THE APPLICATION OF VARIOUS NMR TECHNIQUES TO FREE AND PROTEIN-BOUND FLAVINS

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THE APPLICATION OF VARIOUS NMR TECHNIQUES TO FREE AND PROTEIN-BOUND FLAVINS

An approach to elucidate the active center of flavoproteins

Proefschrift

Ter verkrijging van de graad van

doctor in de landbouwwetenschappen,

op gezag van de rector magnificus,

dr. C.C. Oosterlee,

hoogleraar in de veeteeltwetenschap,

in het openbaar te verdedigen

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des middags te vier uur in de aula

van de Landbouwhogeschool te Wageningen

1. Favaudon en medewerkers kennen in het Desulfovibrio Gigas flavodoxine
de 7,8-methyl resonanties van het flavine toe. De kans dat deze toekenning
juist is, is nihil.
V. Favaudon, J. LeGall, J.-M. Lhoste. In "Flavins and Flavoproteins",
Proceedings of the 6th International Symposium on Flavins and Flavo-
proteins, K.Yagi, T. Yamano ed., Japan Scientific Press Tokyo and Uni-
versity Park Press Baltimore (1980), 373.

2. De correlatie zoals die door Fox en medewerker gelegd wordt tussen de be-
rekende ionisatie energie en de redox potentiaal is principieel onjuist.
M. Teitell, J.L. Fox; Int. J. Quant. Chem. (1982) 22, 583.

3. Wyatt en medewerkers gebruiken bij de interpretatie van hun resultaten ten
onrechte de "studenten t-test". Er bestaat dan ook geen significant ver-
schil in de cortisol spiegel tussen de met Beclometasondipropionaat be-
handelde groep en de onbehandelde groep.
R. Wyatt, J. Waschek, M. Weinberger, B. Scherman; New. Eng. J. Med. (1978)
299, 1378.

4. De door Spahn en medewerkers gegeven verklaring voor de discrepantie tussen
hun klinische bevindingen en de gemeten serum spiegels is in tegenspraak
met de dosis-respons curve van het metoprolol.
H. Spahn, E. Mutscher, W. Kirsch, E.E. Ohnhaus, H.D. Janisch;
British. Med. J. (1983), 286, 1546.

5. Er bestaat een interactie tussen cimetidine en pentobarbitone, maar deze ont-
breekt tussen Ranitidine en pentobarbitone. Brittain en medewerkers ver-
klaren dit verschil aan de hand van de dissociatieconstanten tussen deze
twee geneesmiddelen en het microsomaal cytochroom P-450. Dit kan niet meer
dan de halve waarheid zijn.
R.T. Brittain, M.J. Daly, D. Jack, L.E. Martin, R. Stabels, M. Sutherland; In
"The Clinical use of Ranitidine", eds. J.J. Misiewicz, K.G. Wormsley;
The Medicine Publishing Foundation (1981), 1.

6. De uitspraak van James en medewerkers over de snelheid van elektronen-
overdracht tussen het semiquinone - en het volledig gereduceerd M.elsdenii
flavodoxine is op onvoldoende gegevens gebaseerd en onjuist.
T.L. James, M.L. Ludwig, M. Cohn; Proc.Natl.Acad.Sci. U.S. (1973) 70, 3292.

7. De door Wagner en medewerkers afgeleide formules verklaren niet de door hen weergegeven Perrin curves. De formules zijn dan ook onjuist en de auteurs hadden de appendix in deze vorm beter achterwege kunnen laten. R. Wagner, W. Junge; Biochemistry (1982) 21, 1890.

8. Gezien de implicaties enerzijds en de nonchalance van de auteurs anderzijds zouden brieven naar medische tijdschriften door een onafhankelijk deskundige beoordeeld dienen te worden.

9. Wanneer men de ontwikkeling op het gebied van de praktijk verkleining volgt, dan kan men duidelijk constateren dat bij de meeste mensen het hart links zit maar de portemonaie rechts.

C.G. van Schagen, The application of various N.M.R. techniques to free and protein bound flavins.
9 november 1983.

aan mijn ouders

aan Lida,
Jeroen, Maarten en Johan.

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Chapter 2   C.G. van Schagen and F.Müller, Helv.Chem.Acta 62, 2187-2201 (1980)

Chapter 3   C.G. van Schagen and F. Müller, Eur.J.Biochem. 120, 33-39 (1981)

Chapter 4   C.G. van Schagen and F. Müller, FEBS Lett. 136 75-79 (1981)

Chapter 5   C.G. van Schagen, F. Müller and R. Kaptein, Biochemistry 21, 402-407 (1982)

LIST OF ABBREVIATIONS

|	
Ala	= Alanine
A.vinelandii	= Azotobacter vinelandii
CIDNP	= Chemically Induced Dynamic Nuclear Polarization
C.pasteurianum	= Clostridium Pasteurianum
DSS	= 4,4-dimethyl-4-silapentane-1-1-sulfonate
EDTA	= Ethylene diamine tetraacetic acid
FAD	= Flavin adenine dinucleotide
FMN	= Flavin mononucleotide
$FMNH_2$	= 1,5-dihydro flavin mononucleotide
$FMNH^-$	= 1,5 dihydro flavin mononucleotide in the anionic form
LF	= Lumiflavin
$MeAc_4RF$	= N(3)-Methyl-2',3',4',5'-tetraacetylriboflavin
$MeAc_4RFH_2$	= N(3)-Methyl-2',3',4',5'-tetracetyl-1,5-dihydroriboflavin
$MeAc_4RFH^-$	= N(3)-Methyl-2',3',4',5'-tetraacetyl-1,5-dihydroriboflavin in the anionic state
NMR	= Nuclear Magnetic Resonance
NOE	= Nuclear Overhauser Effect
S.Lividus	= Synechoccus Lividus
TARF	= 2',3',4',5'-Tetraacetylriboflavin
Trp	= Tryptophan
Tyr	= Tyrosine

GENERAL INTRODUCTION

About a century ago a yellow, fluorescent pigment was isolated from whey [1]. In the period till 1930 yellow pigments were extracted from various biological sources. These pigments were called e.g. 'lactochrome', 'ovoflavin', 'lacto-flavin', 'hepatoflavin' or 'verdoflavin'. These names are related either to the source of isolation or the physical appearance of the natural product. In the 1930's it became evident that all the above-mentioned compounds are riboflavin (vitamin B_2). The structure of riboflavin was elucidated and proven by total synthesis in 1934 and 1935 [2,3].

The flavoproteins oxynitrilase and xanthine oxidase were already isolated in 1908 and 1926, respectively, but have not been recognized as flavoproteins at that time [4,5]. The first protein recognized as flavoprotein was 'old yellow enzyme' [6]. The prosthetic group of 'old yellow enzyme' was later identified as ribo-flavin-5'-monophosphate (FMN) [7]. The prosthetic group of D-amino acid oxidase was identified as flavin adenine dinucleotide [8]. Thus in the years before the second world war it was already established that FMN and FAD are derivatives of riboflavin and that they serve as prosthetic groups for certain proteins. In addition it was demonstrated that the prosthetic group in flavoproteins can reversibly be dissociated from the protein. The structure of the common naturally occurring flavins and their photolytic products are shown in Fig. 1.

After the second world war many flavoproteins were isolated, purified and their molecular properties and biological function characterized. Today more than one hundred flavoproteins are known. Many of the flavoproteins are simple biolo-gical molecules in the sense that they contain only flavin as a prosthetic group. Other flavoproteins are much more complex containing also iron (e.g. succinate dehydrogenase [9,10]), iron and molybdenum as in xanthine oxidase [11] or proto-heme (cytochrome b_2 [12]). In addition more recently it became evident that the prosthetic group in some flavoproteins is covalently linked to the apoprotein [13-15]. In these proteins the flavin can only be released by proteolytic treat-ment of the flavoprotein. The thus obtained flavins contain amino acid residues.

The most prominent feature of the chemistry of flavin is its redox properties.

Fig 1

Fig 2
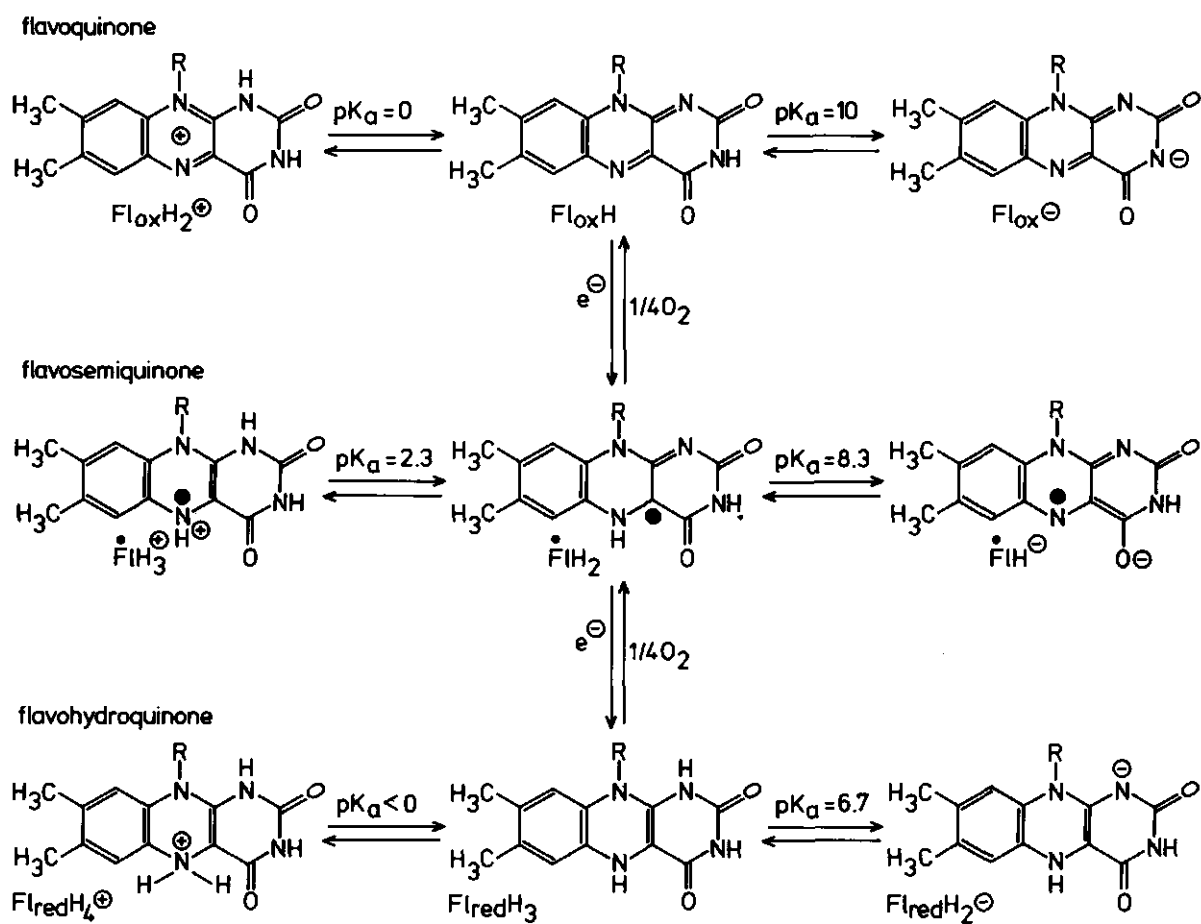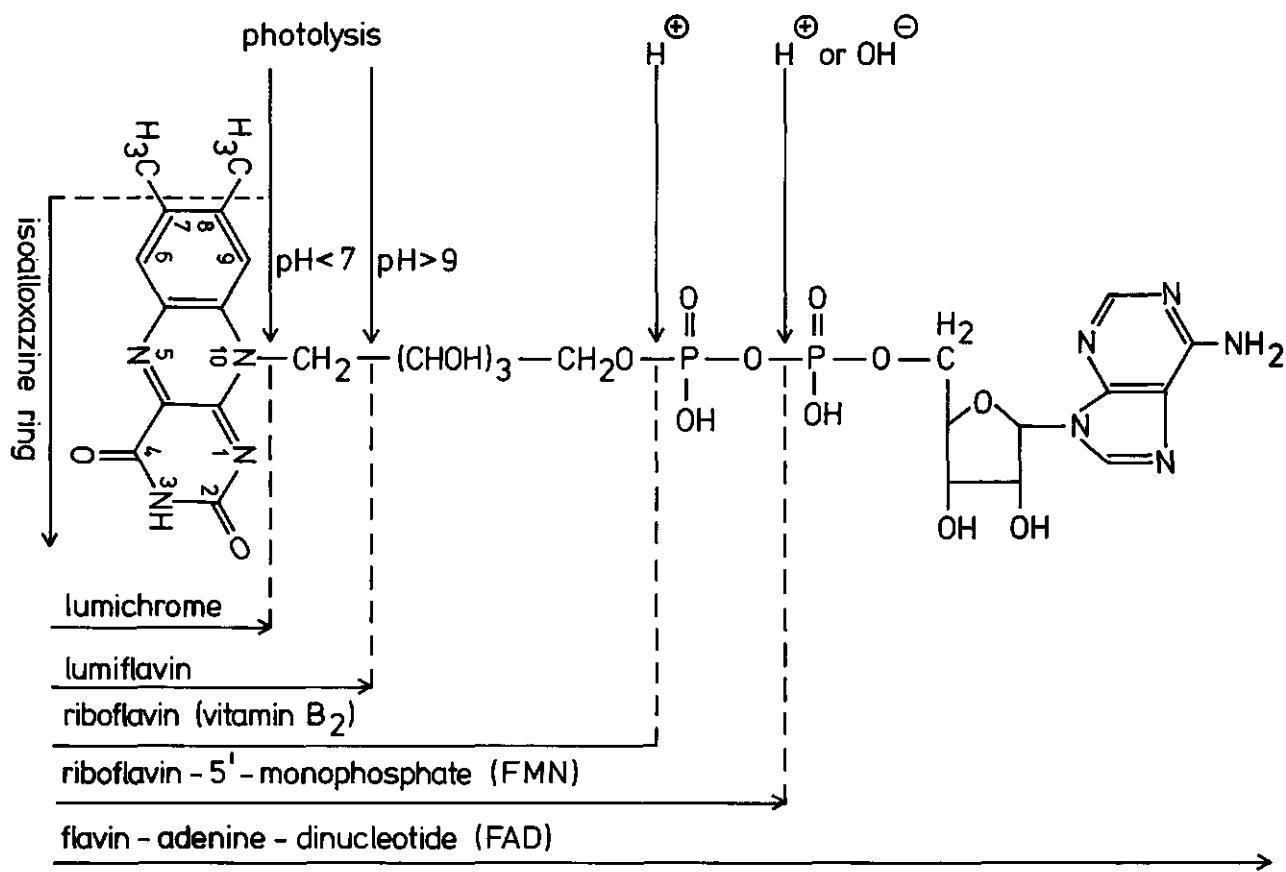
These properties make flavin especially suitable for its broad involvement in biological reactions. The three redox states are: flavoquinone, flavosemiquinone and flavohydroquinone. In all three redox states, flavin is an amphoteric molecule (Fig. 2). The reduction of free flavin by one or two electrons is thermodynamically quantitatively reversible. In flavoproteins only five of the nine structures given in Fig. 2 are possible, because flavoproteins denature at pH values >9 or <2. In addition flavoproteins make selective use of certain redox states depending on their biological function. For instance electron-transferring flavoproteins usually only shuttle between the flavosemiquinone and the flavohydroquinone state, whereas flavoprotein oxidases avoid always the flavosemiquinone state during catalysis and shuttle therefore between the flavoquinone and the flavohydroquinone state.

In the last two decades a wealth of data has been published on the catalytic mechanism and the molecular properties of flavoproteins. Questions like if a certain flavoprotein reacts according to a group transfer, where σ-complexes should be formed as intermediates, or a pure redox mechanism could not be answered, although it has been established in a few cases that the one mechanism is operative in a certain class of flavoproteins and the other in another class of flavoproteins. Nevertheless it is obvious that the apoprotein plays an important role in the determination of the specificity and the reaction mechanism of a certain flavoprotein. Since all flavoproteins contain one and the same chemical entity it has been proposed that specific interactions between amino acid residues and certain atoms of the isoalloxazine moiety of flavin determine to a great extent the chemical reactivity of protein-bound flavin [16].

The aim of this study was two-fold. Firstly, to test the proposal with respect to possible specific interactions between FMN and the apoproteins of two selected flavodoxins and to assign the lines in the ^{13}C NMR spectra of a series of reduced flavin derivatives. The results on the free flavins formed the basis for a more detailed interpretation of the results obtained on flavoproteins. To be able to meausre the ^{13}C chemical shifts of protein-bound flavins several selectively ^{13}C-enriched FMN derivatives were synthesized. Secondly, an attempt was undertaken to elucidate the amino acid residue in the neighbourhood of the protein-bound flavin,

of the flavodoxin from <u>Megasphaera</u> <u>elsdenii</u> by ^{1}H NMR techniques. This protein was selected because of its easy accessibility in large quantities.

Flavodoxins are small flavoproteins which contain one molecule of FMN as prosthetic group and function as electron carriers of low redox potential reactions ctalyzed by crude extracts of a variety of micro-organisms [17-23]. The physico-chemical properties of several flavodoxins have been studied extensively [24-26] and the crystal structure of flavodoxins from <u>Clostridium</u> <u>MP</u> [27], <u>Desulfovibrio</u> <u>vulgaris</u> [28] and <u>Anacystis</u> <u>nidulans</u> [29] have been determined at high resolution. The primary structures of several flavodoxins are also known [30-35]. The small size of these proteins and the ease with which stable apoenzymes can be prepared from them, make the flavodoxins very suitable for NMR studies. In addition these flavoproteins yield stable flavosemiquinones upon one-electron reduction. This allows to prepare solutions of flavoproteins containing an intrinsic paramagnetic probe.

In the field of flavins and flavoproteins the NMR technique has been employed to elucidate the structure of FAD and FMN, and to verify or to support the structure of low molecular weight flavins. Bullock and Jardetzky [36] assigned unequivocally the proton resonance lines due to $CH_3(8)$, H(6) and H(9) of the flavin molecule by deuterium exchange experiments. These assignments facilitated the elucidation of the solution structures of FMN and FAD. The structures proposed by Sarma et al. [37] have been refined by Kotowycz et al. [38] and by Kainosha and Kyogoku [39] employing high resolution ^{1}H- and ^{31}P-NMR spectroscopy. These studies showed that both FMN and FAD associate themselves by way of vertical stacking of the isoalloxazine rings and that the association is dependent on concentration, temperature and solvent. In aquous solution FAD forms an intramolecular complex where the adenine and isoalloxazine rings are stacked 0.3-0.4 nm apart with their long axes perpendicular to each other [39]. With respect to the structure of FAD there exists some contradiction between the work of Kotowycz et al. [38] and that of Kainosho and Kyogoku [39]. This discrepancy can easily be explained considering the ^{1}H NMR results of Grande et al. [40] which show that the position of the resonance lines due to H(6) and H(9) in the ^{1}H NMR spectrum of FAD depend not only

on the concentration but also on the composition of the aqueous buffer.

It has been known for quite a time that flavins in the 1,5-dihydro state are non planar [41]. This finding was later confirmed by crystallography [42]. The conformation and nitrogen inversion of 1,5-dihydroflavin in solution was investigated by Tauscher et al. [43] by ^{1}H NMR. The inversion barrier was found to be between 42 and 63 KJ/mole. This study has been performed using 1,5-dihydroflavins carrying bulky substituents. Therefore, the conclusion of these results cannot be related directly to simple 1,5-dihydroflavins, where the inversion barrier could not be determined.

Breitmaier and Voelter [44] investigated FMN and FAD by ^{13}C-NMR and assigned almost all of the signals. A reinvestigation by Grande et al. [45] showed that five out of the twelve assignments of the resonance lines due to the isoalloxazine moiety of FMN were incorrect. This is mainly due to the fact that the quaternary carbon atoms exhibit weak to very weak signal intensities and are therefore easily overlooked. Furthermore commercial FMN contains a considerable amount of impurities making a real assignment very difficult. The data of Grande et al. [45] were obtained using slectively ^{13}C-enriched flavin derivatives which allowed the investigators an anambiguous interpretation of the ^{13}C-NMR spectra.

NMR spectroscopy has been applied only in a few cases to flavoproteins. In context with the presentation of a general method for computing NMR spectra obtained from random-coil proteins McDonald and Phillips [46] were the first to apply the NMR technique to flavoproteins. The main finding in this study was, investigating the flavodoxin from Clostridium pasteurianum, that the conformation of the native protein differs drastically from that of the apoprotein. An interesting study was conducted by Crespi et al. [47] who prepared uniformly deuterium labelled flavodoxin from Synchococcus lividus by biological synthesis. Replacement of the deuterated FMN by natural FMN allowed them to study the prosthetic group of the protein by ^{1}H-NMR technique without the interference of the otherwise overwhelming absorptions of the protons of the protein. It was found that the methyl groups at position 7 and 8 of the protein-bound FMN are shifted downfield by 0.2-0.3 ppm, while the protons at position 6 and 9 are shifted upfield by about 0.5 ppm relative

to free FMN. The former effect probably results from the interaction of amino acid residues of the protein with positions 2,3 and/or 4 of the prosthetic group, while the latter effect may be caused by interaction (stacking) of the flavin with aromatic amino acid residues. James et al. [48] examined the ^{1}H-NMR (220 MHz) spectra of flavodoxins from M.elsdenii and Clostridium MP for each of the three redox states. In both cases it was found that beside the broadening of some resonances, located at the extremes of the spectra, beyond detection in the semiquinone state no evident differences were observed among the spectra of the flavodoxins in the oxidized, reduced and semiquinone state. A few resonance lines in the spectra of these proteins were assigned tentatively. The flavodoxins from D.vulgaris and D.gigas were investigated at 100 MHz by Favaudon et al. [49]. One main difference between these flavodoxins and those mentioned above is manifested in the spectra obtained from the holo- and apoproteins; i.e. these spectra are similar in the Desulfovibrio series and quite different in the other series. This indicates that the structure of the native and apoprotein forms are very similar in the former series of flavodoxins. Furthermore, the resonance lines at high field are not influenced upon reduction whereas those at low field behave similarly as those in the spectra of M.elsdenii and Clostridium MP. A few resonance lines in the spectra of the flavodoxins from Desulfovibrio species were assigned tentatively. Finally it should be mentioned that selectively ^{13}C-enriched riboflavin was bound to the apoprotein of egg white riboflavin binding protein and studied by NMR spectroscopy [50].

References

1. Blyth, A.W. (1879) J.Chem.Soc. 35, 530.

2. Kuhn, R., Reinemund, K. and Weygand, F. (1934) Ber.Dtsch.Chem.Ges. 67, 1460.

3. Karrer, P., Schöpp, K. and Benz, F. (1935) Helv.Chim.Acta 18, 426.

4. Rosenthaler, L. (1908) Biochem.Z. 14, 235.

5. Dixon, M. and Kodama, K. (1926) Biochem.J. 20, 1104.

6. Warburg, O. and Christian, W. (1932) Biochem.Z. 254, 438.

7. Theorell, H. (1935) Biochem.Z. 278, 263.

8. Warburg, O. and Christian, W. (1938) Biochem.Z. 296, 294.

9. Beinert, H. and Sands, R.H. (1960) Biochem.Biophys.Res.Commun. 3, 41.

10. DerVartanian, D.V. and Veeger, C. (1962) Biochem.J. 84, 65.

11. Green, D.E. and Beinert, H. (1953) Biochem.Biophys.Acta 11, 599.

12. Boeri, E. and Tosi, L. (1956) Arch.Biochem.Biophys. 60, 463.

13. Singer, T.P. and Edmondson, D.E. (1974) FEBS Lett. 42,1.

14. Edmondson, D.E. and Singer, T.P. (1976) FEBS Lett. 64, 255.

15. Müller, F. (1983) in "Topics in Current Chemistry: Radicals in Biochemistry", Vol. 108, Springer-Verlag, Berlin, p. 71.

16. Müller, F. (1972) Z.Naturforsch. 27b, 1023.

17. Smillie, R.M. (1963) Plant Physiol. 38 (suppl.), 28.

18. Knight, E. and Hardy, R.W.F. (1966) J.Biol.Chem. 241, 2752.

19. Hinkson, J.W. and Bulen, W.A. (1967) J.Biol.Chem. 242, 3345.

20. Van Lin, B. and Bothe, H. (1972) Arch.Microbiol. 82, 155.

21. Shetna, Y.I., Wilson, P.W. and Beinert, H. (1966) Biochim.Biophys.Acta 113, 225.

22. Mayhew, S.G. and Massey, V. (1969) J.Biol.Chem. 244, 794.

23. LeGall, J. and Halchikian, F.E. (1967) Compt.Rendus.Ser.D. 264, 2580.

24. Bothe, H. and Falkenberg, B. (1972) Z.Naturforsch 27b, 1090.

25. Dubourdieu, M. and LeGall, J. (1970) Biochem.Biophys.Res.Commun. 38, 965.

26. Mayhew, S.G. (1971) Biochim.Biophys.Acta 235, 276.

27. Burnett, R.M., Darling, G.D., Kendall, D.S., LeQuesne, M.E. Mayhew, S.G., Smith, W.W. and Ludwig, M.L. (1974) J.Biol.Chem. 249, 4383.

28. Watenpaugh, K.D., Sieker, L.C. and Jensen, L.H. (1973) Proc.Natl.Acad.Sci. USA 70, 3857.

29. Smith, W.W., Pattridge, K.A., Ludwig, M.L., Petsko, G.A., Tsernoglou, D., Tanaka, M. and Jasunobu, K.T. (1981) J.Mol.Biol. 121, ...

30. Tanaka, M., Haniu, M., Yasunobu, K.T., Mayhew, S.G. and Massey, V. (1973) J.Biol.Chem. 248, 4354.

31. Tanaka, M., Haniu, M., Yasunobu, K.T., Mayhew, S.G. and Massey, V. (1974) J.Biol.Chem. 249, 4397.

32. Dubourdieu, M., LeGall, J. and Fox, J.L. (1973) Biochem.Biophys.Res.Commun. 52, 148.

33. Dubourdieu, M. and Fox, J.L. (1977) J.Biol.Chem. 252, 1453.

34. Tanaka, M., Haniu, M., Yosunobu, K.T. and Mayhew, S.G. (1974) J.Biol.Chem. 249, 4393.

35. Tanaka, M., Haniu, M., Yasunobu, K.T. and Yoch, D.C. (1977) Biochemistry 16, 3525.

36. Bullock, F.J. and Jardetzky, O. (1965) J.Org.Chem., 30, 2056.

37. Sarma, R.H., Dannies, P. and Kaplan, N.O. (1968) Biochemistry 7, 4359.

38. Kotowycz, G., Teng, N., Klein, M.P. and Calvin, M. (1969) J.Biol.Chem. 244, 5656.

39. Kainosho, M. and Kyogoku, J. (1972) Biochemistry 11, 741.

40. Grande, H.J., van Schagen, C.G., Jarbandhan, J. and Müller, F. (1977) Helv.Chim.Acta 60, 348.

41. Dudley, K.H., Ehrenberg, A., Hemmerich, P. and Müller, F. (1964) Helv.Chim. Acta 47, 1354.

42. Kierkegaard, P., Norrestam, R., Werner, P., Csöregh, I., von Glehn, M., Karlsson, R., Leijonmarck, M., Rönnquist, O., Stensland, B., Tillberg, O. and Torbjörhsson, L. (1971) in Flavins and Flavoproteins (Kamin, H., ed.), University Park Press, Baltimore, p. 1.

43. Tauscher, L., Ghisla, S. and Hemmerich, P. (1973) Helv.Chim.Acta 56, 630.

44. Breitmaier, E. and Voelter, W. (1972) Eur.J.Biochem. 31, 234.

45. Grande, H.J., Gast, P., van Schagen, C.G., van Berkel, W.J.H. and Müller, F. (1977) Helv.Chim.Acta 60, 367.

46. McDonald, C.C. and Phillips, W.D. (1969) J.Amer.Chem.Soc. 91, 1513.

47. Crespi, H.L., Norris, J.R. and Katz, J.J. (1972) Nature New Biology 236, 178.

48. James, T.L., Ludwig, M.L. and Cohn, M. (1973) Proc.Natl.Acad.Sci. USA 70, 3292.

49. Favoudon, V., LeGall, J. and Lhoste, J.-M. (1976) in Flavins and Flavoproteins (Singer, T.P., ed.), Elsevier, Amsterdam, p. 434.

50. Yagi, K., Ohishi, N., Takai, A., Kawano, K. and Kyogoku, J. (1976) in Flavins and Flavoproteins (Singer, T.P., ed.), Elsevier, Amsterdam, p. 775.

Chapter 1

231. A Comparative ^{13}C-NMR. Study on Various Reduced Flavins[1])

by Cees G. van Schagen and Franz Müller[2])

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(17.VII.80)

Summary

Various two-electron reduced flavin derivatives have been investigated by natural abundance ^{13}C-NMR. spectroscopy. Some selectively ^{13}C-enriched compounds were synthesized to ensure the assignment of some of the quaternary C-atoms of the flavin molecule. Addition of two electrons to oxidized flavin leads to upfield shifts of all resonances except for those due to C(5a), C(9) and C(10'a). The largest upfield shift is observed for C(4a). Also some direct and two-bond coupling constants are reported. Theoretical calculations by INDO show that a rather good correlation exists between the calculated π-electron densities and the observed chemical shifts of the two-electron reduced molecule. For the oxidized molecule, the correlation is less satisfactory. Most substitution effects are additive, but some deviations in some compounds are observed indicating structural differences between the compounds in question.

The chemical shifts are also discussed in terms of the chemical reactivity of the oxidized and reduced flavin molecule.

1. Introduction. – Flavins[3]), *i.e.* isoalloxazines, are involved as coenzymes in many biological reactions (see [1]). Although many of these reactions have been studied by kinetic methods the diversity of biological reactions catalyzed by flavoproteins is not well understood. It has been suggested that the particular reaction catalyzed by a given flavoprotein is mainly determined by the specific interaction of the apoflavoprotein with its coenzyme [2] [3]. If this attractive hypothesis is correct then the electron distribution within the flavin molecule should be altered by the interaction with the apoflavoprotein. A method suitable to detect subtle differences of the electron distribution in a molecule is the NMR. technique. Therefore, we have start to investigate free and protein-bound flavins by the NMR. method with

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[1]) Part of this work was presented at the International Symposia on Flavins and Flavoproteins held in Cracow, Poland (September 28–29, 1976) and in Kobe, Japan (March 13–17, 1978).

[2]) To whom correspondence should be addressed.

[3]) Flavin = 7,8-dimethyl-isoalloxazine; isoalloxazine = 10-substituted 2,3,4,10-tetrahydrobenzo[g]pteridine-2,4-dione; lumiflavin = 7,8,10-trimethyl-isoalloxazine.

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the aim to contribute to a better understanding of the underlying physical principles responsible for the diversity of reactions catalyzed by flavoproteins.

In the past oxidized and reduced flavins have mainly been characterized by light absorption [4] and fluorescence techniques [5] [6] yielding only a limited contribution on the desired information. More recently we have reported a detailed account on the ^{1}H- and ^{13}C-NMR. properties of oxidized flavin derivatives [7] [8]. Recently the latter results have been fully confirmed by *Kawano et al.* [9].

In contrast to the oxidized molecule the 1,5-dihydrostate of flavin has not received te same detailed attention although the two-electron reduced state of flavin is biologically of great importance. The main reason for the lack of detailed physical data on the reduced molecule is due to its easy oxidation by molecular oxygen.

In the present paper we describe results on the ^{13}C-NMR. properties of reduced flavin. The main aim of this investigation was to complete the previous study [8] by the assignments of the resonance lines in the spectra of various 1,5-dihydroflavin derivatives. It is expected that these results will provide a basis for a more profound understanding of the chemical properties of reduced flavins. In addition these studies are needed as a comparative basis for an investigation of flavoproteins.

Tentative [10] and preliminary [11] results on this objective have been published. While this paper was in preparation the assignment of the chemical shifts of the spectrum of one particular 1,5-dihydroflavin derivative has been reported by *Kawano et al.* [9] and by *Ghisla et al.* [12].

2. Experimental Part. – ^{13}C-NMR. spectra were recorded on a *Varian* XL-100 spectrometer operating at 25.2 MHz and equipped with a 16 K *Varian* 620-L computer. All spectra were acquired in the *Fourier* transform mode using 12 mm taperlock tubes purchased from *Wilmad.* For the accumulation of the spectra 8 K data points were used in the time domain and converted to 4 K data points in the frequency domain. The spectral width was 5120 Hz for recording full spectra and 2560 Hz for determining the coupling constants. Frequency and proton noise decoupled spectra [13] were recorded mixing the single frequency from a *Schomandl* NO-60 M synthesizer with the gated, noise modulated frequency from the spectrometer. The power ratio was 200:1. The total signal could be amplified by an ENI-3010 amplifier up to a maximum of 15 W. Although the synthesizer was not coupled to the *Master* oscillator of the spectrometer the largest measured variation in the frequency between the two sources was less than 4 Hz. The other instrumental conditions used were: pulse width 10 µs (30° pulse), repetition rate 2 s. The peak positions were determined from the computer generated printout, using TMS as internal standard. The accuracy of the chemical shifts given is better than 4 Hz. The sample temperature was 26°. Internal deuterium (CDCl$_3$) served as a lock signal. To obtain good natural abundance spectra about 25,000 transients per spectrum were accumulated. The compounds were dissolved in CDCl$_3$ (99.8 atom %, *Merck,* Germany). Saturated solutions were prepared resulting in concentrations varying from 10 to 50 mM. Reduction of solutions of the oxidized flavin to the 1,5-dihydro state was effected directly in the NMR. tube by vigorous shaking of a two-phase solution consisting of CDCl$_3$ and an aqueous solution of 0.5M sodium phosphate (pH 8, saturated with KCl) containing a 10–20 fold excess of sodium dithionite with respect to flavin. The clear 1,5-dihydroflavin solutions thus optained exhibit a reddish orange colour in contrast to usually lighter colour of the oxidized solutions. Also the reduced flavin derivatives which can be isolated in crystalline state (e.g. **9**, *cf. Scheme 2*) were kept under the reducing solution to prevent oxidation (radical formation).

The synthesis of the selectively ^{13}C-enriched flavin derivatives was described earlier [8]. The following compounds *(cf. Scheme 2)* were prepared according to published procedures: 1,3-dimethyl-1,5-dihydrolumiflavin (**9**) and its *N*(5)-acetyl derivative (**8**) [14]; 3-methyl-*N*(5)-benzyl-1,5-dihydrolumiflavin (**12**) and 3-methyl-4a-benzyl-4a,5-dihydrolumiflavin (**13**) [15]; tetraacetylriboflavin (**1**) was prepared from riboflavin [16]; *N*(5)-methyl-(**10**) and *N*(5)-ethyl-1,5-dihydrolumiflavin (**11**) [17]; 2a,3-dimethyl-1,5-dihydrolumiflavin (**14**) and its *N*(5)-acetyl derivative (**17**), 3,7-dimethyl-(**15**) and 3,7,8-

trimethyl-N(1,10)-ethano-N(5)-acetyl-1,5-dihydroisoalloxazine **(16)**, $2a,4a$-dimethyl-N(5)-acetyl-1,5-dihydrolumiflavin **(18)** [7]; 3,7,8-trimethyl-N(10) (ε-acetopentyl)-N(5)-acetyl-1,5-dihydroisoalloxazine **(7)** [18]; 3,7,8-trimethyl-N(10)-undecyl-N(5)acetyl-1,5-dihydroisoalloxazine **(6)** [19], **4** and **5** were prepared from their oxidized molecules by known procedures [14]. Theoretical data were obtained using the INDO program described by *Pople & Beveridge* [20]. We used the original program [21] and modified it for use on the DEC-10 Agricultural University Computer.

3. Results. – 3.1. *Assignments.*

To facilitate the assignments of the quaternary C-atoms in the flavin molecule a few selectively ^{13}C-enriched derivatives were synthesized. Also derivatives containing more than one ^{13}C-label in one molecule were prepared in order to obtain from the coupling constants some further information on the submolecular structure of reduced flavin. Spectra of such derivatives in the oxidized and reduced state are given in *Figure 1* (see also *Scheme 1*). The chemical shift most affected upon reduction of oxidized flavin is that due to C(4a) followed by that due to C(10a) *(Fig. 1 A, B, C)*. The chemical shifts due to C(2) and C(4) are affected much less upon reduction.

The through-bond coupling constant between C(4) and C(10a), the direct coupling constant between C(4) and C(4a), and that between C(4a) and C(10a)[4]) are considerably influenced upon addition of two electrons to oxidized *flavin (Table 1)*.

The assignments of the residual C-atoms in the spectrum of reduced flavin are based on experiments as illustrated in *Figure 2* for 1,3,7,8,10-pentamethyl-1,5-dihydro-isoalloxazine **(9)**. The assignments were aided by selective hetero-decoupling techniques and especially by the frequency- and noise-decoupling method [13]. The latter method has the advantage that the NOE[5]) for all resonances is preserved in the spectrum in contrast to the usual, selective single frequency hetero decoupling technique. The assignments are further based on the known fact [22] that two-bond CH-coupling constants are usually small as compared to one- and threebond CH coupling constants. In addition the assignment of some resonances is supported further by the use of various derivatives, *e.g.* **3** *(Table 2)*.

The chemical shifts of all compounds investigated are collected in *Table 2*, and the chemical structures of the compounds are presented in *Scheme 2*.

Some of the resonances due to the N(10) side chain of various models **(1 to 7)** could not be assigned to individual C-atoms due to lack of resolution. Nevertheless

Scheme 1

1   **2**

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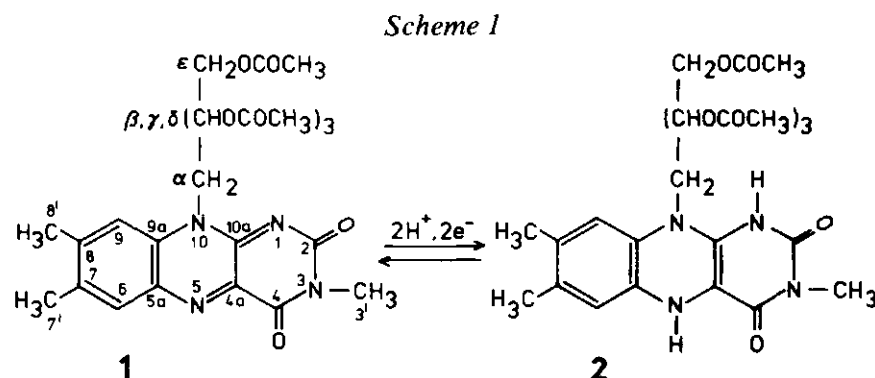
[4]) The coupling constants were verified by homonuclear decoupling experiments.
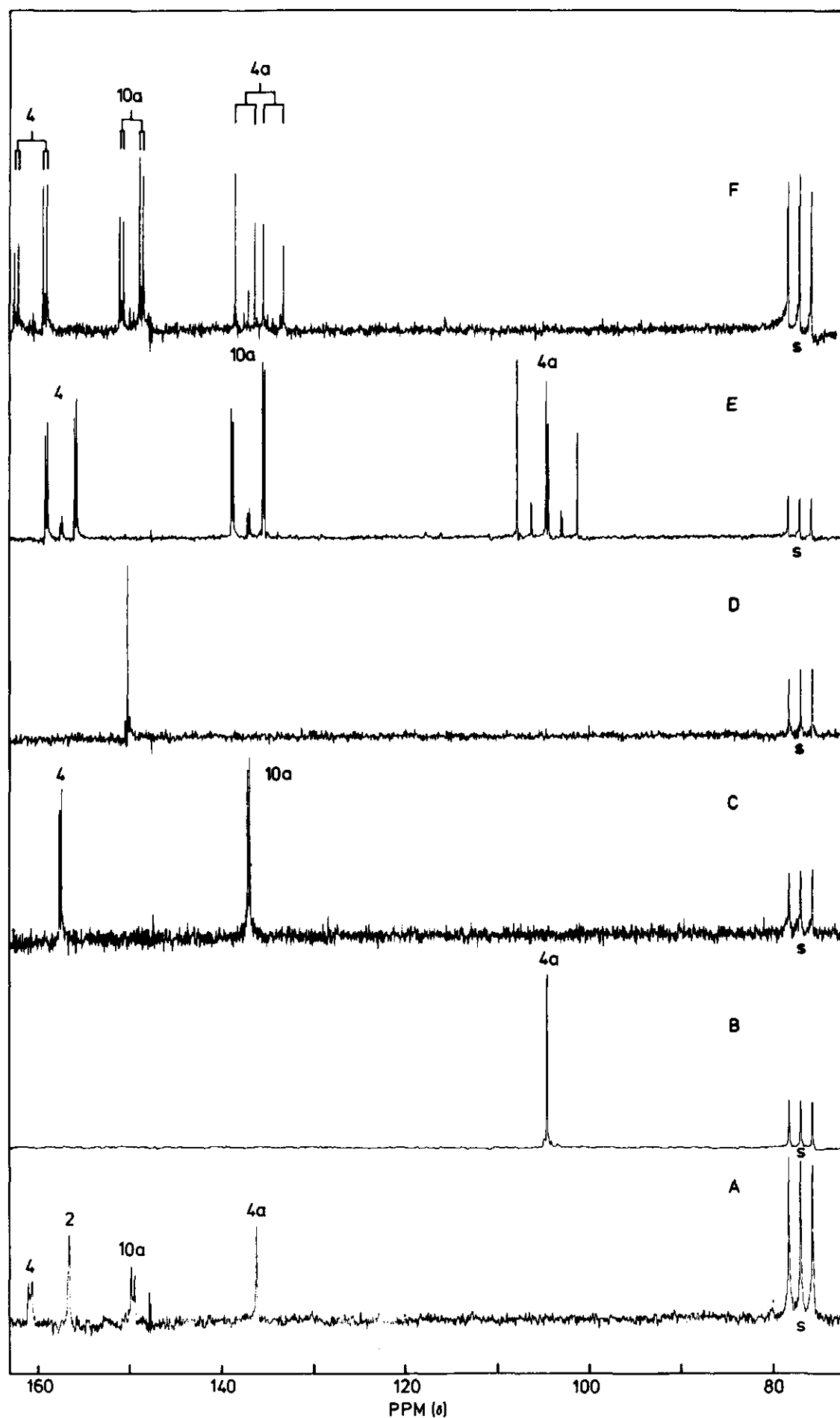[5]) NOE = Nuclear Overhauser Effect.

Fig. 1. *Proton noise decoupled* ^{13}C*-NMR. spectra of various selectively* ^{13}C*-enriched tetraacetylriboflavin* (TARF) *derivatives in the oxidized and reduced state in* $CDCl_3$ *solutions*
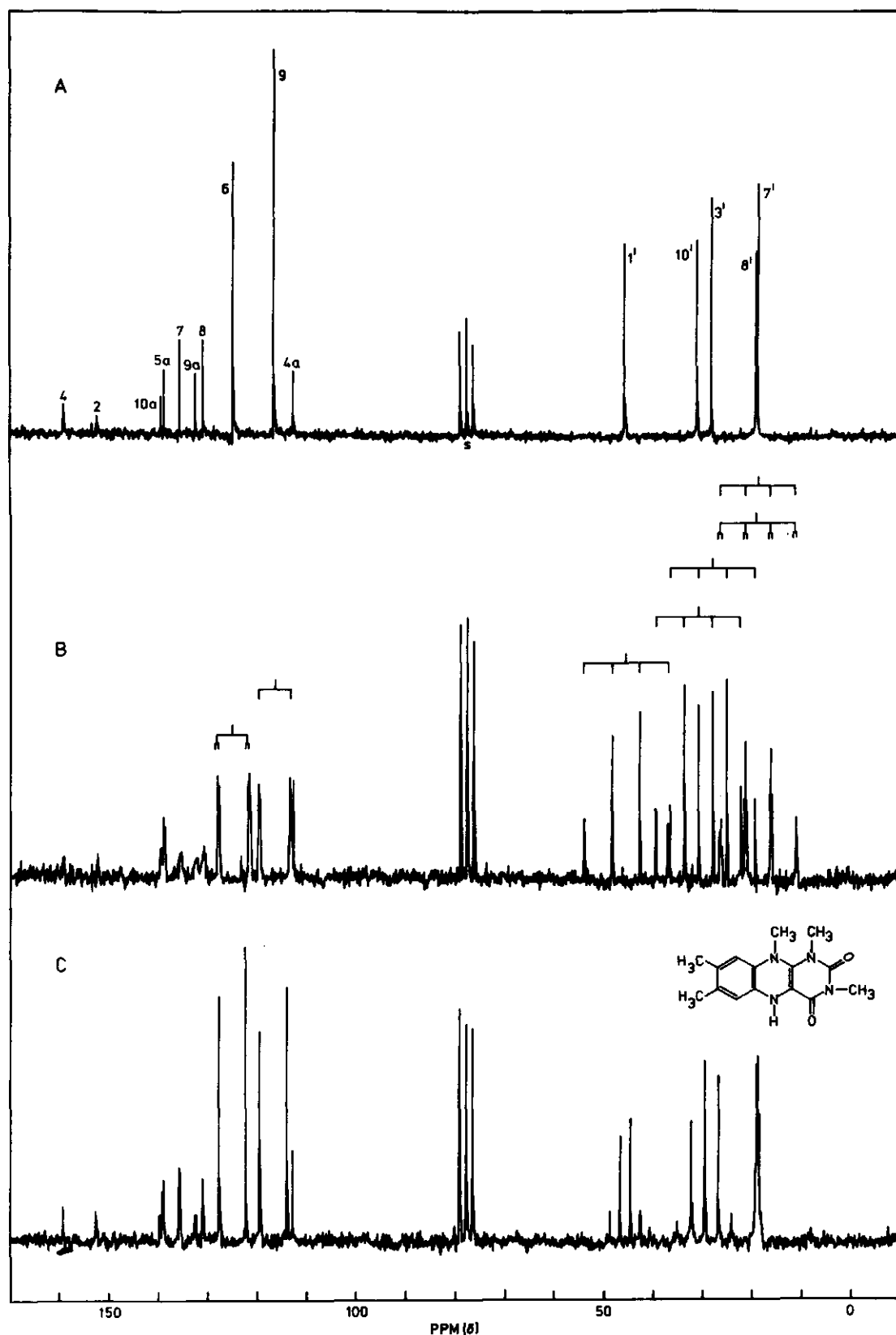
Fig. 2. *Natural abundance ^{13}C-NMR. spectra of 1,3,7,8,10-pentamethyl-1,5-dihydroisoalloxazine* (**9**) *in CDCl₃*

A: Proton noise decoupled; B: gated decoupled; C: off resonance spectrum, irradiation frequency at 2 ppm from TMS in the ^{1}H-NMR. spectrum; s= as in Fig. 1.

A: A mixture of equal concentration of [2-^{13}C]-, [4a-^{13}C]- and [4,10a-^{13}C₂]-TARF in the oxidized state; B: [4a-^{13}C]-TARF in the reduced state; C: [4,10a-^{13}C₂]-TARF in the reduced state; D: [2-^{13}C]-TARF in the reduced state; and [4,4a,10a-^{13}C₃]-TARF in the oxidized (F) and reduced (E) state; s= resonance due to the solvent.

Table 1. *Experimental*[a]) *and Calculated Coupling Constants* (in Hz) *in [4,4a,10a-$^{13}C_3$]- and [4,10a-$^{13}C_2$]-3-methyltetraacetylriboflavin in the Oxidized (1) and Reduced State (2)*

Redox State	Coupling Constant					
	$^1J(C(4),C(4a))$		$^1J(C(4a),C(10a))$		$^2J(C(4),C(10a))$	
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
Oxidized	76.5	100.4	53.3	80.1	10.4	−9.3
Reduced	79.2	98.3	84.5	103.1	5.5	−10.1

[a]) The accuracy of the experimental coupling constants is ±0.2 Hz.

it should be noted that the resonances due to the methyl groups of the acetyl groups become magnetically more equivalent upon reduction of the isoalloxazine ring system. This suggests a small (conformational) perturbation of the side chain upon reduction of the molecule.

3.2. *Comparison of the chemical shifts of various derivatives.* In order to separate possible effects of the side chain acetyl groups on the chemical shifts in the spectrum of **4** compound **5** has been investigated[6]) *(cf. Scheme 2).* Compound **5** possesses only the terminal acetyl group. In the spectrum of **5** the resonance lines due to C(9a), C(5a) and C(10a) are shifted downfield by 5.0, 1.0 and 0.6 ppm, respectively, and that due to C(4a) is shifted upfield by 1.5 ppm, as compared to those of **4**. It should be noted that the chemical shifts of **5** and **6** are identical as far as the C-atoms of the isoalloxazine skeleton are concerned. Therefore, the difference in shifts between **4** and **5** are most probably caused by the dipole of the acetyl group at C(10'β) *(Fig. 3).*

Substitution of H−N(5) in **2** by an acetyl group, giving **4**, leads to large upfield shifts of the resonances due to C(5a) (7.8 ppm), C(4a) (5.3 ppm) and C(9) (2.2 ppm) and to large downfield shifts of the resonances due to C(6) (11.7 ppm), C(10a) (10.0 ppm), C(8) (6.1 ppm) and C(9a), C(2), C(4) (about 2 ppm). Similar shifts, but different in magnitude, are observed by comparing the spectra of **9** and **8**, and those of **14** and **17** except that the resonance due to C(9) in **9** is shifted upfield as compared to the corresponding atom in **8** *(Table 2* and *Fig. 3).* In addition C(9a) in **8** and **17** undergoes a larger downfield shift than that in **4**.

Methylation at N(3) (**7** *vs.* **4**) does not influence the chemical shifts except that the chemical shift of C(10a) in the spectrum of **4** is shifted upfield by 2.5 ppm.

Replacing the methyl groups at N(1,10) by an ethano group (**8** *vs.* **16**) leads to upfield shifts of the resonances due to C(4a), C(5a), C(8), C(9), C(9a), C(10a) and C(2) *(Table 2).*

Methylation of C(2a) instead of N(1) (**14** *vs.* **9**, *Scheme 2*), causes an upfield shift of the resonance lines of C(4), C(4a), C(5a), C(6), C(8), C(9) and C(9a) whereas downfield shifts are observed for C(10a) (8.5 ppm) and C(10') (24.1 ppm). On the other hand, placing the methyl group of N(1) onto N(5) (**9** *vs.* **10**) drastically influences the resonance positions of the peaks of C(4a), C(9a) and C(10a) and, to a lesser degree, those of C(5a), C(6), C(7), C(8) and C(9).

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[6]) It was not possible to compare **2** with the corresponding compound of **5** or **6** because of the low solubility of the later compounds in CDCl₃.

Replacement of the N(5) methyl by an ethyl (**11**) or benzyl group (**12**) has some influence on the resonance lines of C(4a), C(6), C(9a) and C(10a) *(Table 2)*.

The effect of substitution of a particular atom of the flavin molecule on the chemical shift by various substituents is summarized in *Figure 3* for a few compounds. From *Figure 3* it can be concluded that only the chemical shifts of C(2), C(4) and C(7) are relatively insensitive with respect to both the kind of substituent introduced and the place of substitution. The chemical shifts most affected by substitution are those due to C(4a), C(5a), C(9a) and C(10a).

Substitution of C(4a) (**13**) results in a chemically completely different compound which thus cannot be directly compared with **12** or the other flavin derivatives. However, **13** serves as a model compound for postulated, biologically important intermediates [23][7]). For this reason this compound has been included in this study. Compared to the spectrum of **12**, that of **13** exhibits large downfield shifts for C(2), C(4), and C(10a), and a small downfield shift for C(9). The resonances of all other C-atom are shifted upfield. The largest upfield shift is observed for C(4a) in accord with a change of hybridization ($sp^2 \rightarrow sp^3$) of this atom. It is interesting to note that in the spectrum of **13** the following pairs of atoms become magnetically equivalent: C(7) and C(8), C(7') and C(8'), and C(6) and C(9). Moreover, the absolute difference between the chemical shifts of these pairs is much smaller than that of the corresponding pairs in the spectrum of **12**, indicating a more symmetrical charge distribution in the benzene subnucleus of **13** than in that of **12**.

4. Discussion. – It is well documented [25] that ^{13}C-chemical shifts are mainly dependent on the charge and the π-electron density at the C-atom under consideration. This fact should, therefore, be helpful in explaining the chemical reactivity of reduced flavin towards various reactants and to predict which atom most likely could be expected to be the reactive center for a nucleophile or an electrophile. To correlate these properties of the molecule with the chemical shifts observed some theoretical calculations were performed. Using published, theoretical data [26–28] and the established relationship [29]:

$$\delta_C = -159.5\,p + 288.5 \tag{1}$$

where p is the π-electron density, only a rough correlation was found between experimental and calculated chemical shifts. The disagreement is probably caused by the fact that the geometry of the molecules, especially that of reduced flavin, had to be assumed in the calculations due to lack of crystallographic data. In the meantime such data have become available for both the oxidized and reduced flavin molecule, *e.g.* [30]. In our calculations by INDO [20] we used the crystallographic data of *Wang & Fritchie* [31] for the oxidized (coplanar structure) and those of *Norrestam & Glehn* [32][8]) for the reduced (folded structure) molecule. For economic

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[7]) Studies on model compounds support the idea that interaction of 1,5-dihydroflavin with molecular oxygen leads to the formation of a flavinhydroperoxide intermediate [24]. It was shown recently that this interaction occurs at C(4a) of reduced flavin [12].

[8]) The authors mentioned in the text that the proton at N(5) is in axial position which is in contradiction with their actual data. The theoretical data obtained with the structure where the proton is in equatorial position gave also better agreement with the experimental data than using the other stereoisomer (*cf.* also [33]).
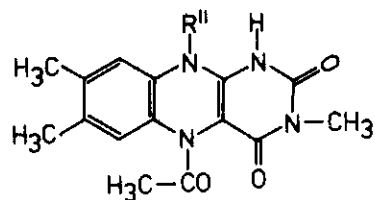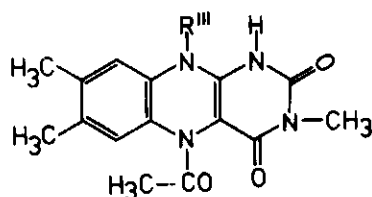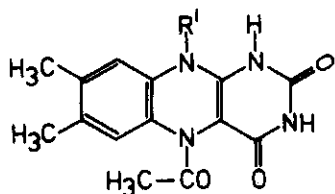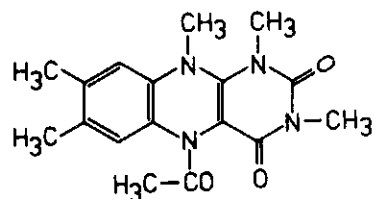
Scheme 2
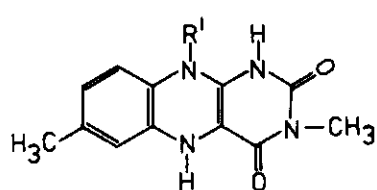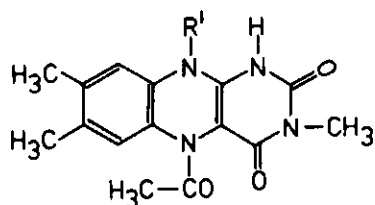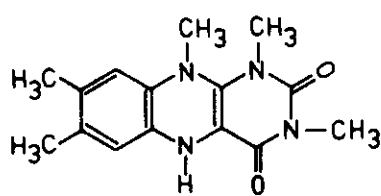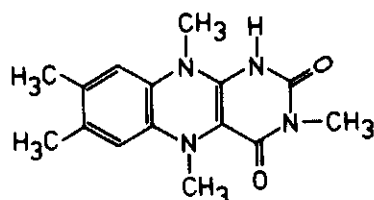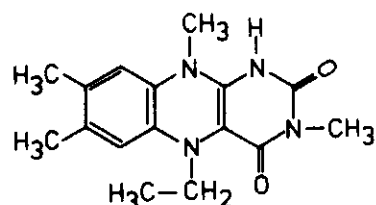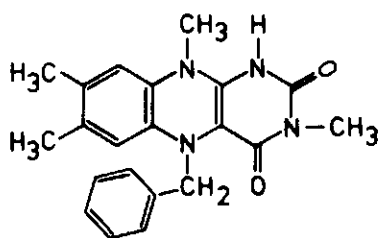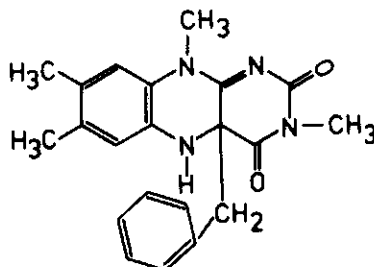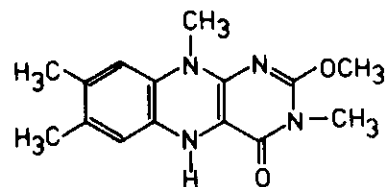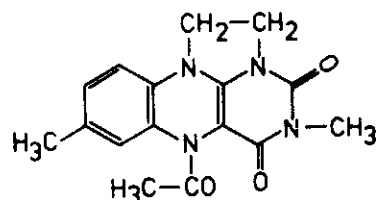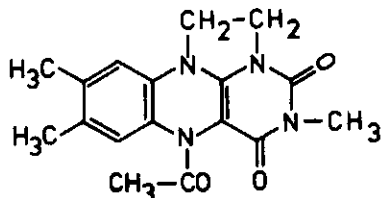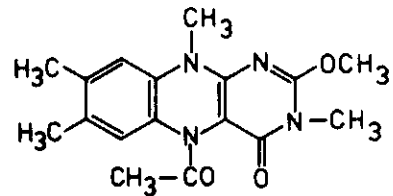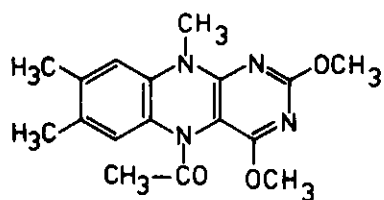
$R^{I} = CH_2(CHOCOCH_3)_3 CH_2OCOCH_3$

$R^{II} = (CH_2)_4 CH_2OCOCH_3$

$R^{III} = (CH_2)_{10} CH_3$

Table 2. ¹³C-Chemical Shifts (in ppm) of Various 1,5-Dihydroflavin Derivatives^a)

No.^b)	C(2)	C(4)	C(4a)	C(5a)	C(6)	C(7)	C(8)	C(9)	C(9a)	C(10a)	C(1')	C(3')	C(7')	C(8')	C(10')	C(10'a)	C(10'e)	C(5'a)	C(5'β)
1	155.9	161.4	136.0	134.9	133.1	136.8	147.8	115.6	131.9	149.2	–	28.7	19.4	21.4	–	45.3	62.0^c)	–	–
2	150.6	157.0	105.2	136.0	116.1	133.6	129.0	118.0	128.2	137.1	–	27.3	18.9	18.9	–	47.4	62.0^d)	–	–
3	150.4	156.9	104.5	137.0	115.1	135.7	121.6	116.0	128.6	138.1	–	27.3	20.7	–	–	47.7	62.1^d)	–	–
4	152.3	158.3	99.9	128.2	127.8	132.9	135.1	115.8	129.9	147.1	–	27.7	19.1	19.5	–	44.1	62.0^d)	171.4	21.8
5	152.3	158.1	98.4	129.2	127.7	132.7	135.2	115.8	134.9	147.7	–	28.3	19.3	20.0	–	44.3	64.0^e)	171.5	21.6
6	152.4	158.2	98.1	129.0	127.6	132.5	135.1	115.8	134.9	147.9	–	27.7	19.3	20.0	–	44.4	14.1^f)	171.6	21.5
7	152.0	158.7	99.4	129.6	127.7	132.7	135.4	116.1	129.7	149.6	–	–	19.3	19.8	–	44.2	62.1^d)	171.0	21.4
8	152.2	158.3	105.8	133.0	126.7	133.2	135.3	119.3	138.6	150.8	40.3	28.8	19.5	19.7	33.8	–	–	171.0	22.0
9	151.5	157.7	111.8	137.7	123.7	134.3	129.8	115.6	131.2	138.4	45.5	28.2	19.0	19.3	31.1	–	–	–	–
10	152.2	158.5	103.8	135.2	122.8	132.7	131.3	114.5	138.2	145.6	–	27.3	19.0	19.4	32.0	–	–	44.5	–
11	152.0	158.8	101.3	135.9	124.4	132.5	131.7	114.4	137.1	147.6	–	27.3	19.0	19.4	31.8	–	–	50.5	11.3
12	151.9	159.0	101.2	n.o.^g)	124.8	132.3	137.5	114.8	n.o.^g)	147.8	–	27.3	19.1	19.5	31.8	–	–	60.6	–
13	161.7	169.1	59.0	n.o.^g)	117.3	110.4	110.0	117.5	n.o.^g)	155.6	–	27.6	19.4	19.4	31.1	–	–	–	–^h)
14	151.3	154.3	106.3	133.8	114.3	133.8	127.9	114.5	127.7	146.9	–	28.9	18.7	19.0	27.7	–	–	–	–^i)
15	150.5	158.4	93.2	127.8	127.3	134.2	128.1	112.3	134.4	148.5	47.0	28.2	20.9	–	44.0	–	–	172.7	22.0
16	150.4	158.4	93.3	124.9	128.3	132.4	134.4	113.8	134.3	148.5	47.0	28.2	19.4	19.7	44.1	–	–	172.8	21.8
17	154.6	157.4	99.9	126.6	127.1	130.8	134.8	114.4	136.8	156.0	–	28.2	19.2	19.9	30.6	–	–	171.2	21.3^k)
18	161.0	161.9	100.1	126.6	127.1	130.8	135.2	114.9	136.8	163.2	–	–	19.2	19.9	30.6	–	–	171.2	21.3^l)

a) Spectra were obtained in CDCl₃.

b) For the structure of the compounds, see Scheme 1 and 2.

c) Chemical shifts of the other side chain C-atoms: 69.2, 69.5, 70.6 (C(10'β,γ,δ)); 21.0, 20.8, 20.7, 20.3 (CH₃CO(10'β,γ,δ,ε)); 170.8, 170.5, 170.1, 169.9 (CH₃CO(10'β,γ,δ,ε)).

d) Chemical shifts of the other side chain carbon atoms: 69.7, 70.0, 70.1 (C(10'β,γ,δ)); 20.6 (CH₃CO(10'β,γ,δ,ε)); 170.8, 170.1, 170.0, 169.8 (CH₃CO(10'β,γ,δ,ε)).

e) Chemical shifts of the other side chain C-atoms: 27.4, 27.8, 28.2 (C(10'β,γ,δ)); 171.5 (CH₃CO(10'ε)); 20.9 (CH₃CO(10'ε)).

f) Chemical shifts of the other side chain C-atoms: 31.9, 29.6, 29.3, 29.1, 26.6, 22.7, 21.4.

g) n.o. = not observed.

h) C(4a'α): 43.5.

i) C(2'β): 55.2.

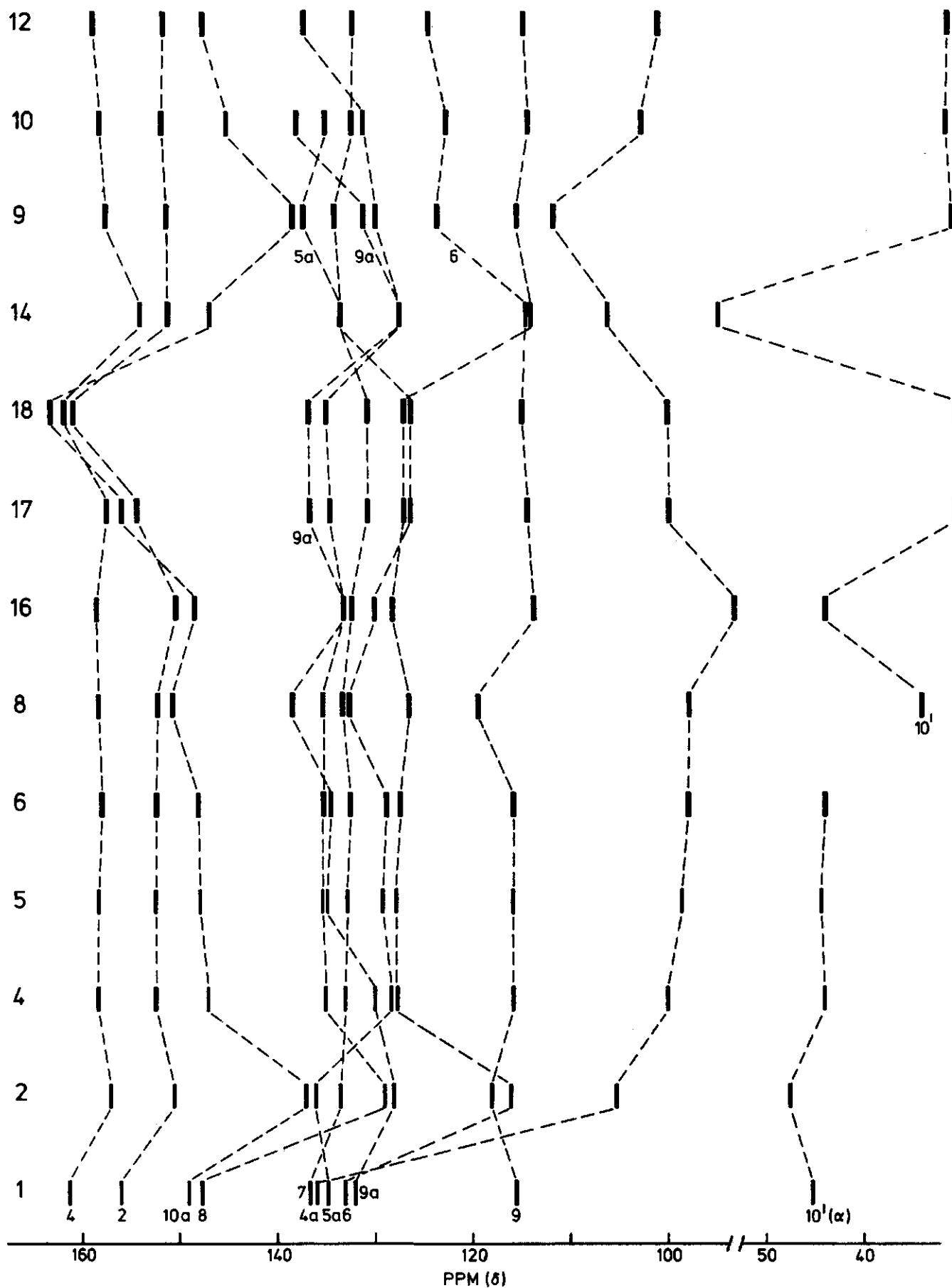k) C(2'β): 55.5.

l) C(2'β): 54.9; C(4'β): 54.2.

Fig. 3. *¹³C-chemical shift correlation diagram for various flavin derivatives.* The numbers on the horizontal axis refer to the C-atoms of the flavin molecule *(cf. Scheme 1),* the numbers on the vertical axis are related to the structure of the individual derivatives *(cf. Scheme 2).*

reasons in our calculations the methyl groups in the structures mentioned were replaced by protons. The calculated electron densities and chemical shifts are given in *Table 3*. The results show that for the oxidized molecule a fair and for the reduced molecule a rather good correlation exists between experiment and theory. Plotting the calculated chemical shifts against the experimental ones gives a correlation coefficient (r^2) of ≥ 0.96 for the reduced and of ≥ 0.85 for the oxidized molecule. The slope of a plot of calculated π-electron densities against the experimental chemical shifts gives a value of 147 ppm/π-electron, in fair agreement with the theory [29]. For the reduced molecule only the chemical shift for C(2) and C(9) are predicted less accurately. The downfield shift of the resonances of C(5a), C(9) and C(10′) (*Table 2* and *Fig. 3*) and the upfield shift of the resonances of all other C-atoms in the flavin molecule observed upon reduction can thus be explained by a decrease and an increase, repectively, of π-electron density at the atom under consideration. The only exception to this statement is observed for C(7) where the theory predicts a downfield shift but an upfield shift is found experimentally. The fact the experimental chemical shifts of C(2) and C(4) of the oxidized and reduced molecule are inversed as compared to the theoretical calculations might indicate that neighbouring bond anisotropy influences the chemical shifts considerably. This suggestion is supported by findings on uracil derivatives where the corresponding C-atoms show an identical behaviour as those in the flavin molecule [34].

From the relationship 160 ppm/π-electron [29] it is estimated that only about 20% of an electron is added to the benzene ring of flavin upon reduction of the molecule. Furthermore, the theoretical data also show a remarkable change in the density matrix of the p_z-orbitals of C(4a) and C(10a). In spite of an increase of the π-electron density at C(4a) and C(10a), the cross terms between the p_z-orbitals of C(4a) and C(10a) are increased as compared to those of C(5a), C(9a), C(6), C(7), C(8) and C(9). This indicates that a fairly localized double bond exists between C(4a) and C(10a), which is in agreement with crystallographic studies [30]. This fact and the π-electron density increase at C(4a) and C(10a) as well as the rather drastic increase of π-electron density at N(5) and N(1) explain reasonably the observed upfield shift of the resonances due to C(4a) and C(10a).

It should be noted that the theory (*Table 3*) predicts a π-electron density decrease at N(10) upon reduction of the flavin molecule. Preliminary ^{15}N-NMR. experiments [35] showed that N(10) indeed undergoes a downfield shift upon reduction whereas the other three N-atoms of flavin shift upfield as expected. This decrease in π-electron density at N(10) can thus explain the downfield shift of C(10′a) and C(9).

The higher magnetic equivalence of the pairs of C(6) and C(9), and C(7) and C(8) in **2** as compared to those in **1** indicates that the conjugation *via* C(8)–C(6)–N(5)–C(10a)–C(2) is decreased in **2** as compared to that in **1** [8].

The finite pertubation theory [36] makes it possible to estimate ^{13}C, ^{13}C-coupling constants. The computed constants are in reasonable agreement with the experimental values (*Table 1*). The latter values are in the range expected for sp^2, sp^2-interactions [37]. Available data suggest that direct ^{13}C-coupling constants are approximately correlated to the s character of the orbitals making up the bond [38]. Neglecting possible influences of the neighbouring heteroatoms on the direct

Table 3. *Calculated Total and π-Electron Densities of Oxidized and Reduced Flavin and their Comparison with Calculated and Observed Chemical Shifts*

Atom	Oxidized Electron density				Reduced Electron density			
	Total	π	δC_{calc}	δC_{obs}[a])	Total	π	δC_{calc}	δC_{obs}[b])
C(2)	3.49	0.80	160.5	155.9	3.48	0.82	157.3	150.6
C(4)	3.60	0.82	157.3	161.4	3.59	0.82	157.3	157.0
C(4a)	3.98	1.03	123.7	136.0	4.09	1.16	102.9	105.2
C(5a)	3.94	1.05	120.5	134.9	4.10	0.96	134.9	136.0
C(6)	3.98	0.97	133.3	133.1	4.04	1.05	120.5	116.1
C(7)	3.99	1.02	125.3	136.8	4.03	0.99	130.1	133.6
C(8)	3.96	0.96	134.9	147.7	4.00	1.02	125.3	129.0
C(9)	4.03	1.05	120.5	115.6	4.00	1.01	126.9	118.0
C(9a)	3.89	0.96	134.9	131.9	3.92	1.02	125.3	128.2
C(10a)	3.72	0.85	152.5	149.2	3.75	0.95	136.5	137.1
N(1)	5.41	1.35			5.24	1.70		
N(3)	5.24	1.70			5.24	1.67		
N(5)	5.09	0.94			5.17	1.73		
N(10)	5.06	1.59			5.19	1.35		

[a]) Chemical shifts of compound 1.
[b]) Chemical shifts of compound 2.

coupling constants the results in *Table 1* indicate that in the oxidized molecule the C(4)–C(4a) bond possesses a higher *s* character than the C(4a)–C(10a) bond. In the reduced molecule the *s* character of both bonds is more similar. Keeping in mind the above mentioned restrictions it can be expected that the experimentally determined coupling constants show a correlation to the bond lengths determined crystallographically [30]. The following bond lengths were published [30]. For the oxidized and reduced molecule, respectively: 0.148 and 0.142 nm for the C(4),C(4a)-bond, and 0.145 and 0.136 nm for the C(4a),C(10a)-bond. Comparing the corresponding values (coupling constant *vs.* bond lengths) of the oxidized with those of the reduced molecule a fair relationship is observed but the values within the oxidized molecule correlate less. This suggests that the effect of the neighbouring polar atoms on the direct coupling constants is probably appreciable.

The experimental two-bond coupling constant is larger in the oxidized molecule *(Table 1)*. Since two-bond coupling constants are also dependent on the bond angles [22] the results are in agreement with crystallographic data, *i.e.* coplanar *vs.* folded structure of oxidized and reduced flavin, respectively.

The assignment presented in this paper for 1 and 2 are in full agreement with the results of *Kawano et al.* [9] who used riboflavintetrabutyrate in their study. The more recent published data of *Ghisla et al.* [12] agree also with our data except for C(4a) and C(7) in 1, where the order must be reversed. In addition these authors could not assign unequivocally the resonance lines due to C(6), C(7), C(8) and C(9) in 2 and 13.

In this work and the published work of others [9] [12] tetraacetylriboflavin (1) was used as a model compound because of the high solubility of its oxidized and reduced form in $CHCl_3$. It has not been realized, however, that the carbonyl groups of the ester functions of the ribityl side chain might influence some of the chemical

shifts of the isoalloxazine ring. The influence of these ester functions is negligible in 1, because the chemical shifts of 1 are identical with those of lumiflavin [8] and the oxidized form of 6 (this work, not shown) as far as the C-atoms of the isoalloxazine ring are concerned. The situation is different for 2 (= the reduced form of 1). A comparison of the chemical shifts of 4 to 6 reveals that the shifts for 5 and 6 are very similar, if not identical, and that the most significant difference between 4 and the compounds 5 and 6 is observed for C(9a) which is shifted upfield by 5.0 ppm in 4 as compared to that in 5 and 6. These results indicate that the upfield shift of C(9a) in 2 to 4 is induced by the electric field effect of one of the carbonyl groups of the side chain ester functions. The small comformational change of the side chain observed upon reduction of 1must, therefore, be related to the upfield shift of C(9a) in 2 to 4 as compared to that in 5 and 6. Hence, one must be careful in relating the chemical shifts of the esterified compounds with those of e.g. reduced riboflavin monophosphate.

The effects of substitution at different atoms in reduced flavin are partly rather complex. But this fact is not surprising considering the complex structural behaviour of reduced flavin. From crystallographic data, describing only static structures, it is known that the conformation of reduced flavin is influenced by substitution at N(1) and/or N(5) [39–41], i.e. the angle between the normals of the benzene and the pyrimidine subnucleus of the flavin molecule is increased upon substitution. In addition the relative orientation of the N(5)-substituent with respect to the pyrazine subnucleus is also altered [40] [41]. Preliminary studies on the temperature dependence of the ^{13}C-chemical shifts of e.g. 4 show that already at –18° at least two different molecular forms are present in solution. These findings are in contradiction with published work of others [42] who showed that the ^{1}H-NMR. spectrum of a similar compound was not influenced at all by a decrease of the temperature down to –107°. Since the ^{13}C-chemical shifts are obviously more sensitive to structural changes than the ^{1}H-chemical shifts we have decided to devote a separate study to the observed temperature dependence of the ^{13}C-chemical shifts in order to elucidate the structural features of reduced flavin in more detail. These results will be published in a forthcoming paper. Nevertheless, at this moment we wish to discuss shortly some of the substitution effects. Methylation of C(2a) of the flavin molecule does not affect the chemical shifts of the C-atoms of the benzene subnucleus whereas the chemical shifts of the C-atoms of the pyrimidine subnucleus are additive (17, 18). Acetylation of N(5)H (cf. e.g. 4 vs. 2) leads to large upfield shifts of C(4a) (5.3 ppm) and C(5a) (7.8 ppm) and large downfield shifts of C(6) (11.7 ppm), C(8) (6.1 ppm) and C(10a) (10.0 ppm). Similar shifts are observed when 17 and 14 are compared with each other. On the other hand comparing 8 and 9 it is learned that the upfield shift of the C-atoms in question and the downfield shift of C(8) and C(10a) is still about the same as mentioned above but the downfield shift of C(6) is decreased by about 9 ppm. Since in 4, 8 and 17 the N(5) substituent is the same and N(1) methylation the chemical shift of C(6) not affects (cf. 8 vs. 6) the observed effect must be related to the relative stereochemical position of the acetyl group with respect to the C(6)-atom[9]). From a comparison of 11 vs. 2 it

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[9]) It is a reasonable assumption that the acetyl group excerts the same electronic influence via the bonds on the shielding of the aromatic C-atoms in all three compounds.

can be concluded that the methyl group of the N(5) ethyl substituent causes a down-field shift of C(6) (δ-effect). Hence the acetyl group in 4 and 17 must be closer to the C(6)-atom than in 8. This interpretation is in agreement with crystallographic data [41] [42] and results obtained with stereoisomers of a partially related system, i.e. N-acetyl-tetrahydrochinolin [43].

Finally the relationship observed between the calculated π-electron densites and the experimental ^{13}C-chemical shifts suggests that a similar correlation can be expected with the chemically active atoms of the flavin molecule. Oxidized flavin in the ground state adds nucleophiles preferentially at the centers N(5) (SO_3^{2-}, $P(C_6H_5)_3$) [44] [3] and C(10a) (CH_3O^-) [45]. The centers C(2) and C(8) are chemically less reactive but undergo nucleophilic substitution reactions [46]. An electrophilic substitution reaction is known to occur at C(9) [47]. The reduced flavin molecule, on the other hand, reacts with electrophiles preferentially at the centers N(5) and C(4a) [17, 12] and probably also at C(10a) [24, 48]. These observations demonstrate that indeed a good correlation also exists between the calculated π-electron densites and the observed chemical shifts, and the experimentally observed centers of chemical reactivity of the flavin molecule. This relationship should, therefore, be very helpful for the chemical characterization of new flavin derivatives and flavoenzymes.

REFERENCES

[1] 'Reactivity of Flavins', K. Yagi, ed., University of Tokyo Press 1975; 'Flavins and Flavoproteins', T.P. Singer, ed., Elsevier Publishing Company, Amsterdam 1976; 'Flavins and Flavoproteins: Physiochemical Properties and Function', W. Ostrowski, ed., Polish Scientific Publishers, Warsaw and Cracow 1977.

[2] V. Massey, F. Müller, R. Feldberg, M. Schuman, P.A. Sullivan, L.G. Howell, S.G. Mayhew, R.G. Matthews & G.P. Foust, J. Biol. Chem. 244, 3999 (1969).

[3] F. Müller, Z. Naturforsch. 27b, 1023 (1972).

[4] K.H. Dudley, A. Ehrenberg, P. Hemmerich & F. Müller, Helv. 47, 1354 (1964).

[5] A.J.W.G. Visser & F. Müller, Helv. 62, 593 (1979).

[6] G. Weber, Biochem. J. 47, 114 (1950).

[7] H.J. Grande, C.G. van Schagen, T. Jarbandhan & F. Müller, Helv. 60, 348 (1977).

[8] H.J. Grande, R. Gast, C.G. van Schagen, W.J.H. van Berkel & F. Müller, Helv. 60, 367 (1977).

[9] K. Kawano, N. Ohishi, A.T. Suzuki, Y. Kyogoku & K. Yagi, Biochemistry 17, 3854 (1978).

[10] C.G. van Schagen & F. Müller, in 'Flavins and Flavoproteins: Physicochemical Properties and Functions', W. Ostrowski, ed., Polish Scientific Publishers, Warsaw and Cracow 1977, p. 9.

[11] C.G. van Schagen & F. Müller, Helv. 61, 3139 (1978).

[12] S. Ghisla, J.W. Hastings, V. Favaudon & J.M. Lhoste, Proc. Natl. Acad. Sci. USA 75, 5860 (1978).

[13] K. Bock & C. Pedessen, J. Magn. Reson. 25, 227 (1977).

[14] K.H. Dudley & P. Hemmerich, Helv. 50, 355 (1967).

[15] J.W.H. Walker, P. Hemmerich & V. Massey, Helv. 50, 2269 (1967).

[16] F. Müller, Ph.D. Thesis, Basel 1964.

[17] *S. Ghisla, U. Hartmann, P. Hemmerich & F. Müller,* Liebigs Ann. Chem. *1973,* 1388.

[18] *R. Gast & F. Müller,* Helv. *61,* 1353 (1978).

[19] *J. K. Eweg, F. Müller, A.J. W.G. Visser, C. Veeger, D. Bebelaar & J.D. W. van Voorst,* Photochem. Photobiol. *30,* 463 (1979).

[20] *J.A. Pople & D.L. Beveridge,* in 'Approximate Molecular Orbital Theory', McGraw-Hill, New York 1970, p.163.

[21] Quantum Chemistry Program Exchange 281.

[22] *P.E. Hansen,* Org. Magn. Reson. *11,* 215 (1978).

[23] *S. Ghisla, B. Entsch, V. Massey & M. Husain,* Eur. J. Biochem. *76,* 139 (1977).

[24] *F. Müller,* in 'Flavins and Flavoproteins', H. Kamin, ed., University Park Press, Baltimore 1971, p.363.

[25] *F.A. Bovey,* 'Nuclear Magnetic Resonance Spectrocopy', Academic Press, New York 1969, p.231.

[26] *B. Grabe,* Acta Chem. Scand. Ser. A. *28,* 363 (1974).

[27] *B. Pullman & A. Pullman,* Proc. Natl. Acad. Sci. USA *45,* 136 (1959).

[28] *P.S. Song & M. Sun,* in 'Chemical and Biochemical Reactivity', The Israel Academy of Sciences and Humanities, Jerusalem 1974, p.407.

[29] *J.B. Stothers,* 'Carbon-13 NMR Spectroscopy', Academic Press, New York 1972. p.91.

[30] *M. Leijonmarck,* 'On the Structures of Flavin Derivatives, Crystallographic Studies and a Review', Chemical Communications, No. 8, University of Stockholm, Stockholm 1977.

[31] *M. Wang & C.J. Fritchi,* jr., Acta Crystallogr. B *29.* 2040 (1973).

[32] *R. Norrestam & M. von Glehn,* Acta Crystallogr. B *28,* 434 (1972).

[33] *J.K. Eweg, F. Müller, H. van Dam, A. Terpstra & A. Oskam,* J. Am. Soc. *102,* 51 (1980).

[34] *A.R. Tarpley, jr. & J.H. Goldstein,* J. Am. Chem. Soc. *93,* 3573 (1971).

[35] *H.-D. Franken, H. Rüterjans & F. Müller,* Hoppe-Seyler's Z. Physiol. Chem. *361,* 246 (1980).

[36] *G.E. Maciel, J. W. McIver, jr., N.S. Ostlund & J.A. Pople,* J. Am. Chem. Soc. *92,* 11 (1970).

[37] See [29], p. 370.

[38] *F.J. Weigert & J.D. Roberts,,* J. Am. Chem. Soc. *94,* 6021 (1972).

[39] *R. Norrstam, M. van Glehn, L.O. Hagmann & P. Kierkegaard,* Acta Chem. Scand. *23,* 2199 (1969).

[40] *R.E. Werner & O. Rönnquist,* Acta Chem. Scand. *24,* 997 (1970).

[41] *R. Norrestam, P. Kierkegaard, B. Stensland & L. Torbjörnsson,* Chem. Commun. *1227,* 1250 (1968).

[42] *L. Tauscher, S. Ghisla & P. Hemmerich,* Helv. *56,* 630 (1973).

[43] *H. Fritz & T. Winkler,* Helv. *59,* 903 (1976).

[44] *F. Müller & V. Massey,* J. Biol. Chem. *244,* 4007 (1969).

[45] *C.G. Van Schagen, H.J. Grande & F. Müller,* Recl. Trav. Chim. Pays Bas *97,* 179, (1978).

[46] *P. Hemmerich, C. Veeger & H.C.S. Wood,* Angew. Chem. *77,* 699 (1965).

[47] *D.B. McCormick,* J. Heterocycl. Chem. *4,* 629 (1967).

[48] *H.I.X. Mager,* in 'Flavins and Flavoproteins', K. Yagi and T. Yamano, eds., Japan Scientific Societies Press, Tokyo 1980 p.455.

CHAPTER 2

A ^{13}C Nuclear-Magnetic-Resonance Study on Free Flavins and *Megasphaera elsdenii* and *Azotobacter vinelandii* Flavodoxin

^{13}C-Enriched Flavins as Probes for the Study of Flavoprotein Active Sites

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Selectively ^{13}C-enriched free and protein-bound flavins in the oxidized and two-electron reduced state were investigated by the ^{13}C nuclear magnetic resonance technique. FMN in aqueous solution and N(3)-methyl-2′,3′,4′,5′-tetraacetylriboflavin in an apolar solvent were used as reference compounds for the protein-bound FMN.

A comparison of the chemical shifts of FMN in aqueous solution with those of N(3)-methyltetraacetylriboflavin in CHCl$_3$ reveals that FMN is strongly polarized yielding a pseudo-ionic molecule stabilized by hydrogen bonding of H$_2$O with C-2α of the isoalloxazine molecule. The interpretation is fully supported by the one-bond (^1J) ^{13}C coupling constants. The chemical shifts combined with the coupling constants indicate the change of charge distribution within the molecule when going from apolar to polar solutions.

Binding of FMN to *Megasphaera elsdenii* and to *Azotobacter vinelandii* apoflavodoxins affects the resonances due to C-2 and C-10a as compared to free FMN. These shifts together with the coupling constants indicate the formation of a hydrogen bond between C-2α of FMN and an amino acid residue of the apoprotein. In *M. elsdenii* flavodoxin such a hydrogen bond also exists with C-4α which is not observed in *A. vinelandii* flavodoxin.

The chemical shifts to the two-electron reduced derivatives of FMN and N(3)-methyl-2′,3′,4′,5′-tetraacetylriboflavin clearly indicate that the FMN derivative possesses a more planar conformation than its analog; it shows pH-dependent chemical shifts of the atoms C-10a, C-2 and C-4a. The chemical shift of C-4 is pH-independent. From the pH-dependence of the chemical shifts an ionization constant of 6.7 is calculated.

The chemical shifts of protein-bound FMN in the reduced state are very similar to those of the anionic, reduced FMN. The data show that in both flavodoxins studied the prosthetic group when reduced is bound in the anionic form an possesses approximately a coplanar conformation.

The results are discussed with respect to the possible biological implications. It is suggested that specific interaction of the prosthetic group with the apoprotein, the ionization state and the degree of planarity of the reduced prosthetic group are factors determining the biological functions of flavodoxins and possibly the functions of flavoproteins in general.

Flavoproteins are involved in the catalysis of a broad spectrum of biological reactions, see e.g. [1]. The question why one and the same chemical entity, i.e. isoalloxazine, embedded in different apoflavoproteins is able to catalyze specifically a particular biological reaction could not be explained by the wealth of kinetic results available on flavoproteins. In fact the approach by chemical kinetics will probably never lead to the information needed to explain this 'mystery'. However, the interaction between the prosthetic group and the apoenzyme (strength of binding, specific interaction between certain atoms of isoalloxazine and amino acid residues) must be an important factor in determining the specificity of a particular flavoprotein. In this context we have put forward a hypothesis deduced from model studies [2]. In order to obtain a better insight into the interactions mentioned and to test the proposed model we have set up a program

devoted to this problem. One of the most powerful tools to achieve this goal is the nuclear magnetic resonance (NMR) technique. This method yields information on the molecular and submolecular structure of the molecule investigated and is, therefore, particularly suited to yield information which can be correlated to the chemical properties of the molecules studied.

The overwhelming numbers of protons in a biomolecule makes it very difficult to observe the resonances of flavin in flavoproteins by the ^{1}H-NMR technique. In addition, the very few proton resonances observable from flavins [3] would contribute little to the desired information. For this reason we made use of selectively ^{13}C-enriched prosthetic groups. Furthermore, ^{13}C-chemical shifts represent, in general, the charge distribution in a molecule much better than other nuclei. This fact should, therefore, allow a correlation of the chemical shift differences between free and protein-bound flavin with the site of interaction of flavin with the apoprotein. Moreover, the use of isotopically rather than chemically substituted prosthetic groups has the advantage that subtle, but important, interactions with the apoprotein are not affected.

In the present paper we report on the NMR properties of free flavin and the flavodoxins from *Megasphaera elsdenii* and from *Azotobacter vinelandii* in the oxidized and two-electron reduced state. The prosthetic group was selectively labelled at positions C-2, C-4, C-4a and C-10a. Some preliminary results were reported elsewhere [4,5]. A similar but less detailed study was conducted by Yagi et al. [6] on the oxidized form of the riboflavin-binding protein from egg white.

MATERIALS AND METHODS

The selectively ^{13}C-enriched riboflavin derivatives were synthesized from ^{13}C-labelled barbituric acids by condensation with 2-(phenylazo)-4,5-dimethyl-*N*-ribitylaniline (gift of Hoffmann-La Roche, Basel, Switzerland) in a mixture of glacial acetic acid and butanol [7]. The barbituric acid derivatives were prepared from [^{13}C]urea and [2-^{13}C]-, [1,3-[13]C$_2$]- and [1,2,3-[13]C$_3$]diethylmalonate (90−92.5 atom-%, Prochem, UK) as described earlier [8]. The yields of riboflavin were 66−71% (cf. 65.8% [7]).

Riboflavin was phosphorylated by the method of Scola-Nagelschneider and Hemmerich [9]. The crude FMN thus obtained was purified on a column (3.2 × 12 cm) of DEAE-cellulose (DE-52) equilibrated with distilled water (20 °C). 280 mg of FMN was dissolved in distilled water (40 ml) and the pH of the solution adjusted to 7.0 with a 5 mM NaOH solution. This solution was poured onto the column and the column exhaustively washed with distilled water (several liters) eluting three bands. The first band consisted of a small amount of lumichrome, the second band contained riboflavin (15 mg) and the third band contained an unidentified, non-fluorescent compound. Elution with a 25 mM solution of ammonium carbonate released first riboflavin 4′-monophosphate (33 mg). The second band contained FMN (160 mg). Further elution of the column with a 100 mM ammonium carbonate solution gave another yellow-green fluorescent compound which is probably a riboflavin diphosphate derivative (67 mg). All handling was carried out in the dark. The collected fractions were freeze-dried yielding salt-free compounds. The purity of the compounds was judged by thin-layer chromatography in the solvent mixture butanol/glacial acetic acid/water (7:2:2, by vol.). Tetraacetylriboflavin was prepared from riboflavin as described earlier [10].

The following flavoproteins were isolated and purified according to published procedures: flavodoxin from *Megasphaera elsdenii* LC1 [11], flavodoxin from *Azotobacter vinelandii* OP [12]. The corresponding apoflavoproteins were prepared by known methods: *M. elsdenii* apoflavodoxin according to Mayhew [13] and *A. vinelandii* apoflavodoxin as reported by Edmondson and Tollin [14]. The reconstitution of the apoflavoproteins with the prosthetic group was conducted in neutral, buffered solutions at 4 °C. Before use the proteins were dialyzed exhaustively against the desired buffer solution.

^{13}C-NMR spectra of free flavins were obtained on a Varian XL-100-15 NMR spectrometer equipped with a 16 K 620-L computer and operating at 25.2 MHz. The spectra were acquired in the Fourier transform mode at 26 °C using 12-mm taperlock tubes (purchased from Wilmad). For the accumulation of the spectra 8 K data points were used in the time domain and converted to 4 K data points in the frequency domain. The spectral width was 2560 Hz resulting in 0.68 Hz/point. Tetramethylsilane or 2,2-dimethyl-2-silapentane-5-sul-

fonate served as internal standards. Internal deuterium (C^{2}HCl$_3$, ^{2}H$_2$O) served as a lock signal. The other instrumental conditions were: pulse width 10 μs (30° pulse), acquisition time 1.6 s, repetition rate 2 s. The concentration of the samples was about 10 mM. Oxidized tetraacetylriboflavin was dissolved in C^{2}HCl$_3$ and reduced to the 1,5-dihydro state in the NMR tube by a two-phase system [15]. FMN was dissolved in a mixture of ^{2}H$_2$O/H$_2$O (1:9, by vol.) and buffered with sodium pyrophosphate (50 mM). For pH-dependent studies the pH of the solution was adjusted directly in the NMR tube by addition of small amounts of 0.1 M HCl or 0.1 M NaOH solutions. The pH was measured by means of a specially constructed microelectrode (Wilmad). Reduction was affected by addition of buffered solution of dithionite ($\approx$ 1 M). Usually a 5 − 10-fold excess of dithionite was used with respect to flavin.

Spectra of proteins were recorded on a Bruker HX-360 NMR spectromer (S.O.N. facility at the University of Groningen) operating at a frequency of 90.5 MHz and equipped with a BNC12 data and a NIC-294 disk system. The spectra were accumulated with 16 K in the time domain and 8 K in the frequency domain. The spectral width was 12000 Hz resulting in 1.5 Hz/point. Dioxan served as an internal standard. The data were converted to the tetramethyl-silane scale using (δ_CC$_4$H$_8$O$_2$ = 67.8 ppm). The instrumental settings were: pulse width 15 μs (90° pulse) and a repetition rate of 2 s. The proteins were dissolved in a mixture of ^{2}H$_2$O/H$_2$O (1:9, by vol.) and buffered with sodium phosphate or sodium pyrophosphate solutions (50 mM). Reduction of the proteins and adjustment of the pH were done as described above for FMN. The concentration of the samples of proteins was 1 − 2 mM. The temperature was 20 °C and 10-mm NMR tubes (Wilmad) were used.

RESULTS AND DISCUSSION

Studies on Oxidized Flavins

In order to facilitate the interpretation of the ^{13}C-chemical shifts of protein-bound flavins the free prosthetic group was also studied. Since FMN is only soluble in highly polar solvents the relevant published results [15,16] on *N*(3)-methyl-2′,3′,4′,5′-tetraacetylriboflavin (MeAc$_4$rF) in chloroform are also given for comparison. These latter results are important for a reasonable interpretation of the results obtained on flavoproteins, i.e. to evaluate the influence of the environment and of possible interaction(s) of the prosthetic group with the apoflavoprotein on the chemical shifts.

The ^{13}C chemical shifts of FMN and MeAc$_4$rF in the oxidized state are summarized in Table 1. The results show that, except for C-4a, the resonances of FMN in aqueous solution appear at lower field than the corresponding resonances of MeAc$_4$rF in chloroform. The largest downfield shift is observed for C-2 in FMN amounting to 2.9 ppm as compared to the corresponding C atom of MeAc$_4$rF in chloroform. The difference in chemical shifts in the two molecules is caused mainly by the larger polarization of the carbonyl functions of the flavin molecule in aqueous than in apolar solvents (cf. Scheme, structure I vs Ia). This is in particular valid for C-2 which is strongly conjugated via C-8 − C-6 − N-5 − C-10a in polar solvents [3,8]. This conclusion is supported independently by recent coherent anti-Stokes Raman scattering spectra [17], and by recent NMR data [16] where we have shown that the chemical shifts in flavin are correlated to the calculated π-electron density distribution in the mole-

Table 1. [13]C chemical shifts of various selectively enriched free and protein-bound flavins

Chemical shifts were measured relative to tetramethylsilane. $\Delta\delta$ = difference in chemical shifts between the oxidized and the (neutral) reduced state; negative values indicate a downfield shift

Compound	Solvent	Oxidation state	Chemical shift of carbon atoms							
			C-2		C-4		C-4a		C-10a	
			δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$
			ppm							
MeAc₄rF	C[2]HCl₃	oxidized	155.9		161.4		136.0		149.2	
				5.3		4.4		30.8		12.1
MeAc₄rFH₂	C[2]HCl₃	reduced	150.6		157.0		105.2		137.1	
FMN	pH 7.5	oxidized	158.8		162.6		135.2		151.2	
				7.7		5.3		32.8		7.2
FMNH₂	pH 5.0[a]	reduced	151.1		157.3		102.4		144.0	
				1.2[c]		5.3[c]		34.1[c]		−3.8[c]
FMNH⁻	pH 9.0[b]	reduced	157.6		157.3		101.1		155.0	
M. elsdenii flavodoxin	pH 7.0	oxidized	159.4		162.6		135.4		153.1	
				2.9		7.7		32.3		−1.1
	pH 7.8[d]	reduced	156.5		154.9		103.1		154.2	
A. vinelandii flavodoxin	pH 8.0	oxidized	159.3		161.7		135.3		154.8	
				1.2		6.8		33.1		−0.1
	pH 8.0	reduced	158.1		154.9		102.2		154.9	

[a] Neutral form.
[b] Anionic form.
[c] Difference in chemical shifts between the oxidized and the anionic, reduced species.
[d] Independent of pH in the range of 7.0−8.5.
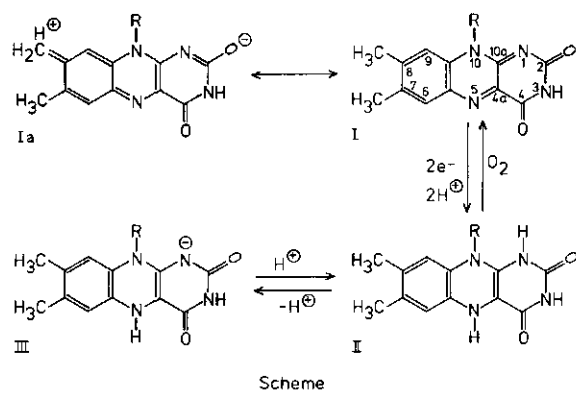
Scheme. Formulae of FMN (I), FMNH₂ (II) and FMNH⁻ (III). R = ribityl 5′-phosphate. In N(3)-methyl-2′,3′,4′,5′-tetraacetylriboflavin (MeAc₄rF), R = ribityl 2′,3′,4′,5′-tetraacetate and the H on N-3 is replaced by CH₃

cule. Hence, the downfield shift of the resonances of C-2 and C-4 in FMN, as compared to those in MeAc₄rF, can be explained by a decreased shielding of these atoms. As a consequence of the partial positive charge on C-2 and C-4 the carbon atom in *meta* position should shift downfield due to mesomeric effects. This is indeed observed for the resonance of C-10a in FMN. The upfield shift of the resonance of C-4a in FMN is caused mainly by the electric field induced by the partial positive charge on C-4 located in *ortho* position to C-4a. A similar, but smaller effect originating from C-2 contributes also to the upfield shift of C-4a *para* to C-2. The explanations offered above are supported by the [13]C-^{13}C coupling constants (Table 2). Published data strongly suggest that one-band ^{13}C coupling constants are approximately

Table 2. Direct [13]C-^{13}C coupling constants in free and protein-bound [4,4a,10a-[13]C₃]flavins in the oxidized and reduced state

The accuracy of coupling constants is ± 0.2 Hz for the free flavins and ± 0.4 Hz for the protein-bound flavin. N(3)-Methyl-2′,3′,4′,5′-tetraacetylriboflavin was measured in C[2]HCl₃, FMN and M. elsdenii flavodoxin in phosphate buffer, pH 7.5

Redox state	Compound	Coupling constants	
		$^1J_{C4-C4a}$	$^1J_{C4a-C10a}$
		Hz	
Oxidized	MeAc₄rF	76.5	53.3
	FMN	75.4	55.9
	M. elsdenii flavodoxin	76.5	58.8
Reduced	MeAc₄rFH₂	79.2	84.5
	FMNH₂ (pH 5.0)[a]	88.0	93.0
	FMNH⁻ (pH 8.0)[b]	74.2	74.2
	M. elsdenii flavodoxin[b]	70.6	79.4

[a] Neutral species, the given coupling constants were calculated from the pH dependence between 5.5−8.5.
[b] Anionic species.

correlated to the s-character of the orbitals making up the bond [18]. It should, however, be noted that these coupling constants could be influenced considerably by neighbouring polar atoms. Nevertheless, it seems a reasonable approximation [18] to estimate the hybridisation state of the binding orbitals by the following equation [19]:

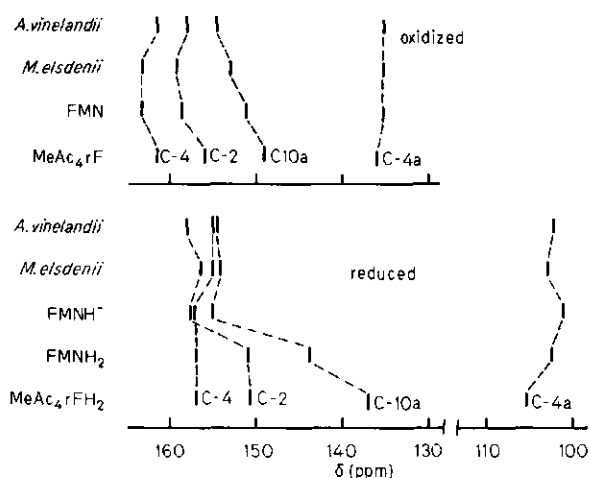$$J = 7.3 \frac{s_1 \cdot s_2}{100} - 17 \text{ Hz} \tag{1}$$

Fig. 1. *Correlation diagram of* [13]*C NMR chemical shifts of* N (3)-methyl-2',3',4',5'-tetraacetylriboflavin, FMN, M. elsdenii *and* A. vinelandii *flavoproteins in the oxidized and reduced state*

where J is the experimentally determined [13]C-^{13}C coupling constant and s the percentage s-character of the orbitals in question. Since the equation can only be solved when we assume that s_1 equals s_2, the calculated values always contain the contributions from two atoms, e.g. C-4a and C-10a, which should be kept in mind. Therefore, no calculated values are presented to avoid the impression that values calculated from Eqn (1) possess a quantitative rather than a qualitative meaning. Nevertheless, the qualitative content of such values is very useful to disclose the general trends needed to support or reject a certain interpretation of the chemical shifts. A comparison of the coupling constants of MeAc₄rF and FMN (Table 2) shows that the values of the coupling of the C-4—C-4a bond vary much less than those of the C-4a—C-10a bond. The small decrease in the coupling constant of the C-4—C-4a bond in FMN, as compared to that in MeAc₄rF, indicates that the chemical shifts of the carbon atoms making up the bond and especially that of C-4a is probably more influenced by inductive effects rather than by the π-electron density. supporting the interpretation given above. On the other hand, the increased coupling constant of the C-4a—C-10a bond in FMN, as compared to that in MeAc₄rF, strongly indicates that the net positive charge of the C-10a atom is enhanced by the inductive effect. This again supports the interpretation given above. For conveniance the chemical shifts are presented as a correlation diagram (Fig. 1).

The difference in chemical shifts and coupling constants between FMN and MeAc₄rF indicates that hydrogen bridge formation between FMN and water clearly influences the charge distribution in the molecule.

Binding FMN to the apoprotein from *Megasphaera elsdenii* leads to a further downfield shift of the resonances of C-2 and C-10a as compared to those of free FMN (Table 1). The relatively large downfield shift of the resonance of C-10a indicates a further decrease of the π-electron density at this position as compared to that in free FMN. The increase of the coupling constant of the C-4a—C-10a bond in protein-bound FMN argues that the inductive effect is larger than that in free FMN (Table 2). The π-electron density decrease on C-10a is most probably due to hydrogen bond formation between an amino acid residue of the apoprotein and C-2α of FMN. This interpretation is in agreement with the ob-

served downfield shift of C-2 of the protein-bound FMN and is supported further by model studies. Since the formation of a hydrogen bond to N-1 or C-2α can be considered as an intermediate stage in a proton-transfer reaction, flavins alkylated ('protonated') at N-1 or C-2α should reveal at least the qualitative trends of the chemical shifts expected upon hydrogen-bond formation to the atoms under consideration in protein-bound FMN. Published data show that alkylation at the two sites in question influences the chemical shifts of the carbon atoms of the pyrimidine subnucleus of the flavin very differently [20]. As compared to neutral flavin, alkylation of C-2α leads to a downfield shift of the resonance signal due to C-2 and a (small) upfield shift of the resonances due to C-4a and C-10a. Both alkylation [20] and protonation [21] at N-1, on the other hand, shift all four ^{13}C resonance signals of the pyrimidine subnucleus upfield, the chemical shift differences with the neutral flavin being much more pronounced than in the case of C-2α alkylation. These results strongly suggest that hydrogen-bond formation in *M. elsdenii* flavodoxin occurs at C-2α and not at N-1. Hydrogen bond formation to N-1 should lead to an upfield shift of C-2, C-10a and C-4a as compared to MeAc₄rF or FMN, which is not observed. The results (Table 1) indicate further that the hydrogen-bond formation is more favoured in the protein-bound than in free FMN in aqueous solution. The chemical shifts of C-4 and C-4a of protein-bound FMN are almost identical with the corresponding chemical shifts of free FMN indicating that both molecules possess very similar π-electron densities around the centers C-4 and C-4a and that in both molecules weak hydrogen bonding exists with C-4α.

In flavodoxin from *Azotobacter vinelandii* the chemical shift of C-10a of the prosthetic group is shifted downfield by 3.6 ppm as compared to that of free FMN (Table 1). With respect to flavodoxin from *M. elsdenii* an additional downfield shift by 1.7 ppm is observed. Besides the relative insensitivity of the chemical shift of C-4a towards solvent and binding interactions of the molecule, the chemical shifts of C-2 and C-4 in *A. vinelandii* flavodoxin appear now at higher field as compared to those in *M. elsdenii* flavodoxin. Moreover the chemical shift of C-4 is more similar to that of MeAc₄rF than to that of free FMN. The increased line width of the resonance lines in the spectrum of *A. vinelandii* flavodoxin and the decreased sensitivity prevented the determination of the coupling constants. This does not, however, jeopardize the interpretation of the results since no drastic change of the charge distribution is expected upon binding of FMN to *A. vinelandii* apoflavodoxin as compared to that in *M. elsdenii* flavodoxin. Nevertheless, the similarity of the chemical shift of C-4 in MeAc₄rF and protein-bound FMN indicates that the environment of both compounds at C-4 is very similar, i.e. a rather hydrophobic environment around C-4 in *A. vinelandii* flavodoxin in contrast to *M. elsdenii* flavodoxin (Fig. 1). The similarity of the chemical shift of C-2 in both proteins suggest very similar interactions with the apoprotein at this center.

One observation needs further attention. The resonance due to C-10a shifts gradually downfield in going from MeAc₄rF to *A. vinelandii* flavodoxin (Fig. 1). This downfield shift is only paralleled by a small upfield shift of the corresponding chemical shift of C-4a (Fig. 1). The large downfield shift of C-10a can probably not be explained solely by hydrogen-bond formation to C-2α. On the other hand, the downfield shift argues against a hydrogen bond to N-1 which would show an opposite effect on the chemical shifts of C-10a. Therefore the downfield shift may contain some contribution caused by the N-10 atom and its side chain. Binding of
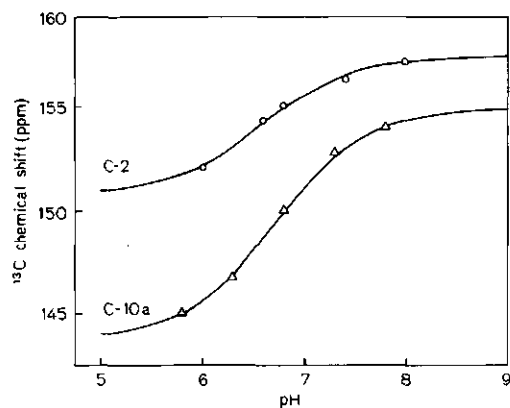
36

Fig. 2. *The pH-dependence of the ^{13}C chemical shifts of reduced [2-^{13}C]-FMN and [4,10a-$^{13}C_2$]FMN in a mixture of $H_2O/^2HO$ (9:1, by vol.). The triangles and circles represent the experimental points, the curve was calculated using pK_a 6.72, $\Delta\delta_{c-2} = 6.5$ ppm and $\Delta\delta_{c-10a} = 11.0$ ppm. Shifts were measured relative to tetramethylsilane*

FMN to apoflavodoxin influences the conformation of the N-10 side chain which is 'locked'. The fixed conformation of the side chain could then influence the configuation of the N-10 atom which in turn could cause a further electron density decrease at C-10a.

Studies on Reduced Flavins

Two-electron reduction of MeAc₄rF or FMN (cf. Scheme, structures II and III) leads to an upfield shift of all resonances when the neutral forms of the two compounds are compared (Table 1). In both compounds the largest upfield shift is observed for C-4a. The pH dependence of the chemical shifts of FMNH₂ is shown in Fig. 2. The experimental points could be fitted by a standard procedure [22] using the following parameters: $pK_a = 6.72$, $\Delta\delta_{C-2} = 6.5$ ppm (difference in chemical shift between neutral and anionic form), $\Delta\delta_{C-10a} = 11.0$ ppm and $\Delta\delta_{C-4a} = 1.3$ ppm. The chemical shift of C-4 is independent of pH. It should be noted that the values for the neutral form of FMNH₂ in Table 1 and 2 are computed values because it was not possible to determine experimentally the chemical shifts at pH values lower than 5.5 because of the formation of a very small amount of flavosemiquinone broadening the resonance lines. The pK_a value of 6.72 is in good agreement with that determined by visible absorption spectroscopy [23]. The fact that the chemical shifts of C-2 and C-10a are affected most when going from FMNH₂ to FMNH⁻ indicates that the negative charge in the anionic form is rather strongly localized on N-1 (cf. Scheme, II ⇌ III). This is supported by the fact that the chemical shift of C-4a is rather insensitive to pH changes. The negative charge on N-1 results in a downfield shift of the resonance of the neighbouring carbon atoms. The negative charge should also lead to an upfield shift of the resonance of C-4a (mesomeric affect) which is probably somewhat counteracted by the electric field of the negative charge resulting in an apparent small upfield shift. Another counteracting effect (i.e. an electron density increase) could also originate from the conformation of the reduced flavin (coplanar vs bent structure).

Comparing the $\Delta\delta$ values between MeAc₄rFH₂ and FMNH₂, some pronounced differences between the two compounds become obvious (Table 1). The chemical shifts of C-10a and C-2 are at lower field in FMNH₂ than in MeAc₄rFH₂. The C-10a resonance of FMNH₂ is much more downfield shifted than that of C-2. The chemical shift of C-4a in FMNH₂ is shifted upfield whereas that of C-4 is almost unaffected in going from an apolar aprotic solvent to an aqueous solution. These differences indicate a conformational difference between the two molecules which, at a first glance, could be related to the difference in solvation of the two molecules. Although the downfield shift of the resonance of C-2 could indicate a hydrogen bond with the oxygen atom of the carbonyl function, the coupling constants (Table 2) should be helpful in revealing the structural differences. The coupling constants of MeAc₄rFH₂ and FMNH₂ increase upon reduction (Table 2). In both cases the coupling constant between C-4a − C-10a increase much more than that between C-4 − C-4a. In addition the increase in coupling constants is much larger in FMNH₂ than in MeAc₄rFH₂. The large increase of the coupling constant of the C-4a − C-10a bond is in accord with the chemical structure of reduced flavin, i.e. double-bond formation between the two centers in the reduced state. The additional increase of about 9 Hz in both coupling constants in FMNH₂ as compared with those in MeAc₄rFH₂ indicates an increase of the s-character of the orbitals making up the bonds between C-4 − C-4a − C-10a in FMNH₂. This decrease in π-electron density in FMNH₂ as compared to MeAc₄rFH₂ manifests itself most clearly in the chemical shift of C-10a which resonates about 7 ppm at lower field than that in MeAc₄rFH₂. This suggests that FMNH₂ possesses a much more planar structure than MeAc₄rFH₂, i.e. the probability of π-electron delocalisation over the whole molecule is higher in FMNH₂ than in MeAc₄rFH₂. Independent support for this suggestion comes from visible absorption spectra [24]. It has been shown that the molar absorption coefficient at a wavelength of 450 nm is correlated with the planarity of the reduced flavin molecule. The published results clearly demonstrate that the molar absorption coefficient of MeAc₄rFH₂ is much smaller (0.89 $mM^{-1} cm^{-1}$) than that of FMNH₂ (1.39 $mM^{-1} cm^{-1}$) [24], in accord with our interpretation. Since the molar absorption coefficients of MeAc₄rFH₂ in aqueous [24] and in an apolar aprotic solvent [25] are identical it must be concluded that the structural difference between MeAc₄rFH₂ and FMNH₂ is caused by the difference in solvation of the two molecules.

Ionization of FMNH₂ drastically influences the carbon-carbon coupling constants (Table 2). Both coupling constants are now identical but smaller than those in FMNH₂. The decrease must be ascribed to two effects. First, the negative charge on N-1 leads to a decrease of the coupling constant where C-10a is involved. A similar effect has been observed with, for example, amino acids upon ionization of the carboxyl group [26,27]. Second, it is known [24] that FMNH⁻ is less planar than FMNH₂ owing to the negative charge in the pyrimidine subnucleus of isoalloxazine leading to a decreased delocalization of the electrons.

It was not possible to determine the chemical shifts of MeAc₄rFH⁻ in CHCl₃ but from published light absorption spectra [24] it can be concluded that the structural differences observed between FMNH₂ and MeAc₄rFH₂ no longer exist for the two anionic species.

Comparing the chemical shift differences between FMN and FMNH⁻ with those of the corresponding values of the flavoproteins (Table 1), it is observed that these differences are of about the same magnitude. On the other hand, the chemical shift differences between FMN and FMNH₂ are much larger as far as the C-2 and C-10a atoms are concerned (Fig. 1). From this it must be concluded that the

prosthetic groups in the two flavoproteins are in the anionic form in the reduced state. This conclusion is in agreement with potentiometric studies where it was shown that both flavodoxins exhibit a pH-independent redox potential between the semiquinone and the dihydro state whereas the redox potential between the oxidized and the semiquinone state is pH-dependent [28,29].

There are some remarkable differences between a few of the chemical shifts of FMNH⁻ and protein-bound FMNH⁻. These differences are manifested especially in the chemical shifts of C-2 and C-10a (Fig. 1). The chemical shift of C-2 gradually increases in going from M. elsdenii flavodoxin to FMNH⁻ and A. vinelandii flavodoxin. These shifts to lower field are almost paralleled by a similar trend of the chemical shifts of C-10a. The corresponding chemical shifts of C-4a show an opposite trend. This suggests that the negative charge of FMNH⁻ in M. elsdenii flavodoxin is somewhat more counteracted by specific interaction(s) of an amino acid residue(s) of the apoprotein with the N-1$-$C-2α region of the prosthetic group than that in A. vinelandii flavodoxin. A positively charged group or a neutral group capable of forming a hydrogen bond could explain these observations. It is possible that the chemical shift differences between M. elsdenii and A. vinelandii flavodoxin could also reflect a difference of the planarity of the two molecules, i.e. A. vinelandii is more planar than M. elsdenii flavodoxin. A combination of both effects is most probable. The direct ^{13}C-^{13}C coupling constants in M. elsdenii flavodoxin (Table 2) support this suggestion, i.e. the coupling constant at the C-10a center increases as compared to that of FMNH⁻. The decrease of the coupling constant at the C-4a center indicates an increase in π-electron density at this center which also becomes obvious by the chemical shift of C-4 of the protein-bound flavin which is shifted upfield as compared to that of FMNH⁻.

Flavodoxin from M. elsdenii is structurally very closely related to Clostridium flavodoxin [30]. The three-dimensional structure of the latter protein is known [30]. These studies revealed that the reduced prosthetic group in Clostridium flavodoxin possesses an almost coplanar structure. Our results strongly indicate that M. elsdenii flavodoxin possesses in the reduced state also a prosthetic group with a high coplanarity. This conclusion has already been drawn from fluorescence studies [25] and is supported by the molar absorption coefficient of the reduced molecule at 450 nm. The molar absorption coefficient $(1.7 \text{ mM}^{-1} \text{ cm}^{-1})$ [25] is larger than that of FMNH$_2$ $(1.39 \text{ mM}^{-1} \text{ cm}^{-1})$ [24]. For the reduced form of A. vinelandii flavodoxin no molar absorption coefficient has been published because it is very difficult to reduce the protein quantitatively. However, from published spectra (Fig. 3 of [31]) we have estimated the molar absorption coefficient of this protein. A value of $1.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was calculated as a lower limit. This value indicates that the two-electron reduced prosthetic group in A. vinelandii flavodoxin also has an almost coplanar structure. This statement is an accord with our nuclear magnetic resonance data.

The flavodoxins are electron-transferring protein shuttling between the semiquinone and reduced state during the biological reaction [30]. They are characterized by their low redox potentials as compared to other classes of flavoproteins. The NMR data presented in this paper lead to the conclusion that the high reducing power of the flavodoxins is primarily due to the coplanar conformation of the reduced, ionized prosthetic group. In free FMNH⁻ the coplanar conformation is energetically not favoured and stabilization of FMNH⁻ is achieved by folding the molecule. In flavodoxins the inter-

action between the prosthetic group and amino acid residue(s) of the apoprotein forces the prosthetic group to acquire a coplanar conformation leading to an energy-rich complex. The degree of coplanarity of the protein-bound prosthetic group, enforced on it by specific interactions with the apoprotein, thus determines the reducing power of a particular flavodoxin. The differences in the chemical shifts observed in the reduced form of M. elsdenii and A. vinelandii reflect most probably subtle differences in the conformation and interaction of the prosthetic group with the apoproteins. The differences should also reflect the reactivity of the reduced flavodoxins by molecular oxygen. It has indeed been found [30] that the reduced M. elsdenii flavodoxin exhibits a higher reactivity towards molecular oxygen than reduced free flavin.

In concluding it follows from our study that the use of ^{13}C-enriched prosthetic groups in flavoproteins allows valuable information on the factors determining the specific biological function of a certain flavoprotein to be obtained. With other projects currently under investigation we hope to communicate in the near future more information with respect to the factors determining the specific biological function of flavoprotein.

REFERENCES

1. Yagi, K. & Yamano, T. (eds) (1980) Proc. 6th Int. Symp. on Flavins and Flavoproteins, Japan Scientific Societies Press, Tokyo, and University Park Press, Baltimore.
2. Müller, F. (1972) Z. Naturforsch. 27b, 1023 – 1026.
3. Grande, H. J., van Schagen, C. G., Jarbandhan, T. & Müller, F. (1977) Helv. Chim. Acta, 60, 348 – 366.
4. van Schagen, C. G. & Müller, F. (1977) in Flavins and Flavoproteins: Physicochemical Properties and Function (Ostrowski, W., ed.) pp. 9 – 27, Polish Scientific Publishers, Warsaw and Cracow.
5. Müller, F., van Schagen, C. G. & van Berkel, W. J. H. (1980) in Flavins and Flavoproteins (Yagi, K. & Yamano, T., eds) pp. 359 – 371, Japan Scientific Societies Press, Tokyo, and University Park Press, Baltimore.
6. Yagi, K., Ohishi, N., Takai, A., Kawano, K. & Kyogoku, Y. (1976) in Flavins and Flavoproteins (Singer, T. P., ed.) pp. 775 – 781, Elsevier Scientific Publishing Company, Amsterdam.
7. Tishler, M., Pfister, K., Babson, R. D., Ladenburg, K. & Fleming, A. J. (1947) J. Am. Chem. Soc. 69, 1487 – 1492.
8. Grande, H. J., Gast, R., van Schagen, C. G., van Berkel, W. J. H. & Müller, F. (1977) Helv. Chim. Acta, 60, 367 – 379.
9. Scola-Nagelschneider, G. & Hemmerich, P. (1976) Eur. J. Biochem. 66, 567 – 577.
10. Müller, F. (1964) Ph.D. Thesis, University of Basel, p. 124.
11. Mayhew, S. G. & Massay, V. (1969) J. Biol. Chem. 244, 294 – 302.
12. Hinkson, J. W. & Bulen, W. A. (1967) J. Biol. Chem. 242, 3345 – 3351.
13. Mayhew, S. G. (1971) Biochim. Biophys. Acta, 235, 289 – 302.
14. Edmondson, D. E. & Tollin, G. (1971) Biochemistry, 10, 124 – 132.
15. Van Schagen, C. G. & Müller, F. (1978) Helv. Chim. Acta, 61, 3139 – 3142.

16. van Schagen, C. G. & Müller, F. (1980) *Helv. Chim. Acta, 63,* 2187–2201.
17. Dutta, P. K. & Spiro, T. G. (1980) *Biochemistry, 19,* 1590–1593.
18. Stothers, J. B. (1972) in *Carbon-13 NMR Spectroscopy,* pp. 313–374, Academic Press, New York.
19. Weigert, F. J. & Roberts, J. D. (1972) *J. Am. Chem. Soc. 94,* 6021–6025.
20. van Schagen, C. G., Grande, H. J. & Müller, F. (1978) *Rec. Trav. Chem. Pays-Bas, 97,* 179–180.
21. Lhoste, J.-M., Favaudon, V., Ghisla, S. & Hastings, J. W. (1980) in *Flavins and Flavoproteins* (Yagi, K. & Yamano, T., eds) pp. 131–138, Japan Scientific Societies Press, Tokyo, and University Park Press, Baltimore.
22. Dwek, R. A. (1973) *NMR in Biochemistry,* pp. 107–108, Clarenden Press, Oxford.
23. Lowe, H. J. & Clark, W. M. (1956) *J. Biol. Chem. 221,* 983–992.
24. Dudley, K. H., Ehrenberg, A., Hemmerich, P. & Müller, F. (1964) *Helv. Chim. Acta, 47,* 1354–1383.
25. Ghisla, S., Massey, V., Lhoste, J.-M. & Mayhew, S. G. (1974) *Biochemistry, 13,* 589–597.
26. London, R. E., Walker, T. E., Kollman, V. H. & Matwiyoff, N. A. (1978) *J. Am. Chem. Soc. 100,* 3723–3729.
27. Fermandjian, S., Tran-Dinh, S., Savrda, J., Salà, E., Mermet-Bouvier, R., Bricas, E. & Fromageot, P. (1975) *Biochim. Biophys. Acta, 399,* 313–338.
28. Mayhew, S. G., Foust, G. P. & Massey, V. (1969) *J. Biol. Chem. 244,* 803–810.
29. Edmonson, D. & Tollin, G. (1971) *Biochemistry, 10,* 133–145.
30. Mayhew, S. G. & Ludwig, M. L. (1975) in *The Enzymes* (Boyer, P. D., ed.) pp. 57–118, vol. XII, 3rd edn, Academic Press, New York..
31. van Lin, B. & Bothe, H. (1972) *Arch. Microbiol. 82,* 155–172.

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Chapter 3

HIGH RESOLUTION ^{1}H NMR STUDY AT 360 MHz ON THE FLAVODOXIN FROM
MEGASPHAERA ELSDENII

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1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy has been applied to flavoproteins in only a few cases. The technique was mainly used as a supporting analytical tool in the elucidation of the structure of low M_r flavins (review [1]). NMR was applied to the apo-flavodoxin from *Clostridium pasteurianum* [2] in a presentation of a general method for the computation of ^{1}H NMR spectra. An interesting study was conducted in [3,4] preparing uniformly deuterium-labelled flavodoxin from the thermophilic blue-green alga *Synechococcus lividus* by biological synthesis. Replacement of the deuterated FMN by the natural prosthetic group allowed an easy study of the protein-bound FMN without interference from the overwhelming proton resonances due to the protein. In [5] the flavodoxin from *M. elsdenii* and that from *Clostridium* MP was investigated at 220 MHz. A few resonances were assigned tentatively. *Desulfovibrio vulgaris* and *D. gigas* flavodoxins were investigated at 100 and 250 MHz in [6,7]. Data on the interaction between riboflavin and egg yolk apoprotein appeared in [8].

The flavodoxins serve as electron carriers in biological reactions. The low M_r and the formation of the relatively stable flavosemiquinone make the flavodoxins suitable for NMR studies. We have undertaken such studies to contribute to a better understanding of the factors governing the interaction between apoflavoproteins and their prosthetic groups and to elucidate the active sites of the proteins. Here, ^{1}H NMR data are reported on *M. elsdenii* flavodoxin. The protein has been studied in the oxidized, the one-electron (semiquinone) and the two-electron (hydroquinone) reduced state. These results combined with those obtained from the apoprotein made it possible to assign some resonance lines to amino acid residues which are part of or in the vicinity of the active site of the flavodoxin. It is also shown that some of the assignments in [5] are incorrect.

2. Materials and methods

Deuterium oxide (99.9 atom%) was purchased from Merck AG (Darmstadt). Solutions of flavodoxin were prepared by dissolving the dry powder in phosphate buffer prepared from deuterium oxide. The solution was then lyophilized and the residue redissolved in deuterium oxide. This procedure was repeated at least 3 times to ensure complete exchange of the exchangeable protons in the protein. Protein was 2–4 mM. The concentration of the protein was determined using the published molar extinction coefficient [9].

Megasphaera elsdenii flavodoxin was isolated and purified as in [9]. The apoprotein was prepared according to [10]. Two-electron reduction of the flavodoxin was achieved by a 2-fold excess of buffered sodium dithionite. When the semiquinone form of the protein was required the 2-electron reduced solution was reoxidized by careful admission of molecular oxygen. The final solutions were kept under argon to prevent oxidation to the flavoquinone state.

Prosthetic groups selectively deuterated at positions C(9) or C(8α) were prepared as in [11].

The NMR spectra were obtained on a Bruker HX 360 spectrometer operating at 360 MHz, equipped with Fourier transform and a NIC-12 data system. The spectra were accumulated under the following instrumental conditions: spectral width 4800 Hz, accumulations 1000, pulse width 5 μs (90°C); 4000

data points were collected in the time domain and
transferred to 8000 data points in the frequency
domain. Before Fourier transformation the spectral
data were treated by the so-called sin-bell routine
[12] or the convolution difference technique [13].
The chemical shifts are reported relative to the inter-
nal standard 2,2-dimethyl-2-silapentane-sulfonate
(DSS). The temperature of the samples was 30°C.

3. Results and discussion

The ^{1}H NMR spectra of *M. elsdenii* flavodoxin are
shown in fig.1. Spectrum A is identical with that in
[5] except for the better resolution of the spectrum
and additional resonances not observed in [5]. The
resolution of the conventional spectrum (spectrum A)
was improved by the convolution difference technique
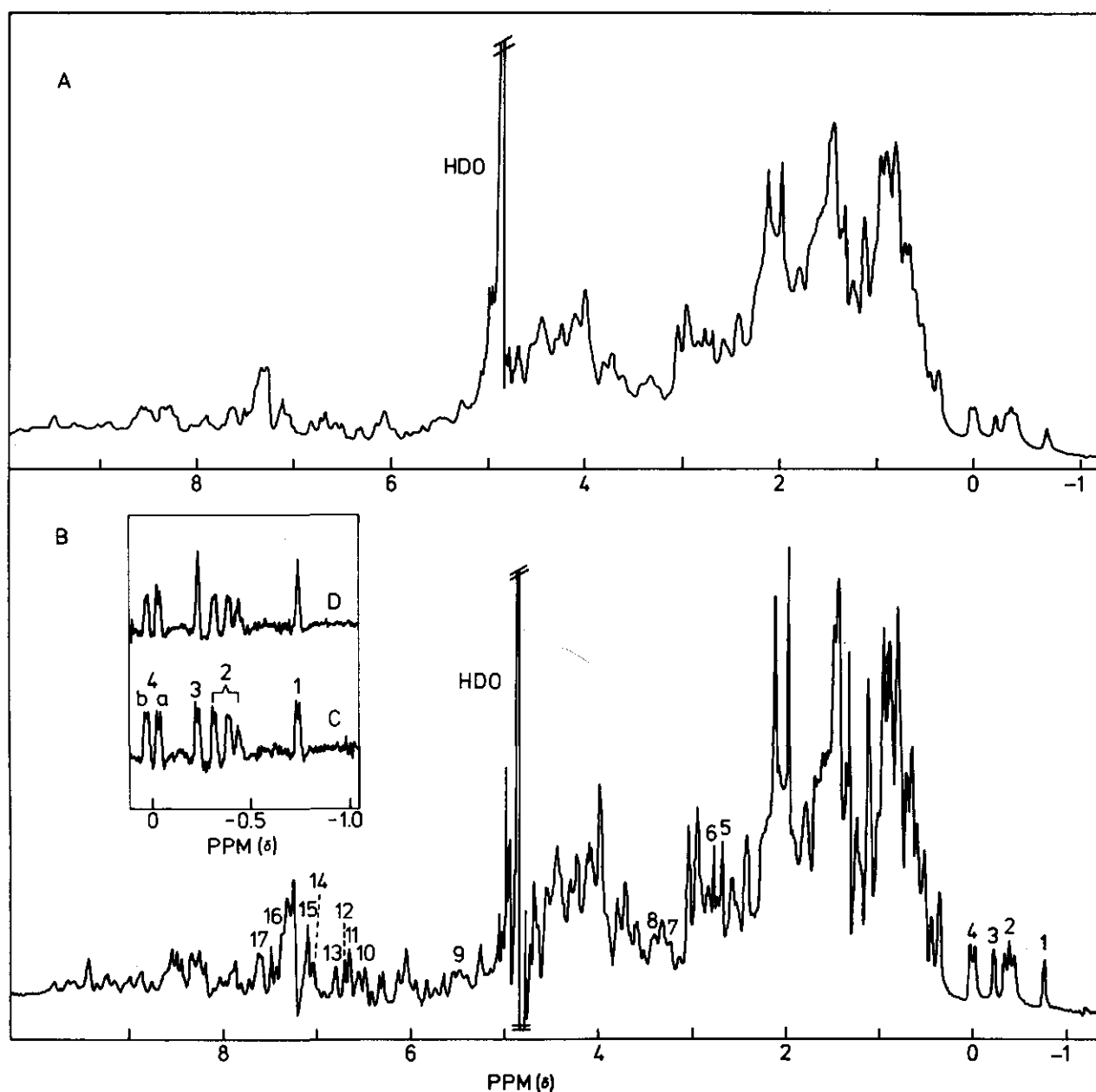
Fig.1. 360 MHz ^{1}H FT NMR spectra of a 2 mM solution of *M. elsdenii* flavodoxin in 0.1 M phosphate, p^{2}H 6.8: (A) conventional
spectrum; (B) obtained by treatment of the original data (A) by the convolution difference technique [14]. The original spectrum
was obtained under the following instrumental settings: 1000 scans, 0.8 s acquisition time, 7 µs pulse width, 4800 Hz spectral
width, 1.17 Hz/point. The higher resolution of the high field peaks (C) was achieved by the application of the sin-bell technique
[12]; (D) double resonance spectrum, irradiation at 1.1 ppm. For the peak numbers see table 1.

(spectrum B) [13]. Remarkably well-resolved are the peaks occurring in the region upfield from the internal standard DSS. The resolution of these peaks can be further enhanced by the sin-bell technique [12] as shown in the inset of the figure (spectrum C). The peaks upfield from DSS arise from methyl groups of amino acid residues of the protein. Such upfield shifts generally occur when protons are in the diamagnetic, secondary field regions produced by ring current effects of aromatic compounds. Appreciable upfield shifts can also be caused by electric fields of ionized groups or by permanent dipoles, e.g., anisotropic shielding by peptide bonds [2,14].

The high field peaks numbered 1–4 represent 7 methyl groups (table 1). Except for the high field peak of cluster 2, which is a triplet, all peaks are doublets (spectrum C). Irradiation at 1.1 ppm transforms the peaks 1 and 3 into singlets (spectrum D). In these double resonance experiments both peaks exhibited the same dependence on both the irradiation frequency and power used. This strongly indicates that both methyl groups belong to one and the same amino acid residue. This leaves valine and leucine as the only possible candidates. Considering the fact that the proton which couples with the 2 methyl groups resonates at 1.1 ppm we prefer to assign the 2 methyl groups to leucine. For a valine

residue the upfield shift of the CH group in question would amount to ~1.1 ppm with respect to the corresponding chemical shift of free valine [2]. For leucine the corresponding upfield shift is ~0.6 ppm. The peaks 1,3 and 4a are absent in the spectrum of the apoprotein (not shown). This strongly indicates that the corresponding amino acid residues are located within a distance of ~1 nm from the prosthetic group. According to the model in [5] Met-57, Ala-56 and Leu-62 are within this distance (0.6 nm) from the flavin. Since no singlet was observed at high field, peak 4a is assigned to Ala-56 and peak 1 and 3 to Leu-62. The magnetic non-equivalence of the 2 methyl groups of Leu-62 suggests that the methyl groups are not free to rotate. It is suggested that the methyl group of Leu-62 which resonates at higher field is under the influence of the ring current of the flavin whereas that at lower field is under the influence of Trp-7. This interpretation is supported by spectra obtained from the semiquinone flavodoxin. In these spectra peak 4a is completely absent, peak 1 loses most of its intensity, whereas peak 3 still shows considerable intensity. In going from the oxidized to the semiquinone state a loss of intensity rather than a broadening of the resonance lines is observed, which indicates that the rate of electron exchange between the 2 states is slow. This is in full agreement with

Table 1

Assignments and patterns of some resonances in the ^{1}H NMR spectrum of *M. elsdinii* flavodoxin

Peak no.[a]	Resonance position[b] (ppm)	Assignment	Pattern[c]
1	−0.72	Leu-62, δ-CH$_3$	d, J = 6 Hz
3	−0.28	Leu-62, δ-CH$_3$	d, J = 6 Hz
4a	−0.07	Ala-56, β-CH$_3$	d, J = 6 Hz
5	2.32	FMN, CH$_3$-C(7)	s
6	2.36	FMN, CH$_3$-C(8)	s
7	3.14	Trp-(96,100), β-CH$_2$	d
8	3.32	Trp-91, β-CH$_2$	d
9	5.47	Trp-91, H-C(5,6)	(m)
10	6.32	Trp-91, H-C(4,7)	(d)
11	6.67	Tyr-6, H-C(3,5)	(d)
12	6.68	FMN, H-C(9)	s
13	6.72	Trp-91, H-C(2)	(s)
14	7.00	Tyr-6, H-C(2,6)	(d)
15	7.07	Trp-(96,100), H-C(5,6)	(m)
16	7.52	Trp-(96,100), H-C(4,7)	(d)
17	7.77	Trp-(96,100), H-C(2)	(s)

[a] See fig.1; [b] relative to internal DSS
[c] s, singlet; d, doublet; m, multiplet; (apparent patterns)

results obtained from the same protein by ^{31}P NMR [15]. These data also suggest that the methyl group of Ala-56 (peak 4a) is located closer to the prosthetic group than that of Leu-62 (peak 1). This interpretation is in excellent agreement with the model in [5] but our assignments are in complete contrast with those [5] where peak 3 was assigned to Ala-56 and peak 1 to Met-57. Clearly, the doublet character of peak 1 and peak 3 disprove these assignments. The other high field methyl resonances, which are shifted upfield by the ring currents of aromatic amino acid residues of the protein, have not yet been identified, but are currently under investigation.

In the spectrum of the reduced protein the resonances 1,3 and 4a are again present as sharp peaks in contrast to that for the flavosemiquinone. In addition, peaks 1 and 4a undergo a small downfield shift upon two-electron reduction of the flavodoxin. The small downfield shifts indicate that the ring current effect of the isoalloxazine ring system on the 2 methyl resonances is somewhat attenuated, probably caused by a slight bending of the reduced FMN. This interpretation is supported by three-dimensional data on the related *Clostridial* flavodoxin [16] and ^{13}C NMR data on *M. elsdenii* [17]. In addition, the small downfield shift of peak 1 in the spectrum of the reduced protein supports our interpretation with respect to one of the methyl groups of Leu-62, although it cannot be excluded that the downfield shift is due to a small conformational change in the binding region of the prosthetic group.

The peaks 5 and 6 (table 1) each represent 3 protons in a difference spectrum between the oxidized minus the semiquinone state of *M. elsdenii* flavodoxin, i.e., these resonances are absent in the spectrum of the semiquinone form. In the reduced state the 2 methyl groups become magnetically equivalent and appear at higher fields (table 1). The fact that these two resonances are not present in the spectrum of the semiquinone and that peak 6 is absent in the spectrum of the oxidized protein, where FMN had been replaced by C^{2}H$_3$C(8)-FMN, leads to the conclusion that the peaks 5 and 6 are due to the two methyl groups of the prosthetic group. The assignment is further supported by the observation that the intensity of the resonance line at 2.08 ppm (table 1) in the difference spectrum (semiquinone minus reduced) was decreased by a factor of 2 when FMN was replaced by C^{2}H$_3$C(8)-FMN. As compared to free FMN the methyl groups of protein-bound FMN resonate at lower fields

(−0.4 ppm) in the oxidized flavodoxin whereas in the reduced state the methyl resonances appear at almost the same field. With respect to oxidized flavodoxin a similar downfield shift was observed for the methyl groups of FMN in oxidized *S. lividus* flavodoxin [4].

Peak 12 can be assigned to H-C(9) of protein-bound FMN based on the fact that this peak is absent in the spectrum of flavodoxin reconstituted with ^{2}H-C(9)-FMN. Compared to free FMN the corresponding resonance line in flavodoxin is shifted upfield by 0.7 ppm. In the spectrum of the reduced protein the resonance line of H-C(9) could not be identified with certainty due to overlap with other resonances. The chemical shift of peak 12 (6.68 ppm) agrees well with that of 6.7 ppm in the spectrum of *S. lividus* [4] which was also assigned to H-C(9) of bound FMN. The proposed interaction between protein-bound FMN and a tyrosine and/or tryptophane residue(s) in *S. lividus* [4] to explain the upfield shift may also be valid for *M. elsdenii* flavodoxin, but further experiments are needed to support this proposal.

The peaks 9,10 and 13 and 15−17 are due to 2 tryptophan residues as has been shown independently by the photo-CIDNP (chemically-induced dynamic nuclear polarization) NMR technique [18]. The peaks 9,10 and 13 are not observable in the spectrum of the semiquinone, whereas the peaks 15−17 are still present in this spectrum. From this observation and the fact that the peaks 9,10 and 13 are shifted upfield by ∼1.5 ppm as compared to those of free tryptophan it can be concluded that this tryptophan residue is in the vicinity of the prosthetic group. The pattern of the peaks was determined by the pulse method developed in [19] (table 1). According to in [5] Trp-91 is located within a few tenths of 1 nm from the prosthetic group. From the X-ray data of the related flavodoxin from *Clostridium* MP it is known that the distance between the prosthetic group and Trp-90 is 0.34 nm [16]. Therefore it is reasonable to propose that the peaks 9,10 and 13 are due to Trp-91. The upfield shift of ∼1.5 ppm observed for Trp-91 cannot be explained solely by the interaction with the isoalloxazine ring system because a crude calculation by the method in [20] yields an upfield shift of 0.5 ppm. It must therefore be concluded that other effects play a more important role, e.g., effects of other aromatic amino acid residues or charged groups in the vicinity. Peak 8 represents the β-CH$_2$ group of Trp-91. The polypeptide chain of *M. elsdenii* flavodoxin contains tryptophan residues at positions 7,91,

96 and 100 [21]. According to [5] Trp-7 is located within a distance from the prosthetic group for which line broadening in the spectrum of the semiquinone is expected, however this was not observed. Therefore, the peaks 15—17 must be assigned to either Trp-96 or Trp-100. The corresponding β-CH_2 group resonates at 3.14 ppm (peak 7). The assignment of peak 10 to Trp-91 is in agreement with [5] but disagrees with respect to the assignment of peak 15 (Trp-96 or Trp-100 in place of Trp-91).

Megasphaera elsdenii flavodoxin contains 2 tyrosine residues (Tyr-6, Tyr-89) [21]. Tyr-89 is located in the FMN binding region of the protein. The resonance lines at 6.67 ppm (peak 11) and at 7.00 ppm (peak 14) are assigned to Tyr-6 (on the surface of the protein) based on the fact that its chemical shifts correspond with those of free tyrosine. The assignment of the peaks 11 and 14 was ascertained independently by the photo-CIDNP technique [18].

Acknowledgements

References

[1] Van Schagen, C. G. and Müller, F. (1977) in: Flavins and Flavoproteins: Physicochemical Properties and Function (Ostrowski, W. ed) pp. 9—27, Polish Scientific Publishers, Cracow, Warsaw.

[2] McDonald, C. C. and Phillips, W. D. (1969) J. Am. Chem. Soc. 91, 1513—1521.

[3] Crespi, H. L., Norris, J. R. and Katz, J. J. (1972) Nature 236, 178—180.

[4] Crespi, H. L., Norris, J. R., Bays, J. P. and Katz, J. J. (1973) Ann. NY Acad. Sci. 222, 800—815.

[5] James, T. L., Ludwig, M. L. and Cohn, M. (1973) Proc. Natl. Acad. Sci. USA 70, 3292—3295.

[6] Favaudon, V., Le Gall, J. and Lhoste, J.-M. (1976) in: Flavins and Flavoproteins (Singer, T. P. ed) pp. 434—438, Elsevier/North-Holland, Amsterdam, New York.

[7] Favaudon, V., Le Gall, J. and Lhoste, J.-M. (1980) in: Flavins and Flavoproteins (Yagi, K. and Yamano, T. eds) pp. 373—386, Japan Sci. Soc. Press, Tokyo.

[8] Lubas, B., Soltysik, M., Steczko, J. and Ostrowski, W. (1977) FEBS Lett. 79, 179—182.

[9] Mayhew, S. G. and Massey, V. (1969) J. Biol. Chem. 244, 294—302.

[10] Wassink, J. H. and Mayhew, S. G. (1975) Anal. Biochem. 68, 609—616.

[11] Bullock, F. J. and Jardestzky, O. (1965) J. Org. Chem. 30, 2056—2057.

[12] De Marco, A. and Wüthrich, K. (1976) J. Magn. Res. 24, 201—204.

[13] Campbell, I. D., Dobson, C. M., Williams, R. J. P. and Xavier, A. V. (1973) J. Magn. Res. 11, 172—181.

[14] Sternlicht, H. and Wilson, D. (1967) Biochemistry 6, 2881—2892.

[15] Moonen, C. T. W. and Müller, F. (1981) Biochemistry, in press.

[16] Mayhew, S. G. and Ludwig, M. L. (1975) in: The Enzymes, 3rd edn, vol. 12, (Boyer, P. D. ed) pp. 57—118, Academic Press, New York.

[17] Van Schagen, C. G. and Müller, F. (1981) Eur. J. Biochem. in press.

[18] Müller, F., Van Schagen, C. G. and Kaptein, R. (1980) Methods Enzymol. 66, 385—416.

[19] Campbell, I. D., Dobson, C. M., Williams, R. J. P. and Wright, P. E. (1975) FEBS Lett. 17, 96—99.

[20] Haigh, C. W. and Mallion, R. B. (1972) Org. Magn. Res. 4, 203—228.

[21] Tanaka, M., Haniu, M., Yasunobu, K. T., Mayhew, S. G. and Massey, V. (1973) J. Biol. Chem. 248, 4354—4366.
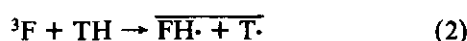
CHAPTER 4

Photochemically Induced Dynamic Nuclear Polarization Study on Flavin Adenine Dinucleotide and Flavoproteins[†]

Cees G. van Schagen, Franz Müller,* and Robert Kaptein

ABSTRACT: Flavin adenine dinucleotide and some flavoproteins ranging in relative molecular mass from 15 000 to 100 000 were investigated by the photochemically induced dynamic nuclear polarization (photo-CIDNP) technique. At neutral pH values, where FAD forms a strong intramolecular complex, no photo-CIDNP signal is observed. Lowering the pH values of an aqueous solution of FAD leads to the gradual appearance of photo-CIDNP signals. The pH dependence of the intensity of the signal follows that of the fluorescence quantum yield of FAD, indicating that in both types of experiments the same mechanism is operating. This is an example of where a photochemically active constituent of a complex is used to induce polarization in its partner. The flavoproteins investigated did not exhibit a photo-CIDNP signal in the absence of external free flavin so that information on aromatic amino acid residues located in the neighborhood of the bound prosthetic group could not be obtained. If intermediate bi-radical complexes are formed in these cases, they are not able to dissociate which is mandatory for the observation of CIDNP signals. Addition of free aromatic amino acids to solutions of flavoproteins yields the corresponding CIDNP signals of the amino acids upon excitation of protein-bound flavins. Addition of free flavins to solutions of flavoproteins leads to the observation of CIDNP signals of aromatic amino acid residues. Various flavodoxins and other flavoproteins were investigated by using as an external dye free flavins carrying different charges. In general positively and negatively charged free flavins give rise to CIDNP signals originating from different aromatic amino acid residues. It is shown that the technique allows assignments of proton resonances of aromatic amino acid residues where the sequence and preferably the three-dimensional structure of flavoproteins are known. This is demonstrated with a few flavodoxins from various sources.

It has been shown that free flavins are valuable as dyes in photo-CIDNP[1] experiments (Kaptein et al., 1978; Müller et al., 1980). The interaction of excited flavin with aromatic amino acids and the resulting photo-CIDNP spectra obtained at 360 MHz were described recently (Kaptein, 1978; Müller et al., 1980). The nuclear spin polarization recorded in photo-CIDNP spectra is induced in the following reactions given here for flavin (F) and tyrosine (TH):

$$F \xrightarrow{h\nu} {}^{1}F \rightarrow {}^{3}F \tag{1}$$

$$^{3}F + TH \rightarrow \overline{FH\cdot + T\cdot} \tag{2}$$

$$\overline{FH\cdot + T\cdot} \rightarrow F + TH^* \tag{3}$$

$$2FH\cdot \rightarrow FH_2 + F \tag{4}$$

$$FH_2 + O_2 \rightarrow F + H_2O_2 \tag{5}$$

After light excitation of the flavin, rapid intersystem crossing occurs to the reactive triplet state. ^{3}F abstracts the phenolic hydrogen atom of TH, yielding a short lived radical pair (eq 2). Upon electron back transfer within the geminate radical pair, the tyrosine is nuclear spin polarized (TH*). This latter species leads to the observation of emission or absorption lines in the photo-CIDNP spectra. For more detailed information with respect to the reaction mechanism and an explanation for the observation of a particular photo-CIDNP spectrum for biologically relevant compounds, the reader is referred to published data (Kaptein, 1971, 1977). During the experiments

the small molecular weight flavin is not destroyed by light and is probably recycled as indicated by eq 4 and 5.

The photo-CIDNP technique has been used to identify amino acid residues in proteins (Kaptein, 1978), facilitating the assignment of resonance lines in ^{1}H NMR spectra. Since the isoalloxazine ring system is part of the prosthetic group in flavoproteins, it was interesting to test the possibility that CIDNP spectra could be detected for amino acid residues located in the neighborhood of protein-bound, photoexcited flavin. In this study we report on the observation of photo-CIDNP spectra of flavoproteins varying in relative molecular mass from 15 000 to 100 000. For generation of photo-CIDNP spectra, various external small molecular weight flavin derivatives were used in order to study the possible influence of charged groups on the interaction with the polypeptide chains. In addition FAD was also studied as a function of pH and solvent polarity. Some preliminary results were published elsewhere (Müller et al., 1980).

Materials and Methods

The following flavin derivatives were used as external dyes and were synthesized according to published procedures: N^3-methyllumiflavin and N^3-carboxymethyllumiflavin (Hemmerich, 1964) and N^3-(ethylamino)lumiflavin (Vass, 1966). FAD was a product of Boehringer, Mannheim, Germany, and was used without further purification.

The flavoproteins were isolated and purified as described in the literature: old yellow enzyme from brewer's bottom yeast (Abramowitz & Massey, 1976), riboflavin-binding protein from egg yolks (Murthy et al., 1979), and D-amino acid oxidase from pig kidneys (Massey et al., 1961). The

[1] Abbreviations: photo-CIDNP, photochemically induced dynamic nuclear polarization; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; NMR, nuclear magnetic resonance; FAD, flavin adenine dinucleotide; Me$_2$SO (DMSO in figures), dimethyl sulfoxide; Lfl, lumiflavin; EDTA, ethylenediaminetetraacetic acid.

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Table I: Relative Photo-CIDNP Signal Intensity of C(6) H and C(8) CH$_3$ of the Isoalloxazine and C(8) H of the Adenine Moiety of FAD as a Function of the pH[a]

| | rel photo-CIDNP signal intensity of | | |
pH	C(6) H	C(8) CH$_3$	C(8) H
1.3	0.4	0.4	1.9
1.6	0.6	1.3	2.7
2.1	1.6	1.8	5.0
3.3	0.9	1.2	3.2
5.7	0	0	0

[a] The intensities are given as ratios of the intensities of the signals obtained with and without illumination of the FAD solutions.

flavodoxin from *Megasphaera elsdenii* was isolated according to the procedure of Mayhew & Massey (1969). The flavodoxins from *Azotobacter vinelandii*, *Desulfovibrio vulgaris*, and *Clostridium MP* were a gift from Dr. S. G. Mayhew. Oxynitrilase and glucose oxidase were products of Boehringer, Mannheim, Germany.

CIDNP spectra were recorded on a Bruker HX 360 NMR instrument operating at 360 MHz. The instrumental setup was described earlier (Kaptein et al., 1978). The experimental conditions were as follows: 0.4-s light pulses and ±10-W laser light intensity; a delay of 7 s was used between the various 90° pulses. As compared to the published conditions (Kaptein et al., 1978), no presaturation pulse but a higher light intensity was used. Alternating "light" and "dark" free induction decays were collected and stored in the NIC-294 disk. After Fourier transformation, they were subtracted to yield the pure CIDNP difference spectrum. This procedure minimizes the effect of slow drift or temperature variations while permitting time averaging for improvement of the signal-to-noise ratio. To obtain the CIDNP spectra, 40–80 accumulations were necessary. The total volume in a flat-bottom 5-mm NMR tube was 0.2 mL. The protein concentration was about 1 mM. The final concentration of externally added flavin was usually also 1 mM. When external amino acids were added in place of small molecular weight flavins, the protein concentration was about 0.5 mM. The samples of flavoproteins with a small relative molecular mass were lyophilized several times in the presence of ^{2}H$_2$O; all other flavoproteins were dialyzed several times against a buffer solution prepared in ^{2}H$_2$O (99.95 atom %, Merck, Darmstadt, Germany). The chemical shifts are reported in parts per million relative to internal DSS.

Results and Discussion

Flavin Adenine Dinucleotide. We have shown previously (Kaptein et al., 1979) that CIDNP can be observed in the photoreaction of flavin with adenosine 5'-phosphate. In contrast, FAD in which the isoalloxazine and adenine moieties are covalently attached does not show a CIDNP signal in aqueous solution at neutral pH. However, a decrease of the pH leads to the gradual appearance of a CIDNP signal. At about pH 2.1, the signal is maximal and decreases again at lower pH values (Table I). The CIDNP spectrum exhibits absorption peaks due to the C(6) H and C(8) H of the adenine moiety and an emission line due to C(6) H of the isoalloxazine moiety of FAD. In addition a strong positive polarization due to CH$_3$(8) of the isoalloxazine moiety of FAD is also observed. Furthermore, a weak positive enhancement is observed at about 4.8 ppm. This latter line is probably due to the N(10) CH$_2$ group of FAD. Addition of about an equal volume of Me$_2$SO to a neutral aqueous solution of FAD yields an identical spectrum (Figure 1) as obtained in aqueous solution at pH values ≳ 2.1. The emission line observed at about 2.1 ppm of the CIDNP spectrum of FAD (Figure 1) is probably due
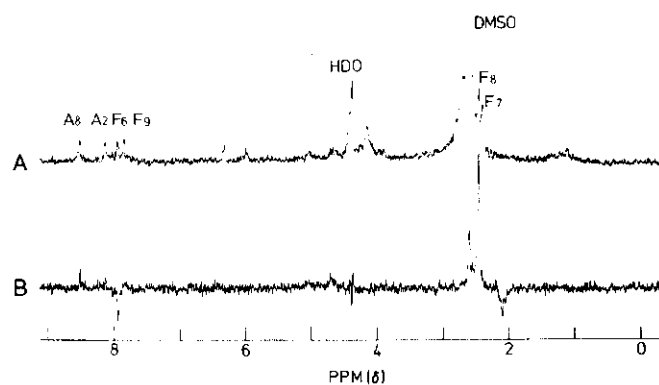

FIGURE 1: 360-MHz ^{1}H FT NMR spectra (25 pulses) of 3 mM FAD in a mixture of ^{2}H$_2$O and Me$_2$SO (3:2 v/v). (A) Dark spectrum; (B) difference spectrum between light and dark spectra. Resonance lines labeled A and F refer to the adenine and flavin moieties of FAD, respectively.

to a photochemical product of FAD and has not been investigated further.

From fluorescence studies it is known that FAD forms a strong intramolecular complex in neutral aqueous solution (Wahl et al., 1974). This complex can be disrupted by either lowering the pH or adding an organic solvent to an aqueous solution of FAD (Tsibris et al., 1965). To observe a CIDNP signal, it is mandatory that the intermediate radical pair formed upon excitation can separate to distances where the exchange interaction between the electron spins is diminished. Thus our results provide independent evidence for the existence of a strong intramolecular complex in FAD and are in accordance with the fluorometric studies. In the presence of organic solvents or at acid pH values, the complex is disrupted so that the conditions for the observation of CIDNP signals is fulfilled. This indicates that the stability of the complex formed is many orders of magnitude smaller than that in neutral aqueous solution. The fact that below pH 2.1 the intensity of the CIDNP signal decreases drastically indicates that an additional mechanism is operating. One possible explanation may be the formation of a small amount of flavin radical cation which is rather stable against oxidation by molecular oxygen (Müller et al., 1981). The protonation reaction of the neutral flavin radical yielding the cation shows a pK_a of 2.3 (Land & Swallow, 1969). The drastic decrease of signal intensity below pH values of about 2 is, however, not caused by the generation of stable radicals since the width of the NMR lines is unaffected. The pH dependence of the fluorescence of all flavin compounds shows a drastic decrease of the quantum yield below pH values of about 2.0 (Tsibris et al., 1965). The explanation for this observation is that increasing concentrations of protons in solution lead to a very efficient radiationless deactivation of the excited singlet state of flavin. This very efficient process affects the intersystem crossing reaction so that the yield of flavin in the triplet state (precursor of the spin polarization reaction) decreases very rapidly with increasing hydrogen ion concentration. The curves of the pH dependence of the photo-CIDNP signal and fluorescence quantum yields of FAD coincide, indicating that in both types of experiments the same initial mechanism is operating. The generation of CIDNP signals upon light excitation of FAD is an example of where flavin as a part of a molecule is used to induce polarization within the molecule via the excited isoalloxazine moiety.

Flavoproteins. A similar, if not identical, situation can be envisaged in a flavoprotein with respect to bound flavin as

described above for FAD. In principle, the excited isoalloxazine moiety of the prosthetic group could induce polarization on neighboring amino acids. However, no flavoproteins studied so far have shown any CIDNP signals. This observation can be explained from the fact that the prosthetic group and neighboring amino acid residues in flavoproteins are not free to associate or dissociate in the same manner as the corresponding free constituents in an aqueous solution. One of the requirements to observe CIDNP signals is the *formation and* dissociation of the intermediate radical pair (cf. above). This latter reaction has to occur within the time scale of the polarization process, i.e., $\sim 10^8$ s^{-1}, which for obvious reasons is not possible in flavoproteins because the constituents are limited in their rotations and translations. It is known that flavoproteins can be reduced photochemically by electron donors, e.g., EDTA (Palmer & Massey, 1966). This reaction occurs via the triplet state of flavin (Visser et al., 1977), showing that the CIDNP active state can still be reached in flavoproteins. So that the proposal could be tested, further experiments were conducted in which the free amino acids Tyr and Trp were added to solutions of flavoproteins. Upon excitation of the protein-bound flavin, CIDNP signals of the corresponding free amino acids were easily detected. The results further indicate that the active centers, i.e., the prosthetic group, of the flavoproteins investigated in this paper are accessible to small molecular weight organic compounds.

The conditions used to excite the prosthetic group of flavoproteins did not lead to measurable destruction of the proteins, except for old yellow enzyme. This enzyme underwent an irreversible photoreaction, yielding a faint purple product. It is suggested that a cysteinyl residue is placed close to the prosthetic group which reacts with the excited flavin in an irreversible reaction.

Addition of external flavin to solutions of flavoproteins led to the generation of CIDNP signals of amino acid residues upon excitation of the flavin. Even with flavoproteins possessing a relatively high molecular mass, CIDNP signals could be observed despite the apparent large line width. For compensation for the light absorbed by the flavoprotein itself, the external flavin was added in about equimolar amounts with respect to bound flavin. The results are summarized in Table II and discussed below.

Flavodoxins. Flavodoxins are electron-transferring proteins possessing a relatively small molecular mass. This property makes them suitable for a NMR study. Such a study is currently under investigation (C. G. van Schagen and F. Müller, unpublished results). The flavodoxins contain a relatively small number of aromatic amino acids. Despite the fact that the flavodoxin from *M. elsdenii* contains only two tyrosines, four tryptophans, and four phenylalanines, the conventional NMR spectrum is still rather complex in the aromatic region, making assignment tedious (van Schagen & Müller, 1977). The photo-CIDNP technique should be very helpful in unraveling the ^{1}H NMR spectrum of *M. elsdenii* flavodoxin (Figure 2A). When the negatively charged lumiflavin derivative was used as an external dye, absorptions were observed in the aromatic region of the photo-CIDNP spectrum (Figure 2B). Since the protein contains no histidine residues, the absorptions must originate from tryptophan residues. An interesting observation is the fact that depending on the concentration of externally added flavin, one or two tryptophan residues were observable in the CIDNP spectrum. Thus when a concentration of external dye which was lower than that of the flavodoxin was used, only one tryptophan residue could be observed (result not shown). The CIDNP

Table II: ^{1}H Chemical Shifts of Amino Acid Residues Observed in Various Flavoproteins by the Photo-CIDNP Technique

flavoprotein	chemical shift (ppm)[a]	amino acid residue	dye
M. elsdenii	7.77	Trp-100	Lfl-CH$_2$COO$^-$ [b]
flavodoxin	7.52	Trp-100	
(M_r 15 000)	7.07	Trp-100	
	3.32	Trp-(β-CH$_2$)	
	7.77	Trp-100	Lfl-CH$_2$COO$^-$ [c]
	7.32	Trp-100	
	7.07	Trp-100	
	6.72	Trp-91	
	6.32	Trp-91	
	5.47	Trp-91	
	3.32	Trp-(β-CH$_2$)	
	7.32	Trp-96	Lfl-CH$_2$CH$_2$NH$_3^+$
	7.07	Trp-96	
	6.67	Tyr-6	
	6.32	Trp-91	
	5.47	Trp-91	
	3.14	Trp-96 (β-CH$_2$)	
	3.32	Trp-91 (β-CH$_2$)	
M. elsdenii	7.65	Trp	Lfl-CH$_3$ or
apoflavodoxin	7.33	Trp	Lfl-CH$_2$COO$^-$
	6.90	Tyr	
	3.30	Trp-(β-CH$_2$)	
	7.59	Trp	Lfl-CH$_2$CH$_2$NH$_3^+$
	7.22	Trp	
	3.51	Trp (β-CH$_2$)	
	3.37	Trp (β-CH$_2$)	
Clostridium MP	7.59	Trp-6 or -95	Lfl-CH$_2$CH$_2$NH$_3^+$
flavodoxin	7.21	Trp-6 or -95	
(M_r 15 800)	6.81	Tyr-88 (−106?)	
	3.13	Trp-(β-CH$_2$)	
	6.8	Tyr-5 (−106?)	Lfl-CH$_2$CH$_2$COO$^-$
Desulfovibrio	7.16	Trp-60	Lfl-CH$_2$CH$_2$COO$^-$
vulgaris	6.94	Trp-60	
flavodoxin	6.72	Tyr-98	
(M_r 16 300)	3.14	Trp-60 (β-CH$_2$)	
Azotobacter	7.56	Trp	Lfl-CH$_3$
vinelandii	7.42	Trp	
flavodoxin	7.00	Tyr	
(M_r 19 900)	3.20	Trp-(β-CH$_2$)	
glucose oxidase	8.0	His	Lfl-CH$_3$
(M_r 186 000)	7.2	His (Trp?)	
	6.9	Tyr	
old yellow enzyme	6.9	Tyr	Lfl-CH$_3$
(M_r 98 000)			
D-amino acid	7.93	His	Lfl-CH$_2$CH$_2$NH$_3^+$
oxidase	7.82	His	
(M_r 80 000)	7.03	Tyr	
	6.79	Tyr	
	8.0	His	Lfl-CH$_2$COO$^-$
	7.9	His	
	7.04	Tyr	
	6.79	Tyr	
	3.08	Tyr-(β-CH$_2$)	
oxynitrilase	6.8	Tyr	Lfl-CH$_3$
(M_r 75 000)			
riboflavin-binding	7.90	His	Lfl-CH$_3$ or
protein	7.80	His	Lfl-CH$_2$COO$^-$
(M_r 36 000)	7.15	Trp[d]	
	6.90	Tyr	

[a] Relative to internal DSS. [b] Concentration of dye was lower than that of the protein. [c] Concentration of dye was equal or higher than that of the protein. [d] The β-CH$_2$ group could not be observed.

spectrum consisted of three absorption and one emission lines (Figure 2B, peaks numbered 1). Increasing the concentration of external dye produced another set of three lines (set 2 in Figure 2B) absorbing at higher fields than those of set 1. The absorption lines of set 1 exhibit chemical shift values closely related to those of free tryptophan whereas the lines of set 2 appear about 1.5 ppm toward higher field compared to those
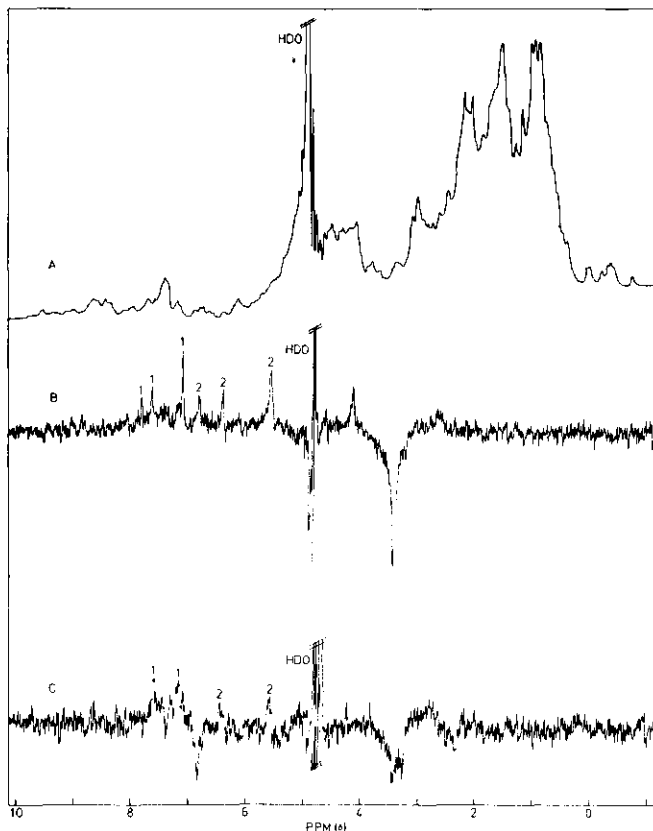
FIGURE 2: 360-MHz ^{1}H FT NMR spectra of 1 mM flavodoxin from *M. elsdenii* in 0.1 M sodium phosphate, p^{2}H 7.1. (A) Conventional spectrum. Photo-CIDNP spectra obtained in the presence of the negatively (B) and the positively (C) charged flavin as an external dye.

of free tryptophan. Moreover the width of the resonance lines of set 1 is smaller than that of the resonance lines of set 2. This suggests that the mobilities of the two tryptophan residues in question are different. It is tempting to suggest that set 1 is due to a tryptophan residue located on the surface of the protein and that the other set is due to a somewhat more buried tryptophan residue. This suggestion would explain the different widths of the resonance lines of the two tryptophan residues and the concentration dependence of the spectrum. This suggestion is supported by conventional ^{1}H NMR experiments where it could be shown that the resonances of set 2 belong to a tryptophan residue located in the neighborhood of the prosthetic group (paramagnetic broadening in the spectrum of the semiquinone form) (C. G. van Schagen & F. Müller, unpublished results). James et al. (1973) assigned in a similar way the proton chemical shifts at 6.32 and 7.07 ppm in the NMR spectrum of *M. elsdenii* to Trp-91. As far as the chemical shift at 6.32 ppm is concerned, the results of James et al. (1973) are in agreement with our data and interpretation. With regard to the value of 7.07 ppm, it must be concluded from our data that this resonance is due to a tryptophan residue other than Trp-91. The polypeptide chain of *M. elsdenii* contains tryptophan residues at positions 7, 91, 96, and 100 (Tanaka et al., 1973). As already mentioned above, the resonance lines at lower field (set 1, Figure 2B) are not broadened in the spectrum of flavodoxin flavosemiquinone (C. G. van Schagen and F. Müller, unpublished results). According to the structure proposed by James et al. (1973) for *M. elsdenii* flavodoxin, Trp-7 is located within a distance of 10 Å from the isoalloxazine ring of FMN, which should lead to broadening of the corresponding resonance lines in the spectrum of the semiquinone. Therefore, the low-field tryptophan resonances must be assigned to either Trp-96 or Trp-

100 in *M. elsdenii* flavodoxin (see also below). The strong emission line at about 3.5 ppm in the spectrum of Figure 2B is due to the β-CH$_2$ group of the tryptophan residues. In addition to the resonance lines of tryptophan residues also an absorption line at about 4.5 ppm is observed in Figure 2B, which could be due to the N(10) CH$_2$ group of FMN, since no CIDNP signals are expected in this spectral region from aromatic amino acid residues (Müller et al., 1980). However, this latter line was not always observed in the CIDNP spectra, and its origin is therefore unclear.

Using a positively charged dye instead of the negatively charged one gave the CIDNP spectrum shown in Figure 2C. Besides the absorption lines due to tryptophan residues (Figure 2B), the spectrum contains additional lines at 6.67 ppm and at about 3.2 ppm. It should be noted that the intensity of the peaks is less than those in Figure 2B leading to the apparent loss of some peaks of the two tryptophan residues. The additional emission line at 6.67 ppm in Figure 2C originates from one of the two tyrosine residues (numbers 6 and 89) present in *M. elsdenii* flavodoxin (Tanaka et al., 1973). The resonance positions of the tyrosine residue are almost identical with those of free tyrosine (Table II). This indicates that this tyrosine residue is positioned at the surface of the protein and is, therefore, assigned to Tyr-6. The fact that the tyrosine residue is not observed in the photo-CIDNP spectrum when the negatively charged dye is used suggests the presence of negatively charged amino acid residue(s) in the neighborhood of the tyrosine residue. The sequence of *M. elsdenii* flavodoxin (Tanaka et al., 1973) shows a glutamic acid residue in position 3. The residues between the glutamic acid and tyrosine residues are isoleucine and valine. It is most likely that the tyrosine and glutamic acid residues are exposed to bulk solvent whereas the other two residues are less exposed to the solvent. This could offer an explanation for the fact that the negatively charged dye cannot interact with the tyrosine residue (repulsion due to the negative charge carried by glutamic acid residue). Tyr-89 is located close to the prosthetic group and can be expected that its chemical shifts would differ from those of free tyrosine. Therefore it is most likely that the CIDNP signal belongs to Tyr-6. The emission line at about 2 ppm in Figure 2C is, by analogy with the corresponding peak in Figure 1B, due to a photochemical product of the dye.

Although the chemical shifts of the low-field peaks of tryptophan shown in parts B and C of Figure 2 are apparently identical, it is possible that these are due to different tryptophan residues, i.e., numbers 96 and 100. The sequence shows (Tanaka et al., 1973) that Trp-96 has two negatively charged neighbors, namely, Glu-95 and Asp-98, whereas Trp-100 has positively charged neighbors, i.e., Lys-101 and Arg-103. Therefore it is reasonable to propose that the low-field resonances observed in the presence of the negatively charged dye are due to Trp-100 and those observed in the presence of the positively charged dye are due to Trp-96.

It would be interesting to obtain CIDNP spectra of *M. elsdenii* flavodoxin in the presence of a neutral dye to support the interpretation given above; i.e., Tyr-6 should be observed in such a spectrum. An attempt to obtain such spectra in the presence of N^3-methyllumiflavin failed, owing to the limited solubility of this dye in aqueous solution. The poorer quality of the spectrum of Figure 2C as compared to that of Figure 2B is a consequence of the limited solubility of the particular dye used.

The three-dimensional structure of *Clostridium MP* flavodoxin is available at 1.9-Å resolution (Burnett et al., 1974). In contrast to the related flavodoxin from *M. elsdenii*, the

CIDNP spectrum of *Clostridium MP* flavodoxin consists of only one emission line in the presence of the negatively charged dye (Table II). This emission line appears toward higher fields compared to that of free tyrosine, which can be taken as an indication that this residue is under the influence of ring-current effects from an aromatic compound, e.g., the prosthetic group of the protein. Inspection of the sequence of *Clostridium MP* flavodoxin reveals that tyrosine residues occur at positions 5, 88, and 106 (Tanaka et al., 1974). According to the X-ray structure (Burnett et al., 1974), Tyr-5 is located within a radius of 10 Å from the prosthetic group, explaining the small upfield shift of the observed resonances as compared to those of free tyrosine. Furthermore, a positively charged amino acid residue (Lys-2) is located close to Tyr-5. This fact could explain the observation that no tyrosine signal is observed in the CIDNP spectrum when the positively charged dye is used (Table II). According to the X-ray structure, Tyr-88 is within 10 Å of the prosthetic group of *Clostridium MP* flavodoxin and is in contact with bulk water. Because of its easy accessibility, Tyr-88 is a good candidate for the observed signal, but Tyr-106 cannot be excluded with certainty as the source of the signal.

The positively charged dye leads to the generation of CID-NP signals due to tryptophan and tyrosine residues of *Clostridium* flavodoxin (Table II). If the suggested correlation between the observation of a CIDNP signal of a particular amino acid residue and the charges of the neighboring amino acid residues is correct, then the tyrosine residues observed in the two sets of experiments might be due to two different tyrosine residues in the sequence. The two resonance lines observed in the CIDNP spectrum (Table II) must be assigned to either Trp-6 or Trp-95 since Trp-90 resonates at 6.39 and 5.95 ppm (James et al., 1973). The final assignment must await further NMR experiments included in our future program.

The CIDNP spectrum of *D. vulgaris* flavodoxin exhibits resonances of one tryptophan and one tyrosine residue. The sequence of the protein (Dubourdieu & Fox, 1977) contains two tryptophan residues, i.e., Trp-60 and Trp-140. Trp-60 and Tyr-98 are located in the isoalloxazine binding pocket of the protein (Watenpaugh et al., 1973). Favaudon et al. (1980) assigned the resonance at 6.82 ppm in the ^{1}H NMR spectrum of *D. vulgaris* to Tyr-98 and a resonance line at 7.12 ppm, observed in the two-electron reduced protein, to Trp-60. The chemical shifts observed in the CIDNP spectrum (Table II) are in good agreement with the values reported by Favaudon et al. (1980), and the upfield shift of the resonance as compared to the corresponding free amino acids suggests that the corresponding amino acid residues are located in the vicinity of the prosthetic group.

The CIDNP spectrum of *A. vinelandii* shows resonances due to tryptophan and tyrosine residues (Table II). The sequence of the protein contains five tyrosine and three tryptophan residues (Tanaka et al., 1977). No other structural data to aid assignment are available. Nevertheless, the resonances observed are identical with those of the corresponding free amino acids, indicating that they probably do not belong to amino residues constituting the active center of the protein.

The conventional ^{1}H NMR spectrum of *M. elsdenii* apoflavodoxin differs from that of the holoprotein at high and low fields (Figure 3A vs. Figure 2A). It is, therefore, interesting to apply the photo-CIDNP technique to the apoflavodoxin. The CIDNP spectrum obtained in the presence of the neutral dye is presented in Figure 3B and shows two absorption peaks due to tryptophan residues and two emission lines at about 7 and 3.5 ppm. The resonance line at about 7 ppm belongs to
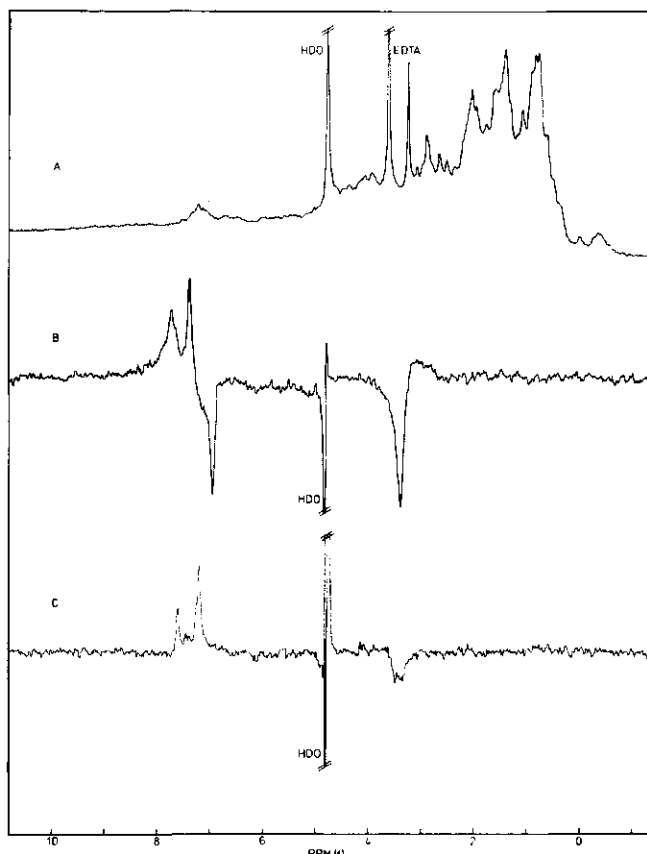

FIGURE 3: 360-MHz ^{1}H FT NMR spectra of 1 mM *M. elsdenii* apoflavodoxin in 0.1 M sodium phosphate, p^{2}H 7.1. (A) Conventional spectrum. Photo-CIDNP spectra obtained in the presence of the neutral (B) and in the presence of the positively charged dye (C).

a tyrosine residue. In the presence of the negatively charged dye, an identical CIDNP spectrum was obtained, whereas in the presence of the positively charged dye, a spectrum was observed exclusively due to tryptophan residues (Figure 3C and Table II). The absence of the high-field peaks of tryptophan (cf. Figure 2B, set 2) in the spectrum of the apoflavodoxin gives independent support to the assignment of the high-field peaks in the spectrum of the holoprotein to Trp-91. It is very difficult to assign the lines in the CIDNP spectra of the apoflavodoxin since the positively and negatively charged dyes discriminate differently between the tyrosine residues in the holo- and apoproteins. This is probably a consequence of conformational changes occurring in the flavin binding site upon preparation of the apoprotein. Nevertheless, the differences observed between the spectrum of Figure 3B and that of Figure 3C suggest that the dyes interact with different tryptophan residues. The observed chemical shifts support this suggestion (Table II). Moreover, the low-field peak (7.65 ppm) in Figure 3B is rather broad, whereas the peak at higher field (7.33 ppm) is rather sharp in comparison. This situation is reversed in Figure 3C. The fact that two tryptophan emission peaks are observed in Figure 3C, in contrast to Figure 3B, is strong evidence for this proposal.

Other Flavoproteins. With increasing molecular weight the line width in a conventional ^{1}H NMR spectrum also increases, leading to unresolved spectra. The "envelope" thus obtained does, however, contain sharp lines from amino acid residues possessing large internal freedom. It seemed of interest to explore the possibility for observing such sharp lines in proteins of molecular weights between 50 000 and 100 000. It was expected that the photo-CIDNP difference technique should make it possible to observe such lines. As demonstrated in Figure 4 for D-amino acid oxidase, this is indeed the case. It
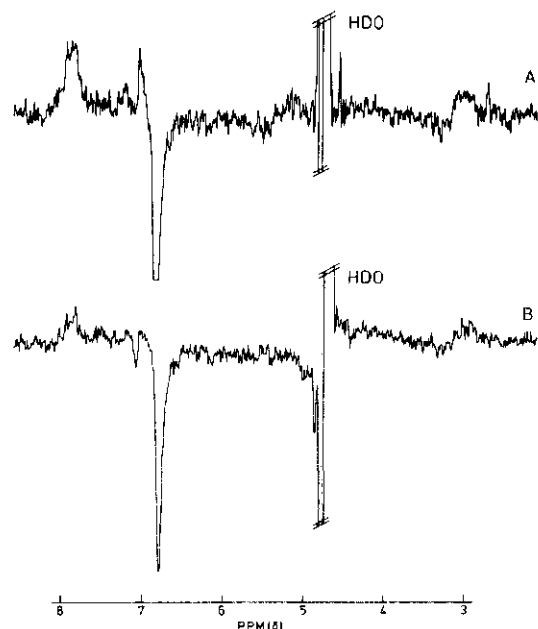
FIGURE 4: 360-MHz ^{1}H FT NMR spectra of 1 mM D-amino acid oxidase in 0.1 M sodium phosphate, p^{2}H 7.2. Difference spectra (light and dark) obtained in the presence of the negatively (A) and the positively (B) charged flavin as an external dye.

should be noted that the signal-to-noise ratio is less favorable than that for the smaller flavoproteins.

The signals observed in the CIDNP spectra of the various flavoproteins are collected in Table II. It is seen that various amino acid residues can indeed be observed in large flavoproteins. Although the resonance lines cannot be assigned yet, this information may be helpful in further studies of flavoproteins. Especially the ease of obtaining CIDNP spectra and the relatively low protein concentrations needed make this technique a valuable tool for chemical protein modification studies. The observation that differently charged dyes (cf. also Figure 4) can lead to CIDNP signals originating from different amino acid residues should make it possible to study the topography of proteins. Furthermore, it should be possible to investigate any influence of chemical modification of particular amino acids on the structure of the protein and to identify possible essential and nonessential amino acid residues. Such studies performed in the presence and absence of substrates or inhibitors may contribute to the unraveling of protein active centers.

Acknowledgments

References

Abramovitz, A. S., & Massey, V. (1976) J. Biol. Chem. 251, 5321–5326.

Burnett, R. M., Darling, G. D., Kendall, D. S., LeQuesne, M. E., Mayhew, S. G., Smith, W. W., & Ludwig, M. L. (1974) J. Biol. Chem. 249, 4383–4392.

Dubourdieu, M., & Fox, J. L. (1977) J. Biol. Chem. 252, 1453–1463.

Favaudon, V., Le Gall, J., & Lhoste, J.-M. (1980) in Flavins and Flavoproteins (Yagi, K., & Yamano, T., Eds.) pp 373–386, Japan Scientific Societies Press, Tokyo.

Hemmerich, P. (1964) Helv. Chim. Acta 47, 464–475.

James, T. L., Ludwig, M. L., & Cohn, M. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3292–3295.

Kaptein, R. (1971) J. Chem. Soc., Chem. Commun., 732–733.

Kaptein, R. (1977) in Chemically Induced Magnetic Polarization (Munds, L. T., Ed.) pp 1–16, D. Reidel Publishing Co., Dordrecht, The Netherlands.

Kaptein, R. (1978) in Nuclear Magnetic Resonance Spectroscopy in Molecular Biology (Pullman, B., Ed.) pp 211–229, D. Reidel Publishing Co., Dordrecht, The Netherlands.

Kaptein, R., Dijkstra, K., Müller, F., van Schagen, C. G., & Visser, A. J. W. G. (1978) J. Magn. Reson. 31, 171–176.

Kaptein, R., Nicolay, K., & Dijkstra, K. (1979) J. Chem. Soc., Chem. Commun., 1092–1094.

Land, E. J., & Swallow, A. J. (1969) Biochemistry 8, 2117–2125.

Massey, V., Palmer, G., & Bennett, R. (1961) Biochim. Biophys. Acta 48, 1–9.

Mayhew, S. G., & Massey, V. (1969) J. Biol. Chem. 244, 794–802.

Müller, F., van Schagen, C. G., & Kaptein, R. (1980) Methods Enzymol. 66, 385–416.

Müller, F., Grande, H. J., Harding, L. J., Dunham, W. R., Visser, A. J. W. G., Reinders, J. H., Hemmerich, P., & Ehrenberg, A. (1981) Eur. J. Biochem. 116, 17–25.

Murthy, S., Sreekrishna, K., & Adigo, P. R. (1979) Anal. Biochem. 92, 345–350.

Palmer, V., & Massey, V. (1966) Biochemistry 5, 3181–3189.

Tanaka, M., Haniu, M., Yasunobu, K. T., Mayhew, S. G., & Massey, V. (1973) J. Biol. Chem. 248, 4354–4366.

Tanaka, M., Haniu, M., Yasunobu, K. T., & Mayhew, S. G. (1974) J. Biol. Chem. 249, 4393–4396.

Tanaka, M., Haniu, M., Yasunobu, K. T., & Yoch, D. C. (1977) Biochemistry 16, 3525–3537.

Tsibris, J. C. M., McCormick, D. B., & Wright, L. D. (1965) Biochemistry 4, 504–510.

van Schagen, C. G., & Müller, F. (1977) in Flavins and Flavoproteins: Physicochemical Properties and Function (Ostrowski, W., Ed.) pp 9–27, Polish Scientific Press, Warsaw and Cracow.

Vass, L. (1966) Ph.D. Thesis, University of Basel, Basel, Switzerland.

Visser, A. J. W. G., Müller, F., & Van Voorst, J. D. W. (1977) Biochem. Biophys. Res. Commun. 77, 1135–1142.

Wahl, P., Auchet, J. C., Visser, A. J. W. G., & Müller, F. (1974) FEBS Lett. 44, 67–70.

Watenpaugh, K. D., Sieker, L. C., & Jensen, L. H. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3857–3860.

SUMMARY

The subject of this thesis is the application of high resolution NMR techniques to study the structure of free and protein-bound flavins. The main part of the thesis deals with low molecular weight flavoproteins, especially with the flavodoxins from M.elsdenii and A.vinelandii. The model studies served as a basis for the interpretation of the corresponding results obtained with flavoproteins.

Chapter 2 describes the ^{13}C NMR properties of the two-electron reduced free flavins in apolar solvents. It was found that a correlation exists between the calculated and observed ^{13}C chemical shifts. This correlation relates the ^{13}C chemical shifts to the π-electron density in a given molecule. This empirical observation allows to draw a cautious conclusion with respect to the perturbation of the π-electron density upon binding of flavin to a particular apoflavoprotein (see Chapter 3). Reduction of the oxidized flavin by two electrons leads to an upfield shift of the resonances due to C(8), C(6), C(4a) and C(10a). The resonance most affected by reduction is that due to C(4a) (upfield shift of ~35 ppm). From the two electrons added to oxidized flavin 80% is accomodated in the pteridine subring whereas the residual 20% is distributed over the benzene moiety of the flavin molecule. The results further support the view that flavin is a bent molecule in the reduced state.

Selectively enriched ^{13}C flavins and prosthetic groups, free and bound to apoflavoproteins, were also studied (Chapter 2). It is shown that the chemical shifts due to C(2), C(6), C(8) and C(10a) in the oxidized free molecule are strongly influenced by the polarity of the solvent. This observation is ascribed to polarization of the molecule in aqueous solutions. This interpretation is supported by the observed direct (one-bond) ^{13}C coupling constants measured in polar and apolar solvents. Similarly it could be shown that the conformation of reduced flavin depends also on the polarity of the solvent, i.e. the molecule is less bent in polar than in an apolar solvent. Comparison of the ^{13}C chemical shifts of free with those of flavin bound to M.elsdenii and A.vinelandii apoflavodoxins revealed that in the oxidized form of the two flavoproteins the fla-

vin forms a hydrogen bond with an amino acid residue of the apoprotein. The interaction with the apoprotein occurs via the $O(2\alpha)$ atom of flavin. This interpretation is in accord with the observed chemical shift due to C(10a). In contrast to the $O(2\alpha)$ atom, the $O(4\alpha)$ atom in M.elsdenii flavodoxin shows a weak interaction with the apoprotein whereas the same atom in A.vinelandii flavodoxin seems to be placed in a rather hydrophobic environment. In the reduced state both flavodoxins possess a more planar structure than free flavin in aqueous solution. In addition these flavodoxins are in the anionic state in the pH range 5.5 to 8.5. Free flavin, on the other hand, shows an ionization constant of 6.7. The facts that the protein-bound flavin is negatively charged, possesses an almost planar structure stabilized by specific interactions with the apoprotein and the relatively apolar environment of the active center indicate that these elements form the basis for the strong reductive power of flavodoxins.

In chapter 4 a ^{1}H NMR study on M.elsdenii flavodoxin is described. The ^{1}H NMR spectrum shows some well resolved resonances at high field (upfield from the internal standard). These resonances are due to methyl groups being under the influence of ring current effects. Some of these resonance lines could be assigned. It follows from oxidation-reduction experiments that the resonances at -0.72, -0.28 and -0.07 ppm are due to methyl groups located in the neighbourhood of the prosthetic group. The resonance line at -0.07 ppm is assigned to alanine-57 and the lines at -0.72 and -0.28 ppm to the S-methyl groups of leucine-62. Since the latter two methyl groups are magnetically non-equivalent it must be concluded that the isopropyl moiety of leucine-62 is not free to rotate around the $CH-CH_2$ bond of leucine. The resonance lines of the three methyl groups are broadened in the radical state supporting the idea that they are located in the active center of the protein. In the two-electron reduced state the resonances due to the methyl groups are slightly downfield shifted indicating attenuation of the ring current effect, probably caused by a slight bending of the isoalloxazine moiety of the prosthetic group. In addition the resonances due to the C(7) and C(8) methyl groups of the prosthetic group could be assigned in the spectrum. These resonances differ only

by about 0.5 ppm from the position of those of free flavin. Tryptophan-91, a constituent of the active site of the protein, could be identified in the ^{1}H NMR spectrum by a combination of various techniques. From the ^{1}H NMR study it follows that the protein does not undergo a gross conformational change upon reduction.

To aid in the assignment of the resonances due to aromatic amino acid residues in the ^{1}H NMR spectrum of the protein the photochemically induced dynamic nuclear polarization (CIDNP) technique was applied to small size flavoproteins (chapter 5). It was found that the protein-bound flavin did not yield any CIDNP signals. To generate CIDNP signals external free flavins had to be added to solutions of flavoproteins. An interesting observation is that protein-bound flavin could act as a sensitizer in the CIDNP reaction when external, free aromatic amino acids were added to solutions of flavoproteins. This strongly indicated that the active centers of the flavoproteins tested must be accessible to the free amino acids. Several amino acid residues in various flavoproteins could be identified, although the assignment of the aromatic amino acid residues in the sequence of some flavoproteins could not be made.
A CIDNP study on free FAD revealed that this molecule exists as an internal complex at neutral pH. This complex is gradually destroyed in solutions of decreasing pH. This result is in agreement with published fluorescence studies.

SAMENVATTING

Dit proefschrift behandelt de toepassing van hoge resolutie NMR bij de studie van vrij en eiwit-gebonden flavine. De bestudeerde flavoproteïnen waren voornamelijk de flavodoxinen van M.elsdenii en A.vinelandii. De studie aan de modelverbindingen dienden ter interpretatie van de resultaten verkregen met deze twee flavodoxinen.

Hoofdstuk 2 beschrijft de resultaten zoals die met de modelverbindingen in apolair milieu verkregen werden. Het was mogelijk om uitgaande van de berekende π elektronen dichtheden een goede correlatie te leggen tussen de berekende en de gemeten koolstof-13 chemische verschuiving. Op grond van deze emiprische relatie tussen de waargenomen chemische verschuiving en de π elektronen dichtheid konden conclusies getrokken worden over de π elektronen dichtheidsverdeling bij de verschillende modelverbindingen en het gereduceerde flavine. Na de reductie tot het volledig gereduceerd flavine bleken de koolstof atomen op de 8, 6, 4a en 10a plaats en grotere π elektronen dichtheid te verkrijgen. Die op de 4a positie nam het sterkst toe. Op grond van het feit dat van de π elektronendichtheid die aan de geoxideerde structuur wordt toegevoegd 80% in de pteridine ring van flavine terechtkomt, mag geconcludeerd worden dat het volledig gereduceerde flavine een gebogen struktuur heeft.

Hoofdstuk 3 behandelt de resultaten met selectief verrijkt flavine. Bovendien wordt het eiwit-gebonden flavine besproken. De polariteit van het oplosmiddel bleek de chemische verschuiving op de centra 2, 6, 8 en 10a het sterkst te beïnvloeden. Deze chemische verschuiving werd verklaard door de polarisatie van het molecuul onder invloed van het oplosmiddel. De toename van de ^{13}C-^{13}C koppelingsconstanten wijzen in dezelfde richting. Op grond van deze waarnemingen mag geconcludeerd worden dat de conformatie van de flavine afhankelijk is van de polariteit van zijn omgeving.

Het protein-gebonden flavine is via waterstofbruggen gebonden aan het apoflavodoxine van zowel het M.elsdenii als het A.vinelandii. De belangrijkste

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is die tussen de O(2α) van het flavine en een aminozuur van het apoflavo-doxine. De chemische verschuiving van het koolstofatoom op de 2 positie en dat op de 10a positie tonen dit aan.

De omgeving van de twee gebonden flavines blijkt sterk te verschillen. Bij het A.vinelandii flavodoxine bevindt de pteridine ring van het flavine zich geheel in een hydrofobe omgeving terwijl in het M.elsdenii flavodoxine de O(4α) veel meer met water in contact staat. Het verschil in chemische verschuiving tussen de C(4) kernen van de twee eiwitten wijst in die richting.

In de gereduceerde vorm zijn de flavodoxines in de anionische vorm bij een pH van 5,5 - 8,5. De ionisatie constante van het vrije flavine is 6,7. Bovendien blijkt uit de vergelijkingen van de chemische verschuiving tussen het vrije gereduceerde anion en het gebonden anion dat de laatste vlakker is dan het vrije anion. Dit is ook de verklaring voor het grote reducerende vermogen van de flavodoxines.

Hoofdstuk 4 omvat een ^{1}H NMR studie aan het M.elsdenii flavodoxine. Opvallend in dit spectrum zijn de lijnen die boven de interne standaard liggen. Deze resonanties behoren toe aan een aantal methylgroepen die door de invloed van hun omgeving naar hoger veld verschoven zijn. Uit de oxidatie reductie experimenten mag geconcludeerd worden dat de resonanties op -.72, -.28 en -.07 ppm in de buurt van de flavine liggen. De eerste twee blijken aan de 2 methylgroepen van Leu-62 te behoren, de derde resonantie lijn is toegekend aan Ala-57. Uit het niet equivalent zijn van deze twee resonanties blijkt dat de leucine gehinderd is in zijn rotatie mogelijkheden.

In de volledig gereduceerde vorm verschuiven de resonanties van de methyl-groepen naar laag veld. Dit wordt veroorzaakt door de afname van de ringstroom in de gereduceerde vorm. Deze is ook meer gebogen dan de geoxideerde vorm, bovendien geladen. Deze effecten dragen ook bij aan de grote verschillen tussen de resonanties van vrij en het gebonden flavine, maar kunnen dit niet geheel verklaren. De resonanties van Trp-91, een onderdeel van het actieve centrum, konden worden toegekend. Uit het spectrum mag verder geconcludeerd

worden dat de tertiaire structuur sterk afhankelijk is van de redox toestand.

In hoofdstuk 5 wordt een techniek behandeld waarbij fluorescentie en ^{1}H NMR gecombineerd worden. Het CIDNP is het gevolg van een reactie tussen flavine en een aminozuur nadat de flavine door een lichtpuls aangeslagen is. Het was een interessante waarneming dat het eiwit-gebonden flavine met een vrij aminozuur een CIDNP-signaal geeft. Dit ondanks het feit dat het gebonden flavine niet fluoresceert.

Via extern flavine was het mogelijk om enkele aminozuren in verschillende flavoproteinen aan te tonen. Deze techniek bleek toepasbaar tot een molekuulgewicht van 100.000 Dalton.

Een CIDNP studie van het FAD toont aan dat bij neutrale pH het FAD een intramoleculair complex vormt. Bij lagere pH bestaat dit complex niet en zijn de flavine en de adenine ring van elkaar verwijderd. Dit is de bevestiging van de suggestie gebaseerd op de fluorescentiestudies.

Vanwege een intramoleculair complex geven de flavoproteïnen zonder externe toevoeging van vrij flavine geen CIDNP-signaal.

NAWOORD

Nu het gebed tot een end gekomen is, past een dankwoord. Allereerst aan al diegenen op de afdeling Biochemie die daar zowel de eerste vier jaar als later een positieve bijdrage aan leverden. Maar natuurlijk ook zij van de afdeling Moleculaire Fysica die de Varian XL-100 aan de gang hielden, en aan bijzondere wensen altijd voldeden.

Het meten in Groningen was steeds weer een avontuur. Knalde niet een kwiklamp uit elkaar, dan ging de koeling van de probe wel los of viel er 's nachts gewoon een buisje om. De mensen van Fysische Chemie bij wie ik zoveel 'geleend' heb en die altijd wel een oplossing wisten voor de technische problemen wil ik hier met ere noemen.

En dan wil ik tenslotte nog een dankwoord richten aan mijn promotor Franz, zonder jouw inzet en motivatie was al dit werk in de vergetelheid geraakt.

CURRICULUM VITAE

Na het behalen van mijn HBS-B diploma aan het Christelijk Lyceum

Zandvliet (1968) begon ik mijn universitaire loopbaan in september van

dat jaar aan de Rijksuniversiteit te Leiden. In november 1971 werd het can-

didaatsexamen S1 behaald. Dit werd drie jaar later gevolgd door het doc-

toraal examen dat in november 1974 afgelegd werd. Het hoofvak was analytische

chemie, de bijvakken, conformatie analyse en numerieke wiskunde. De leiding

gedurende de hoofdvakperiode was in handen van wijlen prof.dr. J.B. Schute.

Dr. C. Altona en Prof.dr. J.M. Spijker waren verantwoordelijk voor mijn

bijvak perioden.

In datzelfde jaar 1974, werd een aanvang gemaakt met het promotie

onderzoek op de afdeling Biochemie van de LH te Wageningen.

Op 1 januari 1979 trad ik in dienst bij Glaxo BV waar ik nu sinds

1 jaar de functie van district manager, ofwel districtsverkoopleider,

vervul.