestingly, three ^{31}P NMR resonances are seen in the region around -12 ppm i.e. at -10.5 ppm, -12.1 ppm and at -12.9 ppm. The peak at -10.5 ppm is the least intense. The spectra of the native and the reconstituted enzyme are identical. This indicates and it is also supported by CD spectra (unpublished results) and activity measurements, that the structure of both proteins is

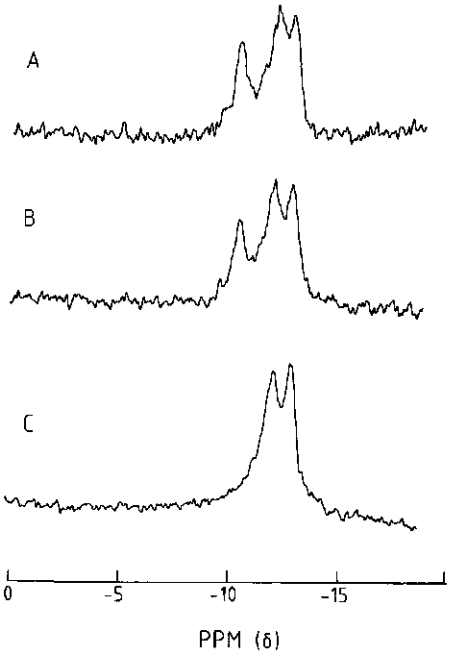

Figure 1. ^{31}P NMR of mercuric reductase in 20 mM HEPES pH = 7.5 + 100 mM potassium chloride.
A. Native unrecombined mercuric recuctase (0.5 mM), 407000 transients
B. Mercuric reductase recombined with C(4) and C(10a) ^{13}C enriched FAD (0.8 mM), 17700 transients.
C. Mn^{2+} (final conc. 200 μM) added to spectrum B, 44360 transients.

the same (Fig. 1A and 1B). It also is clear from these data that mercuric reductase contains another covalent attached phosphorus residue besides the two phosphate groups of the pyrophosphate moiety of FAD. This resonance line cannot be attributed to $NADP^+$ because of the absence of the ^{31}P NMR resonance of the 2' phosphate group of $NADP^+$. This phosphorus residue must be covalently bound to the protein because it does not disappear on preparation of the apoprotein and it does not disappear after repeated gelfiltration. So far no covalently bound phosphorus residue with a reso-

nance around -11 ppm has been observed in flavoproteins. The question whether it is important for catalysis remains open.

Adding Mn^{2+} to the protein solution causes the resonance at -10.5 ppm to broaden beyond detection (Fig. 1C). Mn^{2+} with a rather long electron spin relaxation time (1 ns) causes phosphorus resonances which are accessible for solvent to broaden due to dipolar coupling to the ^{31}P centers. Consequently the phosphorus residue at -10.5 ppm is exposed to solvent.

We conclude from these results that the residue at -10.5 ppm is the covalently bound phosphorus residue and that the two other resonances arise from the pyrophosphate group of bound FAD. These resonances are both shifted upfield by about 2 ppm compared to free FAD. Small changes in the P-O bond angles could give rise to these differences in chemical shift. Unfortunately it is not possible yet to assign the two resonances to the phosphate group of the FMN moiety and to that of the AMP moiety of FAD.

^{13}C NMR

Figure 2 shows a series of ^{13}C NMR spectra of different redox states of mercuric reductase, with C(2) and C(4a) ^{13}C enriched FAD. It is evident that on two-electron reduction (EH_2) the C(4a) resonance shifts downfield and that the C(2) resonance does not change much. On four electron reduction (EH_4) the C(4a) resonance shifts upfield by more than 40 ppm to 98.9 ppm. Figure 3 shows a series of ^{13}C NMR spectra of mercuric reductase with C(4) and C(10a) ^{13}C enriched FAD and the dependence on the redox states. No large changes can be seen, only in the four electron reduced state (EH_4) slight shifts are observed compared to the two other states. The C(4) resonance shifts upfield and that of the C(10a) shifts downfield. These shifts are more evident in the difference spectrum in Fig. 3D.

Table 1 shows the chemical shift in independence of the four carbon atoms on the redox state. For comparison also the ^{13}C chemical shifts of lipoamide dehydrogenase, glutathione reductase and free flavin (FMN) in water are given. The chemical shifts of FMN are used because the redox-active group, the isoalloxazine ring, is identical in FAD and FMN. In the oxidized state the chemical shifts of both C(2) and C(4) are not much different from FMN, which indicate that these carbonyl groups are polarized to

Table 1. ^{13}C chemical shift of the carbon atoms C(2), C(4), C(4a) and C(10a) of FAD bound to mercuric reductase, lipoamide dehydrogenase and glutathione reductase in the oxidized (E), two-electron reduced (EH_2) and four-electron reduced state (EH_4). For comparison the chemical shifts of FMN in water are added. The accuracy of the reported chemical shifts is ±0.1 ppm.

Atom	Oxidized (E)			
	Mer. red.	Glut. red.	Lip dH	FMN
C(2)	159.6	160.4	158.3	159.8
C(4)	163.6	163.3	163.2	163.7
C(4a)	137.5	135.7	137.6	136.2
C(10a)	152.5	152.3	152.2	152.1
		Two-electron reduced (EH_2)		
C(2)	160.1	161.6	158.6	159.8
C(4)	164.5	163.9	163.8	163.7
C(4a)	140.7	139.9	140.3	136.2
C(10a)	153.8	153.8	153.0	152.1
		Four-electron reduced (EH_4)		
	Mer. red.	Glut. red.	Lip dH	$FMNH^-$
C(2)	160.1	161.0	159.4	158.2
C(4)	161.8	162.0	159.7	157.7
C(4a)	98.9	99.7	98.7	101.4
C(10a)	158.6	159.1	157.2	155.5

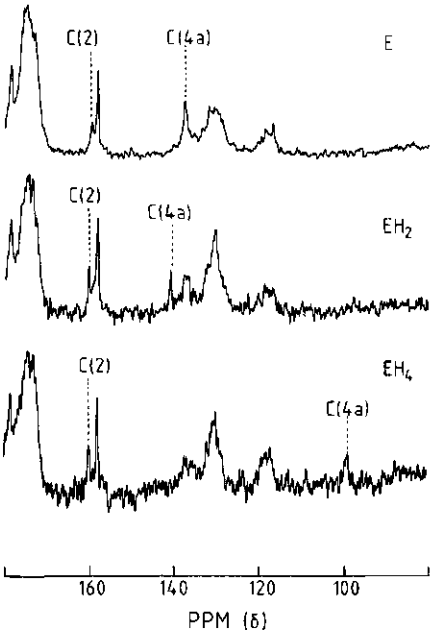

Figure 2. ^{13}C spectra of mercuric reductase with C(2) and C(4a) ^{13}C enriched FAD in 100 mM potassium phosphate pH = 7.0 + 0.3 mM EDTA in the three oxidation states (0.8 mM). A, 19000 transients; B, 8640 transients; C, 7000 transients.

the the same extent as FMN water (7). Studies of hetero-aromatic compounds show that deprotonation of a pyrrole-type nitrogen shifts the resonance of the atom in the α-position downfield and protonation of a pyridine-type nitrogen shifts the resonance upfield [8,9]. Thus, a downfield shifted C(4a) indicates that either N(10) is a weak electron donor because it is out of the molecular plane or that there is no hydrogen bond to N(5) giving rise to a small downfield shift for C(4a) (7). The C(10a) is shifted slightly downfield compared to free flavin probably as a result of a weak hydrogen bond to N(1). Overall, it can be concluded from the ^{13}C NMR results that the isoalloxazine is strongly polarized.

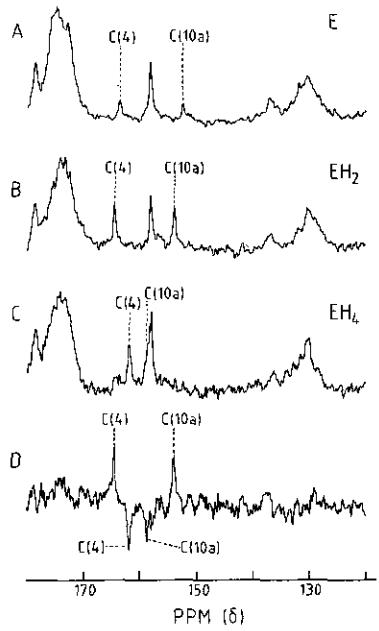

Figure 3. ^{13}C NMR spectra of mercuric reductase with C(4) and C(10a). ^{13}C enriched FAd in 100 mM potassium phosphate pH = 7.0. + .3 mM EDTA in the three oxidation states (0.8 mM).
A: oxidized state, 16300 transients.
B: two-electron reduced state, 17850 transients.
C: four-electron reduced state, 11290 transients.
D: difference spectrum between two-electron reduced (upper) and four-electron reduced (bottom) enzyme.

In the two electron reduced state all four resonances shift downfield. The resonance of C(4a) shifts most, followed by the C(10a) and C(4). The C(2) shifts only by 0.5 ppm. These results demonstrate that in the two-electron reduced state the flavin is still oxidized. The downfield shifts are surprising because we would have expected upfield shifts as the result of a charge transfer complex existing between oxidized flavin and a thiolate anion. An increase in charge density in the prosthetic group should lead to an upfield shift of the resonances. This is clearly not

observed. The explanation for this phenomenon is that the negative charge of the thiolate group induces these downfield shifts. The electric field effect dominates the charge transfer interaction by far. The thiol anion must be very close to the C(4a) because this atom shifts most. The negative charge is also closer to the C(10a) than to the C(4), all in agreement with the crystal data of the related protein glutathione reducase (10).

In the EH_4 the (C4a) resonances shifts by more than 40 ppm upfield. The other three resonances shift less, the C(10a) 4.8 ppm downfield and the C(4) 2.7 ppm upfield. The C(2) does not change. The shifts resemble those of $FMNH^-$ (7), so apparently the flavin is reduced and carries a negative charge at N(1). The negative charge on the N(1) atom has been found in all flavoproteins sofar investigated, but ^{15}N measurements will have to be made for final confirmation. Whether the thiol group still exists in its anionic form is not certain. The chemical shifts do not really support this idea. The downfield shift of the C(10a) atom relative to free reduced anionic flavin is the result of the increased sp^3 hybridization of the N(5) atom, decreasing its π electron donating character (7).
The upfield shift of the C(4a) resonance indicates a high π electorn density at this atom. This high π electron density may be explained by a combination of effects: (a) the very strong deshielding of the C(4) and C(2) at the ortho and para position to C(4a); (b) the more sp^3-hybridized character of N(5) and (c) the probably increased sp^2 hybridization of the N(10) atom.

If we compare the results obtained on mercuric reductase with those of lipoamide dehydrogenase and glutathione reductase (3), we see that the overall picture looks the same. But, there remain marked differences among these three enzymes. Overall, mercuric reductase and glutathione reductase resemble each other more in the three redox states than lipoamide dehydrogenase. A small exception is the C(4a) resonance in the oxidized state. The polarization of the isoalloxazine ring system in lipoamide dehydrogenase seems to be less in the three redox states than in the other two enzymes. This might be due to a higher hydrophobicity of the active site in lipoamide dehydrogenase.

Interesting results are obtained by adding $NADP^+$ to the two electron reduced state at 25°C. Fig. 4A shows the spectrum of EH_2 in the absence of $NADP^+$. Fig. 4B is obtained on adding $NADP^+$ in a 1:1 ratio to the sample of

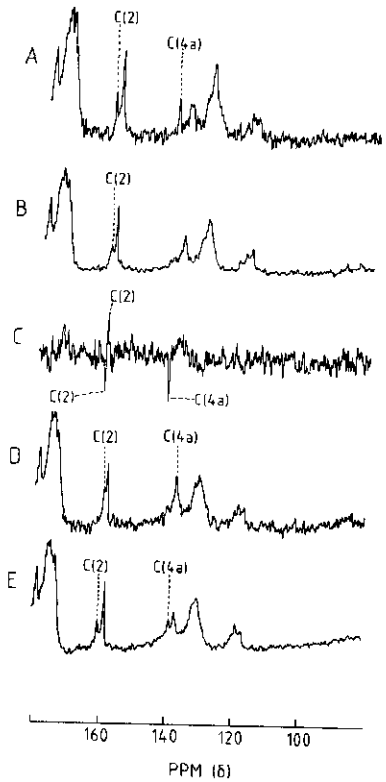

Figure 4. ^{13}C NMR spectra of mercuric reductase with C(2) and C(4a) ^{13}C enriched FAD.
A: two-electron reduced enzyme (0.8 mM), 8640 transients.
B: two-electron reduced enzyme (0.8 mM) + $NADP^+$ (1.6 mM), 60000 transients.
C: difference spectrum between B (upper) and A (bottom).
D: oxidized enzyme (0.8 mM) + $NADP^+$ (1.6 mM), 7300 transients.
E: two-electron reduced enzyme (0.8 mM) + $NADP^+$ (1.6 mM) + DTT (7 mM), 33800 transients.

Fig. 4A. The colour of the solution changes from red to brown. It is known from absorbance spectra that the long-wavelength band is shifted by about 40 nm to the red (11). In the difference spectrum (Fig. 4C) between the two spectra it can be seen that the C(4a) resonance of the EH_2-$NADP^+$ complex is broadened beyond detection.

The broadening process for this C(4a) resonance could be an exchange of the protein-bound $NADP^+$ (or of only the nicotinamide part of the $NADP^+$ molecule). It depends on the differences between the two resonance positions (with and without $NADP^+$) and on the exchange rate if one intermediate resonance, a very broad resonance or two separate resonances are observed. If the broadening process results from the exchange reaction then the rate has to be between 100-1000 Hz.

Another possibility is that the electrons are going back and forth between the disulfide and either flavin and/or $NADP^+$. The same rules can be applied for the broadening process. If the electrons are only going back and forth between the disulfide and flavin then the exchange rate needs to be greater than 1000 Hz. If the electrons are going back and forth between the disulfide and $NADP^+$ then the rate will be between 100 and 1000 Hz. It is impossible to tell from these data which mechanism is responsible for the broadening process.

The interaction between the two-electron reduced enzyme and $NADP^+$ was also studied at 4°C. It can be expected that exchange processes are much slower at 4°C compared to 25°C (11). The C(2) resonance is not clearly visible in Fig. 4B, and is probably split up into a doublet, indicating that the electron exchange is below 100 Hz. The (4a) resonance perhaps also is split up into a doublet with resonances at 140 ppm and at 136 ppm, but the quality of the data is not enough to make a strong statement about this. Taking the results of the latter experiment into account, the explanation we favour is an electron shuttle between the reduced disulfide and the $NADP^+$ molecule. This would also be in agreement with kinetic data (12). In contrast to glutathione reductase and lipoamide dehydrogenase, $NADP^+$ remains bound in the oxidized state. As is shown in Fig. 4D and Table 2 the resonances are slightly different from the oxidized state without $NADP^+$. These differences could reflect changes in the hydrophobicity of the active site when $NADP^+$ is bound.

Table 2. ^{13}C chemical shifts of C(2), C(4), C(4a) and C(10a) of FAD bound to mercuric reductase in the oxidized (E) and two electron reduced state (EH_2) with and without bound pyridine nucleotide.

	Oxidized (E)			
			C(2)	C(4a)
Mercuric reductase			159.6	137.5
Mercuric reductase + $NADP^+$			159.0	137.0
	Two-electron reduced (EH_2)			
	C(2)	C(4)	C(4a)	C(10a)
Mercuric reductase	160.1	164.5	140.7	153.8
Mercuric reductase + NADPH	159.9	163.9	138.3	153.7

The addition of NADPH to the two electron reduced state gives upfield shifts for all resonances (Table 2) compared to the EH_2 state. Fig. 4E shows the spectrum otained by adding DTT to the oxidized enzyme with $NADP^+$ bound. The chemical shifts are the same as when NADPH is added to the EH_2 state. Therefore we conclude that $NADP^+$ is being reduced by the enzyme. The redox couple of oxidized DTT-reduced DTT is more negative than that of the $NADP^+$-NADPH couple. In the *in vivo* and also in the *in vitro* reactions electrons are being translocated from NADPH to the oxidized substrate. Here we observe the reverse pathway.

Comparing the chemical shift of the EH_2-NADPH complex with EH_2 it can be seen that C(4a) shifts 2.4 ppm upfield and that the other resonances shift less than 1 ppm. These effects resemble very much the results obtained on the charge transfer complex of old yellow enzyme (1). Charge transfer complexes between pyridine nucleotides and oxidized flavin have been suggested (1). Perhaps we are dealing also in mercuric reductase with a charge transfer complex between oxidized flavin and reduced NADPH. An alternative explanation could be that on addition of NADPH to the two-

electron reduced enzyme the charge transfer interaction between the thiolate anion and the oxidized isoalloxazine ring is stronger and consequently leads to an upfield shift of the C(4a). In old yellow enzyme the charge is specifically allocated to the N(5)-C(4a) bond in the charge transfer complex. Because ^{15}N results are not yet known, it is not possible to tell if similar effects occur in mercuric reductase.

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Chapter 9. ^{17}O NMR on free flavin

Introduction

Oxygen is undoubtedly one of the most important atoms in biological systems and is often involved in hydrogen bond formation. In the isoalloxazine ring of flavin coenzymes two oxygen atoms are found. Moonen et al. (1984) showed that these two oxygen atoms are very important in the formation and stabilization of mesomeric structures, found in both the oxidized and reduced state. On polarization of the isoalloxazine ring electron density is reallocated from e.g. the N(10) atom towards the C(2) and C(4) carbonyl groups.

One of the most powerful methods to study the molecular and submolecular structure of a compound is Nuclear Magnetic Resonance (NMR). Four out of the five different nuclei present in the flavin molecule can easily be studied by NMR, i.e. ^{1}H, ^{13}C, ^{15}N and ^{31}P (Grande et al., 1977; Moonen and Müller, 1982; Moonen et al., 1984). All four isotopes have a nuclear spin of ½. The fifth atom, oxygen, i.e. ^{17}O, can also be measured by NMR, although there are several disadvantages. The major two are: a low NMR sensitivity, natural abundance is only 0.037% and ^{17}O possesses a nuclear quadrupole moment with a nuclear spin I=5/2. The problem of the low natural abundance content can be overcome by isotopic enrichment. The problem of the quadrupole moment remains. As in all nuclei, except ^{1}H, the screening constant is the sum of a paramagnetic term $\sigma(p)$ and a diamagnetic term $\sigma(d)$ i.e. $\sigma = \sigma(p) + \sigma(d)$. The relative contribution of Lamb's term $\sigma(d)$, is relatively small for the total screening constant and is usually neglected for ^{17}O (Kidd, 1967).

It was shown by Delseth et al. (1980) that in unsaturated carbonyl compounds an increase of the charge density at the oxygen atom implies an expansion of the 2p orbitals which leads to a reduction of $\sigma(p)$ and therefore to an ^{17}O shielding. In contrast electron enrichment in the oxygen atom in aliphatic ethers is accompanied by a downfield shift of the ^{17}O resonance (Beraldin et al., 1982). Another important factor besides electron density is hydrogen bond formation. Schwartz et al. (1980) showed that hydrogen bond formation towards a carbonyl group leads to an upfield shift of 30-50 ppm. Here we report on some preliminary data obtained on flavins enriched with ^{17}O (50%) at both carbonyl groups in the isoalloxazine ring.

Materials and Methods

Synthesis ^{17}O enriched flavins

[2α-^{17}O]Riboflavin: 2-Thioriboflavin (a generous gift from Dr. S. Ghisla, Konstanz, FRG) was transformed to 2-thiomethylriboflavin according to the procedure of Müller and Hemmerich (1966). 250 mg of 2-thiomethylriboflavin was suspended in 1.5 ml $H_2{}^{17}O$ (50 atom %, Amersham, U.K.), brought to 2 N HCl by gaseous HCl, and kept at 70°C until the hydrolysis to riboflavin was completed (several days, followed by thin layer chromatography). Then the mixture was evaporated to dryness under vacuum at 40°C yielding 190 mg crude product. It was partially purified by dissolving it in hot pyridine and filtering the mixture. The residue weighted 90 mg which was transformed into the tetraacetylriboflavin derivative (Müller, 1971) and purified by gel chromatography (Grande et al., 1977). To the pyridine solution ether was added allowed to stand at 4°C overnight and the precipitate collected by filtration and washed by ether, yielding 110 mg of quite pure riboflavin. This was phosphorylated by the method of Plexser and Farkas (1953).

[2α,4α-$^{17}O_2$]Riboflavin: N(5)-Acetyl-1,5-dihydro-tetraacetylriboflavin was prepared from 2,5 g tetraacetylriboflavin virtually according to the procedure of Dudley and Hemmerich (1967) for the preparation of the lumiflavin analogue using a mixture of conc.acetic acid, acetic acid anhydride and Zn powder. After completion of the reaction the solvent was evaporated under reduced pressure at 40°C. To the residue 20 ml H_2O was added and let stand overnight at 4°C to hydrolyze residual acetic acid anhydride. Then the aqueous solution was extracted two times with 50 ml $CHCl_3$, the organic phase dried over Na_2SO_4 and evaporated to dryness yielding 2.5 g N(5)-acetyl-1,5-dihydrotetraacetylriboflavin. This was alkylated in DMF, using diethylsulphate and K_2CO_3 (Dudley and Hemmerich,1967) at 70°C. After 3½ h the reaction was completed as judged by thin layer chromatography (precoated thin layer plates, Merck, Darmstadt, FRG) using acetonitrile as a mobile phase. Two isomers are formed, i.e. O(2α), O(4α)-diethyl and O(2α), N(3)-diethyl-N(5)-acetyl-1,5-dihydrotetraacetylriboflavin. The reaction mixture was then filtered and the solvent evaporated under vacuum at 40°C. To the residue 50 ml H_2O was added and the suspension allowed to stand overnight at 4°C. The precipitate was collected by filtration, washed with water and air-dried, yielding 3.0 g of crude product. The two isomers

were separated by gel chromatogaphy (Grande et al., 1977). The isomer mixture was dissolved in 5 ml $CHCl_3$ and applied to the column. Then the column was washed with CH_2CL_2 eluting most of the O(2α),O(4α)-diethyl isomer. Further washing of the column with the solvent mixture $CH_2Cl_2/CHCl_3$= 1:1 eluted the residual amount of the isomer. Evaporation of the solvent yielded 1.6 g of the pure O(2α),O(4α)-diethyl isomer as a viscous oil, which we were not able to crystallize. The other isomer was eluted using $CHCl_3$ containing 2% methanol. After evaporation of the solvent 1.2 g of pure compound was obtained as a viscous oil. Attempts to crystallize the compounds also failed.

Both compounds were transformed to the corresponding flavinium salts: 0.8 g of the O(2α), N(3)-diethylisomer was dissolved in 25 ml methanol and 25 ml 2 N HCl_4 added. Solid $NaNO_2$ was added in portion under vigorous stirring until the red intermediate colour (flavosemiquinone) had disappeared. When the reaction was completed the reaction mixture diluted with H_2O to 100 ml, extracted two times with $CHCl_3$, the organic phase dried over Na_2SO_4, and the solvent evaporated. Dissolving the residue in ethylacetate and evaporating the solvent, and repeating this procedure several times led to crystals in the round bottom flask. The yield was 0.8 g of light brown crystals.

In the same way 1.2 g of the O(2α), O(4α)-diethyl isomer was dissolved in 10 ml methanol to which 20 ml 2 N $HClO_4$ was added. The other steps were identical with those just described above. The yield was 1.2 g of dark brown crystals.

400 mg of the O(2α), O(4α) flavinium salt was suspended in 1.5 ml $[^{17}]H_2O$ containing 2N HCl. The suspension was kept at 60°C for 2 days and occasionally shaken. The solution was then ten-fold diluted with H₂O, neutralized with solid $NaHCO_3$ and let stand overnight at 4°C. The crystals formed were collected by filtration and air dried. This yielded 160 mg of [2α,4α-$^{17}O_2$]riboflavin. Part of this was phosphorylated (see above) to give [2α,4α-$^{17}O_2$]FMN. The mother liquor was taken to dryness and the residue acetylated. This gave 60 mg of [2α,4α-$^{17}O_2$]TARF.

The ^{17}O NMR measurements were performed on a Bruker CXP 300 spectrometer operating at a frequency of 40.7 MHz. The spectra were recorded employing a spectral width of 41 KHz, 90 degree pulse, 8K data points and a pulse repetition rate of 7/second. Wilmad 10 mm NMR tubes were used. The

sample volume was 1.6 ml. The concentration of flavin was about 50 mM. Broadband decoupling of 1 W was applied. The temperature of the measurements was 30°C. The ^{17}O chemical shifts are reported relative to H_2O. All ^{17}O NMR spectra were recorded without locking the magnet.

Reduction of oxidized flavin solutions in $CHCl_3$ to the 1,5 dihydro state was affected directly in the NMR tube by vigorous shaking of a two-phase solution consisting of the flavin solution in $CHCl_3$ and an aqueous solution of 1 M potassium phosphate (pH=7.5) saturated with sodium chloride containing about a ten-fold excess of sodium dithionite with respect to flavin.

Results and Discussion

A nucleus with a spin greater than ½ possesses an electric quadrupole moment eQ. The electric quadrupole moment eQ arises from the deviation of the globular charge distribution of the nucleus. The dominant relaxation mechanism for quadrupole nuclei comes from the interaction of the electric quadrupole moment eQ with an electric field gradient eq at the nucleus and the modulation of these interactions by rotational motion (James, 1975). In the extreme narrowing limit, i.e. $\omega\tau_c<<1$ where ω is the angular precession frequency of the ^{17}O nucleus and τ_c is the rotational correlation time the contribution of the nuclear quadrupole to the relaxation rate can be expressed as (Abragam, 1961):

$$\frac{1}{T_q} = \frac{1}{T_1} = \frac{1}{T_2} = \frac{12\pi^2}{40}\left(\frac{2I+3}{I^2(2I-1)}\right)\left(1+\frac{\eta^2}{3}\right)\left(\frac{e^2qQ}{h}\right)\tau_c$$

where eqQ/h is the nuclear quadrupole coupling constant (QCC), η is a measure of how much the electric field gradient deviates from axial symmetry and τ_c is the rotational correlation time. The effect of the term $(1 + \eta^2/3)$ is rather small and generally ignored. Fig. 1A shows the ^{17}O NMR spectrum of TARF enriched with ^{17}O at the O(2α) position (50%). Two resonances can be seen, a very broad one at 350 ppm (linewidth 2500 Hz) and a relatively sharp one at 0 ppm (linewidth 50 Hz). The latter resonance is from water present in a coaxial capillary. The correlation time of TARF in

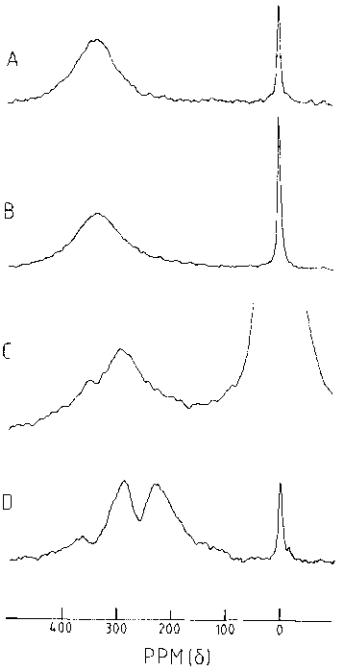

Figure 1. ^{17}O NMR spectra of tetraacetylriboflavin (TARF) in chloroform and FMN in water (pH=7). The ^{17}O chemical shifts are reported relative to H_2O. The temperature of the measurements was 30°.
a) $(2\alpha\text{-}^{17}O)$TARF in the oxidized state. 7000 transients.
b) $(2\alpha,4\alpha\text{-}^{17}O_2)$TARF in the oxidized state. 82000 transients.
c) $(2\alpha,4\alpha\text{-}^{17}O_2)$FMN in the oxidized state. 450000 transients.
d) $(2\alpha\text{-}^{17}O)TARFH_2$ in the reduced state. 228000 transients.

The concentration of the flavins is about 50 mM.

chloroform can be estimated to be about 0.1 ns. It can therefore be concluded that this is in the limit of extreme narrowing i.e. very fast molecular motion with respect to the resonance frequency and equation 1 can be used to calculate the nuclear quadrupole coupling constant. It can be calculated with equation 1, ignoring the contribution of $1 + \eta^2/3$, which

is a safe assumption for carbonyl groups (Cheng and Brown, 1979), that the nuclear quadrupole coupling constant is about 9.1 MHz. It has been suggested that the formal net charge on the oxygen atom can be calculated by using the QCC (Cheng and Brown, 1979), but Delseth et al. (1980) pointed out that the model used was too simplified.

The ^{17}O chemical shift for the oxygen atom of the O(2α) carbonyl group is in the range found in esters and amides (Delseth et al., 1980). These ^{17}O resonances are upfield shifted by about 200 ppm from the ones found in carbonyl groups in aldehyde and ketones. Delseth et al. (1980) explained the upfield shift for the carbonyl groups found in esters and amides to a reduced π bond order and hence to a change in polarity of the carbonyl-oxygen bond.

Fig. 1B shows the spectrum of TARF enriched with ^{17}O (50%) at both carbonyl oxygens. Virtually the same spectrum as Fig. 1A is obtained. The linewidth of the ^{17}O resonance (3000 Hz) is however slightly larger than that in Fig. 1A. The virtually identical chemical shifts for the two carbonlyl groups indicate that the hybridization order is about the same. When the methyl group on the 3 position is exchanged against a hydrogen an upfield shift is observed to 336 ppm. Very likely this upfield shift comes from intramolecular hydrogen bonding or from a small contribution of the iminol tautomer. It has been shown that carbonyl groups can shift upfield by 30-50 ppm on hydrogen bonding (Schwartz et al. 1980). Upon changing the solvent from chloroform to methanol an upfield shift of only 10 ppm is observed (data not shown). In contrast, in water the resonances shift strongly upfield and are found at 291 ppm (Fig. 1C). The two resonances of the carbonyl groups are not resolved. The strong upfield shift in water compared to methanol indicates that not only the protic character of the solvent is important but also, and even more important, is the dielectric constant of the medium.

Moonen et al. (1984) concluded from ^{13}C and ^{15}N NMR data that a protic solvent with a high dielectric constant is needed to fully polarize the isoalloxazine ring. They concluded that in water the N(10) possesses a higher sp^2 character than in an apolar solvent and as a result of this change in hybridization the N(10) becomes a π electron donor. The two carbonyl groups are the main π electron acceptors. The ^{17}O NMR data fully support this view. The linewidth of the resonances of FMN in Fig. 1C (4700 Hz) is much larger than those found in TARF in chloroform (Fig. 1B). This

increase in linewidth can be fully ascribed to the increase of the viscosity of the solvent used (water compared to chloroform) and hence to the slower rotational correlation time (see equation 1).

On reduction of the oxidized flavin to the 1,5-dihydroflavin the ^{17}O NMR spectrum changes drastically. The resonance of the 170 (2α) is split up into a doublet. The same result was obtained with the twofold labeled flavin. Both resonances are upfield shifted from the one found in the oxidized state, indicating an increase in π electron density (Delseth et al. 1980). A possible explanation for the appearance of a doublet in the reduced state might be that the peak at 229 ppm is from the iminol tautomer of the dihydroflavin and the one at 288 ppm from the normal 1,5 dihydroflavin (Fig. 2). The fact that a splitting is observed also implies that the conversion from one isomer to another is slow on the NMR timescale i.e. the lifetime of each isomer must be much larger than 20 ms.

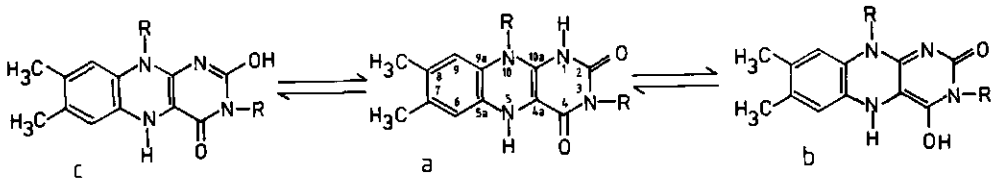

Figure 2. The possible mesomeric structures of N(3)-methyl-1,5-dihydrotetraacetylriboflavin in chloroform.

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Summary

High resolution ^{13}C, ^{15}N, ^{17}O and ^{31}P NMR techniques have been applied to study the structure of free and protein-bound flavins. These techniques yield information on the molecular and sub-molecular level and hence on the mechanism by which the flavin coenzyme is tuned to its specific function. A large part of the thesis deals with the interaction between FMN and apoflavodoxins from several sources i.e. Megasphaera elsdenii, Clostridium MP, Azotobacter vinelandii and Desulfovibrio vulgaris. It is shown in these studies that subtle differences in the interaction between apoflavodoxin and FMN reveal themselves by different ^{13}C, ^{15}N or ^{31}P chemical shifts.

It is shown in chapter 2 that on binding of FMN to the apoflavodoxin from Desulfovibrio vulgaris the isoalloxazine ring of FMN becomes strongly polarized in the oxidized state. Electron density is reallocated from the benzene ring through mesomeric structures towards the C(2) carbonyl group. The pyrimidine part of the molecule is buried in the protein and is not accessible for bulk solvent. On two-electron reduction the isoalloxazine ring maintains its coplanar structure. The isoalloxazine ring is ionized, i.e. carrying a negative charge at N(1), in the pH region between 6.0 and 8.5. Two-electron reduction of the protein leads to a large π electron density increase in the benzene subnucleus of bound FMN compared to free FMN. This increase in π electron density is most likely due to the electron-donating effect of the N(5) and N(10) atoms.

In chapter 3 the interaction between FMN and the apoprotein of three other flavodoxins is studied, i.e. from Azotobacter vinelandii, Megasphaera elsdenii and Clostridium MP. The results show the virtually identical interaction between the prosthetic group and the apoflavodoxins from M.elsdenii and C.MP. Subtle but significant differences are found between these two flavodoxins and the flavodoxins from A.vinelandii and D.vulgaris, e.g. in A.vinelandii flavodoxin the ^{15}N chemical shift indicates that the N(10) atom is out of plane in both redox states. This in contrast to the other three flavodoxins. In all four flavodoxins the pteridine part of the molecule is shielded from solvent in both redox states. The N(5) atom, forms a weak hydrogen bond in the oxidized state in A.vinelandii and D.vulgaris flavo-

doxin, a rather surprising result. No hydrogen bond towards N(5) is found in C.MP and in M.elsdenii flavodoxin. The ^{13}C and ^{15}N results suggest that in A.vinelandii flavodoxin the prosthetic group is bound in a more hydrophobic environment than in the other flavodoxins.
In the two-electron reduced state all four flavodoxins are ionized. A strong hydrogen bond towards N(5) is observed in all four flavodoxins. The isoalloxazine ring is coplanar, except in A.vinelandii flavodoxin where the N(10) atom is slightly out of plane. The C(4) carbonyl group does not form a hydrogen bond with the apoprotein and as a result the O(4α) cannot allocate electron density to such an extent as free flavin. Possibly Coulomb repulsive forces from a carbonyl group of a peptide group of the apoprotein, which forms a strong hydrogen bond with the N(5) atom of the isoalloxazine ring, play also an important role. No clear correlation between redox potential and either ^{13}C or ^{15}N chemical shifts in the reduced state was found with the four flavodoxins although they differ by about 120 mV in redox potential for the semiquinone-hydroquinone transition.

Nuclear Overhauser Effect measurements reveal that the isoalloxazine ring is rigidly bound in both the oxidized and in the two-electron reduced state.

The interaction between a modified flavocoenzyme, i.e. riboflavin 3',5'-bisphosphate, and Megasphaera elsdenii apoflavodoxin was studied in the three redox states (chapter 4). This flavin analog binds rather well to the apoprotein. It was expected that the introduction of an extra phosphate group might influence the redox potentials and this was indeed observed albeit less than expected. This is explained by the observation that the 3'-phosphate is protonated on binding to the apoprotein. The interactions between the isoalloxazine ring and the apoprotein are hardly influenced by the introduction of the extra phosphate group.

In chapter 5 it is shown that when FMN is bound to bacterial luciferase the benzene part of the isoalloxazine ring is in a hydrophobic environment. The polarization of the ring system is much weaker than in the flavodoxins. The N(5) atom is strongly hydrogen bonded in the oxidized state. On two-electron reduction the flavin becomes anionic in the physiological pH region. The negative charge at N(1) is

possibly counteracted by a positively charged group on the protein. The N(5) atom in luciferase-bound $FMHN^-$ is highly sp^2 hybridized rendering an almost planar structure of the prosthetic group. There is one specific strong binding site for $FMNH^-$ per dimeric luciferase molecule. Excess reduced flavin can, however, bind to luciferase in a nonspecific manner.

In chapter 6 the true ^{13}C spectrum of intermediate II of luciferase is identified. In contrast to published results the C(4a) atom of the intermediate resonates at 82.5 ppm, and not at 74 ppm. The intermediate possesses an almost planar structure as deduced by comparison with model studies with only the C(4a) displaced out of plane. The resonance at 74 ppm, previously assigned to the intermediate, is due to a contamination contained in ethylene glycol-d_6.

As an example of the class of hydroxylases, para-hydroxybenzoate hydroxylase from Pseudomonas fluorescens was studied by ^{13}C, ^{15}N and ^{31}P NMR (chapter 7). In the substrate-free enzyme the isoalloxazine ring is probably solvent accessible. Upon binding of substrate the isoalloxazine ring becomes shielded from water. The N(1) and C(2) shift strongly upfield, probably as a consequence of the altered position of a helix dipole in the enzyme-substrate complex. In the reduced state the isoalloxazine ring is not solvent accessible in the substrate-free enzyme. The flavin-molecule carries a negative charge at N(1). The isoalloxazine ring is coplanar when bound to the protein. The binding of substrate causes the resonances of the N(1), C(10a) and N(10) to shift strongly upfield due to the interaction with a helix dipole.

As an example of the class of dehydrogenases, mercuric reductase from Pseudomonas aeruginosa was investigated by ^{13}C NMR and ^{31}P NMR (chapter 8). In the oxidized state the ^{13}C chemical shifts are not much different from those found in free flavin in water. The N(5) atom in mercuric reductase probably lacks a hydrogen bond in the oxidized state.

The protein consists of an oxidized flavin and a reduced disulfide in the two-electron reduced state. The thiolate anion is located in the immediate neighbourhood of the C(4a) atom. The binding of NADPH to the two-electron reduced enzyme leads to an upfield shift of the ^{13}C

resonances, which may indicate charge transfer from the bound NADPH to the isoalloxazine molecule. The binding of $NADP^+$ to the two-electron reduced enzyme leads to a fast intramolecular electron transfer between (probably) the reduced disulfide and the $NADP^+$ molecule. In the four-electron reduced state the isoalloxazine ring is ionized and bent at the N(5) position.

^{31}P NMR studies revealed that mercuric reductase has an extra covalently bound, solvent accessible, phosphate group besides the pyrophosphate group of the protein-bound FAD.

^{17}O NMR studies on free flavins show that besides hydrogen bonding also a high dielectric constant is needed to polarize both carbonyl groups (chapter 9). The spectrum of the two-electron reduced [2α-^{17}O] $TARFH_2$ shows a doublet, which indicates the existence of isomers in reduced flavin in an apolar solvent.

SAMENVATTING

Hoge resolutie ^{13}C, ^{15}N, ^{17}O en ^{31}P kern-spin-resonantie technieken zijn toegepast om de structuur van flavinen, vrij in oplossing en eiwit gebonden, te bestuderen. Deze technieken geven informatie op moleculair en sub-moleculair niveau en kunnen dus inzicht verschaffen over het mechanisme waarmee de flavine coenzymen afgestemd worden op de uiteindelijke functie in het flavine-eiwit. Een groot gedeelte van dit proefschrift behandelt de interactie van FMN met apoflavodoxinen uit diverse bronnen; te weten Megasphaera elsdenii, Clostridium MP, Azotobacter vinelandii en Desulfovibrio vulgaris.

In hoofdstuk 2 wordt aangetoond dat, wanneer FMN gebonden wordt aan het apoflavodoxine van Desulfovibrio vulgaris, de isoalloxazine ring sterk gepolariseerd wordt in de geoxideerde toestand. Electron dichtheid wordt vanuit de benzeenring door middel van mesomere structuren op de C(2) carbonyl groep gebracht. Het pyrimidine gedeelte van het molecuul is diep opgeborgen in het eiwit en niet bereikbaar voor het oplosmiddel. In de twee-electronen gereduceerde toestand blijft de isoalloxazine ring bijna vlak. De isoalloxazine ring heeft dan een negatieve lading op de N(1) positie in het pH gebied van 6.0 tot 8.5. Twee-elektronen reductie van het eiwit geeft aanleiding tot een sterke toename in electronen dichtheid in de benzeenring van het gebonden FMN vergeleken met vrij FMN. Deze toename in π electronen dichtheid is het gevolg van het electronen afgevend effect van de N(5) en de N(10) atomen.

In hoofdstuk 3 is de interactie tussen FMN en de apoflavodoxinen van drie andere bronnen, Azotobacter vinelandii, Megasphaera elsdenii en Clostridium MP, bestudeerd. De flavodoxinen van M.elsdenii en C.MP zijn nagenoeg identiek wat betreft hun interactie tussen het flavinmolecuul en het apoflavodoxine. Kleine maar significante verschillen worden gevonden tussen deze twee flavodoxinen en de flavodoxinen van A.vinelandii en D.vulgaris. In A.vinelandii flavodoxine is bijvoorbeeld het N(10) atoom iets uit het vlak van het molecuul in de twee gemeten redox-toestanden. In de andere drie flavodoxinen is het N(10) atoom in het vlak van het molecuul. In alle flavodoxinen is het pteridine gedeelte van het molecuul afgeschermd van oplosmiddel in de beide redox-toestanden. Het N(5) atoom heeft een zwakke waterstofbrug in de

geoxideerde toestand in A.vinelandii en in D.vulgaris flavodoxine, een verrassend resultaat. De ^{13}C en ^{15}N NMR resultaten geven aan dat het flavine molecuul in A.vinelandii flavodoxine gebonden wordt in een meer hydrofobe omgeving dan in de andere flavodoxinen.

In de twee-electronen gereduceerde vorm zijn de vier flavodoxinen geïoniseerd. Een sterke waterstofbrug naar de N(5) amide groep wordt gevonden in deze eiwitten. De isoalloxazine ring is bijna vlak. De C(4) carbonyl groep heeft geen waterstofbrug met het apoflavodoxine en dientengevolge kan de O(4α) niet zo sterk electronendichtheid opnemen als in vrij flavine. Mogelijkerwijze speelt een Coulombse dipool-dipool interactie tussen een carbonylgroep van een peptide binding van het eiwit, die een sterke H-brug met de N(5) amide groep vormt, ook een belangrijke rol. Er is geen duidelijke correlatie van de redox-potentiaal met de ^{13}C en ^{15}N chemische verschuivingen in de gereduceerde toestand in de vier flavodoxinen, hoewel deze eiwitten 120 mV in redoxpotentiaal verschillen in de semiquinon-hydroquinon overgang.

Nuclear Overhauser Effect metingen geven aan dat de isoalloxazine ring stevig gebonden is in zowel de geoxideerde als de gereduceerde toestand.

De interactie tussen een gemodificeerd flavinemolecuul, riboflavine 3'-5'-bisfosfaat, en M.elsdenii apoflavodoxine is bestudeerd in de drie redox-toestanden (Hoofdstuk 4). Dit flavine analoog bindt redelijk goed aan het apoflavodoxine.

De verwachting was dat de introductie van een extra fosfaat groep de redox potentiaal zou kunnen beïnvloeden en dit werd inderdaad waargenomen, echter minder sterk dan verwacht. Dit wordt verklaard door de waarneming dat de 3'-fosfaat groep geprotoneerd is bij binding aan het apoflavodoxine. De interacties tussen de isoalloxazine ring en het apoflavodoxine worden nauwelijks beinvloed door de introductie van de extra fosfaat groep.

In hoofdstuk 5 wordt aangetoond dat wanneer FMN gebonden wordt aan luciferase, de benzeenring van de isoalloxazine ring zich in een hydrofobe omgeving bevindt. De polarisatie van het ringsysteem is veel zwakker dan in de flavodoxinen. Het N(5) atoom heeft een sterke waterstofbrug in de geoxideerde toestand. In de twee-electronen gereduceerde toestand is het flavine anionisch in het fysiologische pH

gebied en heeft dus een negatieve lading op het N(1) atoom.

Deze negatieve lading wordt mogelijkerwijze gecompenseerd door een positief geladen groep in het eiwit. Het N(5) atoom in het luciferase gebonden $FMNH^-$ is in hoge mate sp^2 gehybridizeerd wat tot gevolg heeft dat de isoalloxazine ring bijna vlak is. Er is één specifieke sterke bindingsplaats voor $FMNH^-$ per luciferase dimeer. Overmaat gereduceerd flavine kan echter op een aspecifieke manier aan luciferase binden.

In hoofdstuk 6 wordt het enige ware ^{13}C NMR spectrum van intermediair II van de luciferase reactie getoond. In tegenstelling tot gepubliceerde resultaten resoneert het C(4a) atoom van het intermediair op 82.5 ppm en niet op 74 ppm. Het intermediair heeft een bijna vlakke structuur, wat afgeleid wordt uit de ^{13}C chemische overschuivingen. De resonantie op 74 ppm, welke voorheen werd toegekend aan het intermediair II, is van een verontreiniging in ethyleenglycol.

Als een voorbeeld van de klasse van hydroxylases is para-hydroxybenzoaat hydroxylase uit Pseudomonas fluorescens bestudeerd met behulp van ^{13}C, ^{15}N en ^{31}P NMR (hoofdstuk 7). In het substraatvrije enzym is de isoalloxazine ring waarschijnlijk water toegankelijk. De binding van substraat leidt tot de afscherming van de isoalloxazine ring van het oplosmiddel. De N(1) en C(2) verschuiven sterk naar hoogveld, waarschijnlijk ten gevolge van de veranderde positie van een helix dipool in het enzym-substraat complex. De isoalloxazine ring is niet toegankelijk voor water in de gereduceerde toestand in het substraatvrije enzym. Het flavine molecuul heeft een negatieve lading op de N(1) positie. De isoalloxazine ring is vlak wannneer het gebonden zit aan het eiwit. De binding van substraat leidt tot een sterke hoogveld verschuiving van de resonanties van de N(1), C(10a) en N(10). Twee mogelijke verklaringen worden aangedragen, ofwel er is een pK shift van het N(1) atoom, ofwel er is een interactie met een helix dipool.

Als een voorbeeld van de klasse van dehydrogenases is kwik reductie uit Pseudomonase aeruginosa bestudeerd met behulp van ^{13}C en ^{31}P NMR (hoofdstuk 8). In de geoxideerde toestand zijn de ^{13}C chemische verschuivingen niet veel verschillend van die van vrij flavine in water. Het N(5) atoom in kwik reductase heeft geen waterstofbrug in de

geoxideerde toestand.

Het eiwit heeft een geoxideerd flavine en een gereduceerd disulfide in de twee-electronen gereduceerde toestand. Het thiolaat anion bevindt zich in de directe omgeving van het C(4a) atoom. De binding van NADPH leidt tot een hoogveld verschuiving van de ^{13}C resonanties, wat mogelijkerwijze wijst op ladingsoverdracht van het gebonden NADPH naar het isoalloxazine molecuul. De binding van $NADP^+$ aan het twee-electronen gereduceerde enzym leidt tot een snelle intramoleculaire electron transfer tussen (waarschijnlijk) de gereduceerde disulfide en het $NADP^+$ molecuul. In de vier electronen gereduceerde toestand is de isoalloxazine ring geioniseerd en gebogen op de N(5) positie.

^{31}P NMR studies geven aan dat kwik reductase een extra covalent gebonden, water toegankelijke, fosfaat groep heeft naast de pyrofosfaat groep van het eiwit gebonden FAD.

^{17}O NMR studies aan vrije flavinen tonen aan dat behalve waterstofbrugvorming ook een hoge dielectrische constante nodig is om de twee carbonylgroepen van de isoalloxazine ring te polariseren (hoofdstuk 9). Het spectrum van de twee-electronen gereduceerde vorm in chloroform geeft een doublet te zien voor het [2α-^{17}O] verrijkt monster. Dit kan verklaard worden door de aanwezigheid van twee isomeren in oplossing.

LEVENSOVERZICHT

De auteur van dit proefschrift werd geboren op 26 oktober 1954 te Horst. In 1973 behaalde hij het diploma Gymnasium-β aan het Boschveld College te Venray. In 1975 werd de studie Moleculaire Wetenschappen aan de Landbouwhogeschool te Wageningen begonnen. De doctoraalfase omvatte de hoofdvakken Biochemie en Moleculaire Fysica en het bijvak Natuurkunde. De praktijktijd werd doorgebracht op de afdeling Biochemie van de Universiteit van Georgia, Athens, Georgia U.S.A., onder begeleiding van prof.dr. J. Lee. De studie werd in september 1982 afgerond.

Op 1 september 1982 werd aan deze dissertatie begonnen, in eerste instantie in dienst van de Stichting voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.). Vanaf 1 september 1985 is de auteur in dienst van de Landbouwhogeschool te Wageningen.