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

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Received 29 October 1981

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 apoflavodoxin 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

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

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 internal 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

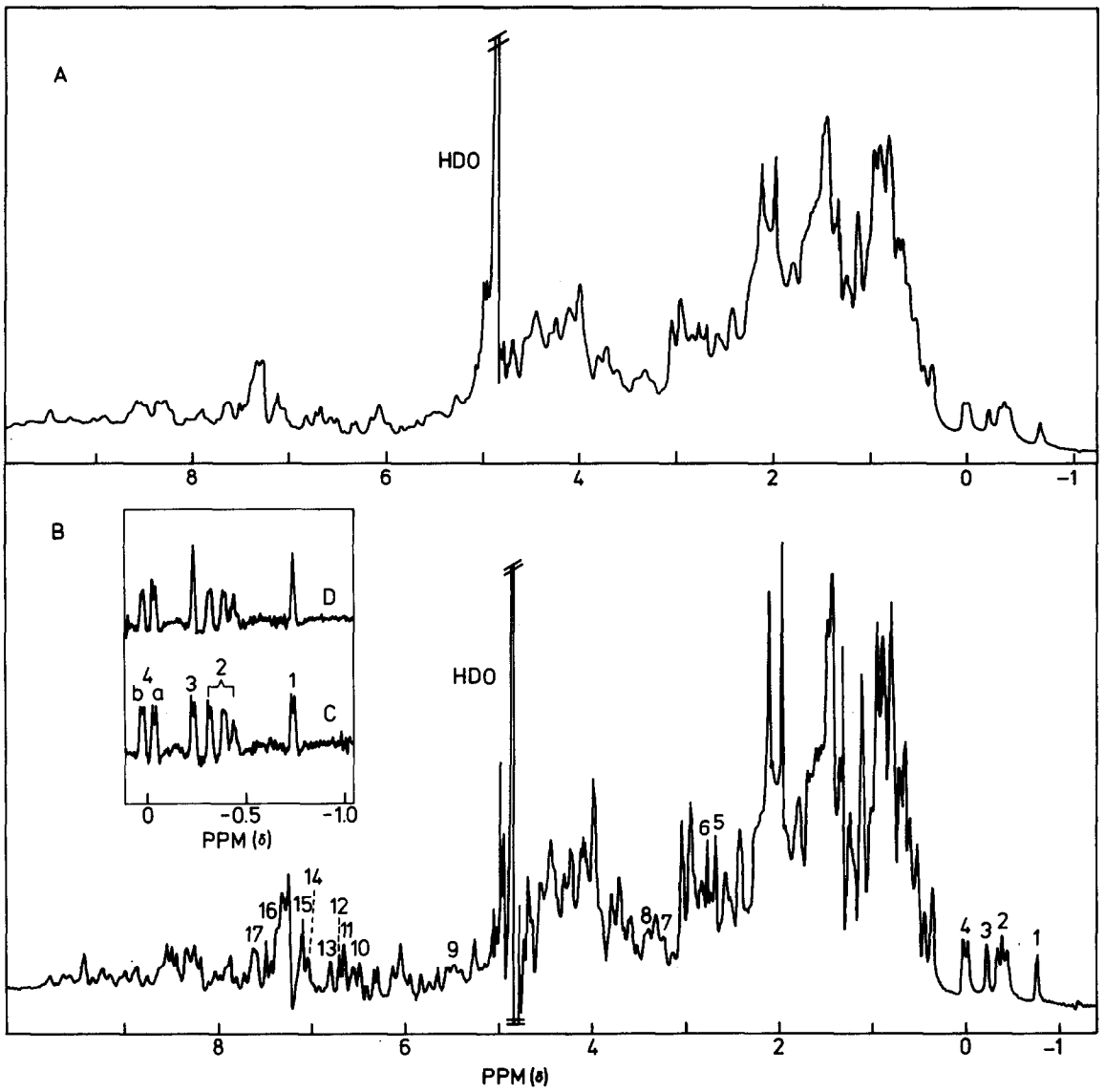

Fig.1. 360 MHz ^{1}H FT NMR spectra of a 2 mM solution of *M. elsdenii* flavodoxin in 0.1 M phosphate, p^{2}H 6.8: (A) conventional spectrum; (B) obtained by treatment of the original data (A) by the convolution difference technique [14]. The original spectrum was obtained under the following instrumental 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-57, 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 attentuated, 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 $C^2H_3C(8)$-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 $C^2H_3C(8)$-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 ^2H-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

We thank Mr M. M. Bouwmans for the preparation of the figure, Mr W. J. B. van Berkel for the isolation of the protein and Mrs J. C. Toppenberg-Fang for typing the manuscript and to Dr G. F. W. Searle for linguistic advice. This work was supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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