237. Electron-Nuclear Double Resonance Study of Flavodoxin from *Peptostreptococcus Elsdenii*

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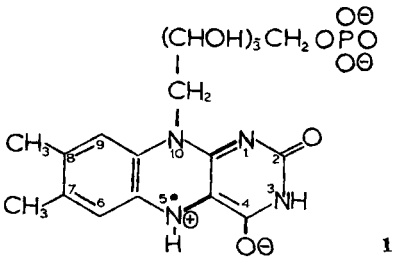
Summary. The proton electron-nuclear double resonance (ENDOR) of the semiquinone of *Peptostreptococcus elsdenii* flavodoxin has been studied at $-155°$. Studies with flavodoxin derivatives in which the natural coenzyme has been replaced by C(8)–CD_3–, C(9)–D–, C(6,9)–D_2–, and *iso*-FMN[2]) have made it possible to unequivocally assign the ENDOR lines to C(8)–CH_3 and C(6)–H.

Introduction. – Flavoprotein radicals exhibit poorly resolved electron spin resonance (ESR.) spectra due to anisotropic broadening [1]. Recently it has been shown that electron-nuclear double resonance (ENDOR) spectra of frozen solutions of free and protein-bound flavin radicals yield valuable information about the submolecular structure of the isoalloxazine moiety [2–4]. Data obtained with the anionic radical of yeast NADPH dehydrogenase and the neutral radical of *Azotobacter* flavoprotein [2–3] indicate that the spin distributions in free and protein bound flavin semiquinones are very similar. A line due to C(8)–CH_3 of flavin was found at about 20 MHz in the ENDOR spectrum of NADPH dehydrogenase, but a line expected for the proton at C(6) was not identified.



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This paper describes ENDOR spectra of a different flavoprotein, flavodoxin. Flavodoxin is an electron transfer protein which contains one molecule of FMN as prosthetic group [5–6]. At half reduction it yields large amounts of the neutral flavin radical [7] (**1**), a property which makes it suitable for ENDOR studies. In order to

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[2]) FMN = Flavin mononucleotid.

assign the individual ENDOR lines in the spectrum unequivocally, the natural prosthetic group has been replaced by a number of FMN derivatives.

Methods and materials. – Flavodoxin was prepared from *Peptostreptococcus elsdenii* [6]. The methods used to prepare apoflavodoxin and to combine apoflavodoxin with derivatives of FMN have been described [8]. Samples in D_2O were prepared by freeze drying the protein, dissolving the dry powder in D_2O and repeating this procedure three times. For ENDOR measurements, 0.3 ml of a solution containing $3–4 \times 10^{-3}$ M flavodoxin, 0.1 M potassium phosphate pH 7 and 0.1 M EDTA, was placed in a quartz sample tube. Air was removed from the tube with a vacuum pump and replaced with nitrogen before sealing the top of the tube in a flame. The tube was then illuminated [6] until maximum flavin radical had formed. Details of the ENDOR effect and instrumentation have been described elsewhere [9] [10].

C(8)–CD_3–FMN was prepared from a solution of 0.05 M FMN in 99.8% D_2O (from *ICN Corporation*) at pH 6.8–6.9 [11]. An NMR.-spectrum (measured in a *Varian* T60 spectrometer) showed that isotopic substitution was greater than 85%.

C(9)–D–FMN was synthesized according to the procedure of *Bullock & Jardetzky* [11]. The hydrochloride of 3,4-dimethylaniline was refluxed twice in D_2O for 50 h to give 2,6-dideuterio-3,4-dimethylaniline. This was condensed with D-ribose in ethanol to form a *Schiff's* base which was then catalytically reduced to 2,6-dideuterio-3,4-dimethyl-N-ribytylaniline [12]. The aniline derivative was diazotized and the dye formed was then condensed with barbituric acid to yield C(9)–D-riboflavin [13]. The FMN analog of C(9)–D-riboflavin was made according to *Flexser & Farkas* [14]. The deuterium content of C(9)–D–FMN was determined by NMR. to be greater than 90% of the theoretical value.

6,9-Dideuterio-FMN was made from 2,5,6-trideuterio-3,4-dimethylaniline. The procedure of *Eriksson et al.* [15] for the synthesis of 2,5,6-trideuterio-3,4-dimethylaniline was modified because the original procedure caused much sulfonation of the starting material. 3,4-Dimethylaniline (3 g) or its dideuterio analog (made as described above) was dissolved in a mixture of 7 g conc. D_2SO_4 (from *ICN Corporation*) and 3 ml D_2O, and heated at 95–100° for $4^1/_2$ days. The deuterated product was isolated and treated again in the same way. The dark brown mixture was cooled, diluted with 150 ml H_2O and adjusted the pH to 1 adding solid $NaHCO_3$. A black amorphous precipitate was removed by filtration, the filtrate was treated with activated charcoal and filtered again. The almost colorless solution from this second filtration was neutralized with solid $NaHCO_3$ and extracted with $CHCl_3$. The organic phase which contained 2,5,6-trideuterio-3,4-dimethylaniline was evaporated to dryness to yield 2.2 g of product. NMR.-spectroscopy showed that the deuterium substitution exceeded 95%. 6,9-Dideuterio-FMN was then prepared as outlined above [13] [14]. The deuterium content of the final product was 90–95% in position 9 and 50–60% in position 6.

Results and Discussion. – The proton ENDOR-spectrum of native flavodoxin in H_2O consists of three main peaks centred at 13.9, 16.5, and approximately 18 MHz respectively (Fig. 1a). The absorption at 13.9 MHz, the *Zeeman* frequency for protons, is called 'matrix ENDOR' [16], and is due to those electrons which interact weakly with the unpaired electron spin. The intensity of this line is decreased when the protein is soaked in D_2O (Fig. 1b), and the effect is of about the same magnitude as was found previously with *Azotobacter* flavoprotein [3]. Although care must be taken in trying to draw accurate quantitative conclusions from the spectra shown in this paper, since the relative proton intensities are strongly dependent on the magnetic field setting, this effect of D_2O supports the conclusion that the neutral semiquinone of flavoproteins is in contact with either water molecules or hydrophylic protein side chains with exchangeable protons. A similar conclusion has been drawn from other physical measurements [17–19]. It has been suggested that in contrast, the anionic semiquinone of NADH dehydrogenase from yeast is much less accessible to water, and that possibly its environment consists partly of hydrophobic side chains [3].

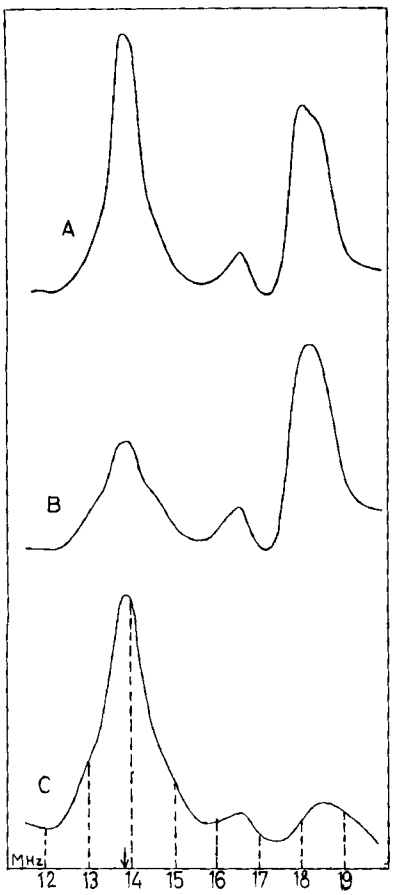

Fig. 1. *ENDOR-spectra of flavodoxin semiquinone*
a) Native flavodoxin in H_2O; free proton frequency 13.85 MHz. b) Native flavodoxin in D_2O; free proton frequency 13.9 MHz. c) C(8)—CD_3-FMN flavodoxin in H_2O; free proton frequency 13.9 MHz. Protein concentrations were 3 mM and samples were dissolved in 0.1 M potassium phosphate, pH 7, and 0.01 M EDTA. The semiquinone was generated by photoreduction. The microware power was 6 mW. Temp. −155°.
Experimental field setting = ESR crossing point.

The absorption at around 18 MHz in the ENDOR-spectrum of flavodoxin is assigned to the methyl group at position 8 of the isoalloxazine ring. This assignment is based on the fact that the line is decreased when the native flavin of flavodoxin is replaced by C(8)-CD_3-FMN (Fig. 1c). Some of the residual absorption in the high frequency spectrum of C(8)-CD_3-FMN flavodoxin is due to incomplete deuterium exchange on the methyl group. However, a part of it may be due to coupling of the CH_2' group of the ribityl side chain [3], as indicated by model studies [20], and may also contribute to the shoulder on the high frequency side of the 18 MHz peak in Fig. 1a. These results are similar to those obtained earlier with the anionic semiquinone of NADH dehydrogenase [3].

The weak line at 16.5 MHz (Fig. 1a) is assigned to the proton at C(6) of the flavin. This assignment was verified by comparing native flavodoxin with C(6,9)-D_2-FMN and C(9)-D-FMN flavodoxin all soaked in D_2O. The ENDOR-spectra of native and C(9)-D-FMN flavodoxin are identical in the 15.5–19 MHz range, in accord with ESR.-data from model studies [20], which indicated that there is only a very small spin density at C(9). The spectrum of C(6,9)-D_2-FMN on the other hand has de-

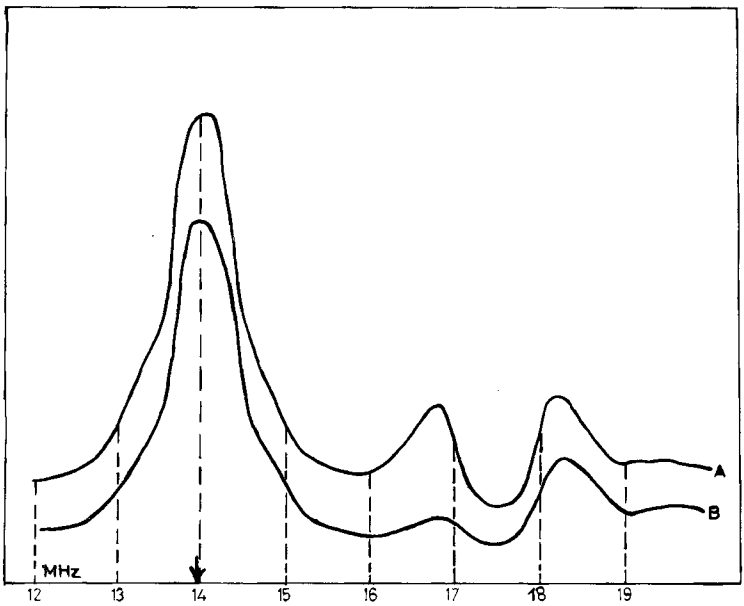

Fig. 2. *ENDOR-spectra of* a) *native flavodoxin semiquinone and* b) *C(6,9)—D_2-FMN flavodoxin semiquinone in H_2O.* Conditions were as described in the legend to Fig. 1. Free proton frequency 13.8 MHz.

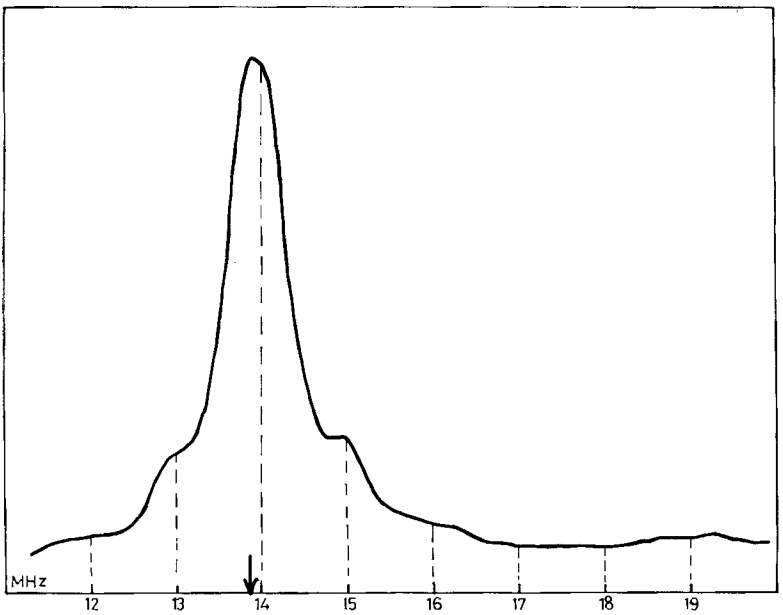

Fig. 3. *ENDOR-spectrum of* iso-*FMN flavodoxin in H_2O.* Conditions as described for Fig. 1, except for the microwave power which was 0.6 mW. The free proton frequency was 13.95 MHz.

creased absorption at 16.5 MHz, as seen by comparison of Fig. 2a and 2b (spectra in these two Fig. were recorded under identical instrumental settings). This proves that the absorption is due to the C(6) proton, as tentatively suggested earlier for *Azotobacter* flavoprotein [3]. The remaining absorption of C(6,9)–D_2–FMN flavodoxin at 16.5 MHz is almost certainly due to residual protons at position 6; the deuterium content of this position was between 50 and 60%.

We initially used *iso*-FMN flavodoxin (C(6)–CH_3, C(7)–CH_3, C(8)–H) in an attempt to find the C(6)–H absorption. It is known that the isotropic part of the hyperfine constant for a methyl group or a proton bonded to a six membered ring in a small molecule are nearly the same [21]. Thus one would expect to find an intense line for C(6)-CH_3 in flavodoxin. The ENDOR-spectrum of *iso*-FMN flavodoxin (Fig. 3) clearly shows that this line is absent. Moreover, the altered matrix ENDOR of this molecule seems to indicate that the spin density distribution is different from that of native flavodoxin.

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REFERENCES

[1] *A. Ehrenberg*, Ark. Kemi *19* (1962) 97–117.
[2] *A. Ehrenberg, L. E. G. Eriksson & J. S. Hyde*, Biochim. biophys. Acta *167* (1968) 482–484.
[3] *L. E. G. Eriksson, A. Ehrenberg & J. S. Hyde*, European J. Biochemistry *17* (1970) 539–543.
[4] *L. E. G. Eriksson & A. Ehrenberg*, Biochim. biophys. Acta *293* (1973) 57–66.
[5] *E. Knight Jr. & R. W. F. Hardy*, J. biol. Chemistry *242* (1967) 1370–1371.
[6] *S. G. Mayhew & V. Massey*, J. biol. Chemistry *244* (1969) 794–802.
[7] *V. Massey & G. Palmer*, Biochemistry *5* (1966) 3181.
[8] *S. G. Mayhew*, Biochim. biophys. Acta *235* (1971) 289–302.
[9] *J. S. Hyde*, J. Chem. Physics *43* (1965) 1806.
[10] *J. Fritz, R. Anderson, J. Fee, G. Palmer, R. H. Sands, J. C. M. Tribris, I. C. Gunsalus, W. H. Orme-Johnson & H. Beinert*, Biochim. biophys. Acta *253* (1971) 110–133.
[11] *F. J. Bullock & O. Jardetzky*, J. Org. Chemistry *30* (1965) 2056–2057.
[12] *J. P. Lambooy*, J. Amer. chem. Soc. *80* (1958) 110–113.
[13] *C. H. Shunk, J. B. Lavigne & K. Folkers*, J. Amer. chem. Soc. *77* (1955) 2210–2212.
[14] *L. A. Flexser & W. G. Farkas*, U.S. Patent 21610176 (1952).
[15] *L. E. G. Eriksson & A. Ehrenberg*, Acta chem. scand. *18* (1964) 1437–1453.
[16] *J. S. Hyde, G. H. Rist & L. E. G. Eriksson*, J. Phys. Chemistry *72* (1968) 4269–4276.
[17] *F. Müller, M. Brüstlein, P. Hemmerich, V. Massey & W. H. Walker*, European J. Biochemistry *25* (1972) 573–580.
[18] *G. Palmer & A. S. Mildvan*, Fed. Proc. *29* (1970) 914 Abs.
[19] *G. Palmer, F. Müller & V. Massey*, in Flavins and Flavoproteins (edited by H. Kamin), University Park Press, Baltimore, 1971, pp. 123–136.
[20] *F. Müller, P. Hemmerich, A. Ehrenberg, G. Palmer & V. Massey*, European J. Biochemistry *14* (1970) 185–196.
[21] *R. W. Tessenden & R. H. Schuler*, J. Chem. Physics *39* (1963) 2147.