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

Chrit T.W. Moonen, Peter J. Hore[+,*], Franz Müller, Robert Kaptein[+] and Stephen G. Mayhew[†]

Department of Biochemistry, Agricultural University, De Dreijen 11, 6703 BC Wageningen, [+]Department of Physical Chemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands and *[†]Department of Biochemistry, University College, Belfield, Dublin 4, Ireland*

Received 30 September 1982

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

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

1. INTRODUCTION

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

* Present address: Physical Chemistry Laboratory, South Parks Road, Oxford, England

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

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

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

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

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

2. MATERIALS AND METHODS

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

3. RESULTS AND DISCUSSION

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

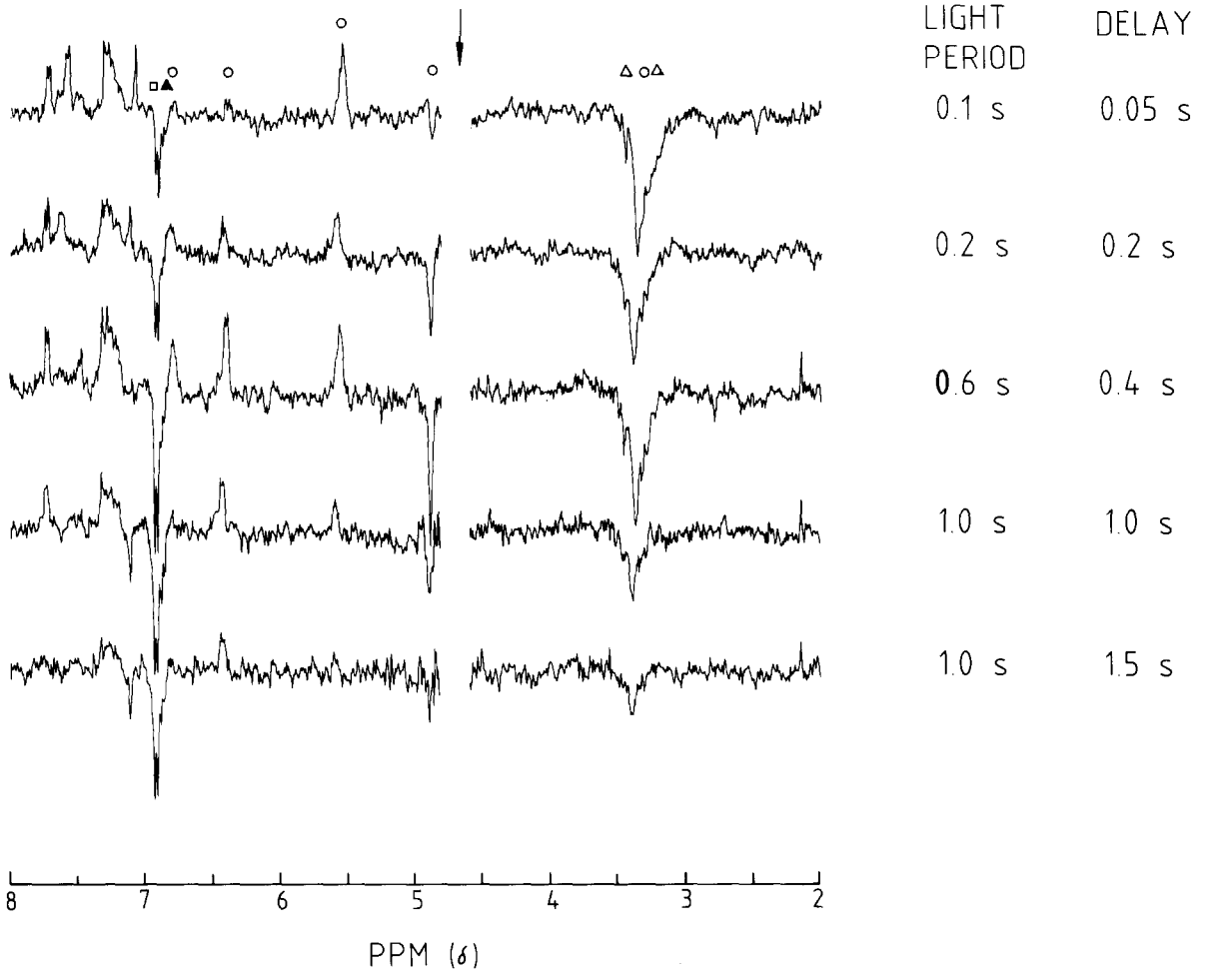

Fig. 1. Photo-CIDNP spectra of *M.elsdenii* flavodoxin with 0.5 mM flavin II as a function of the light period and delay time, as indicated: Trp I (○); Trp II (△); Tyr I (□); Tyr II (▲). The HDO resonance at 4.8 ppm (arrow) has been omitted.

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

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

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

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

Table 1

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

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

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

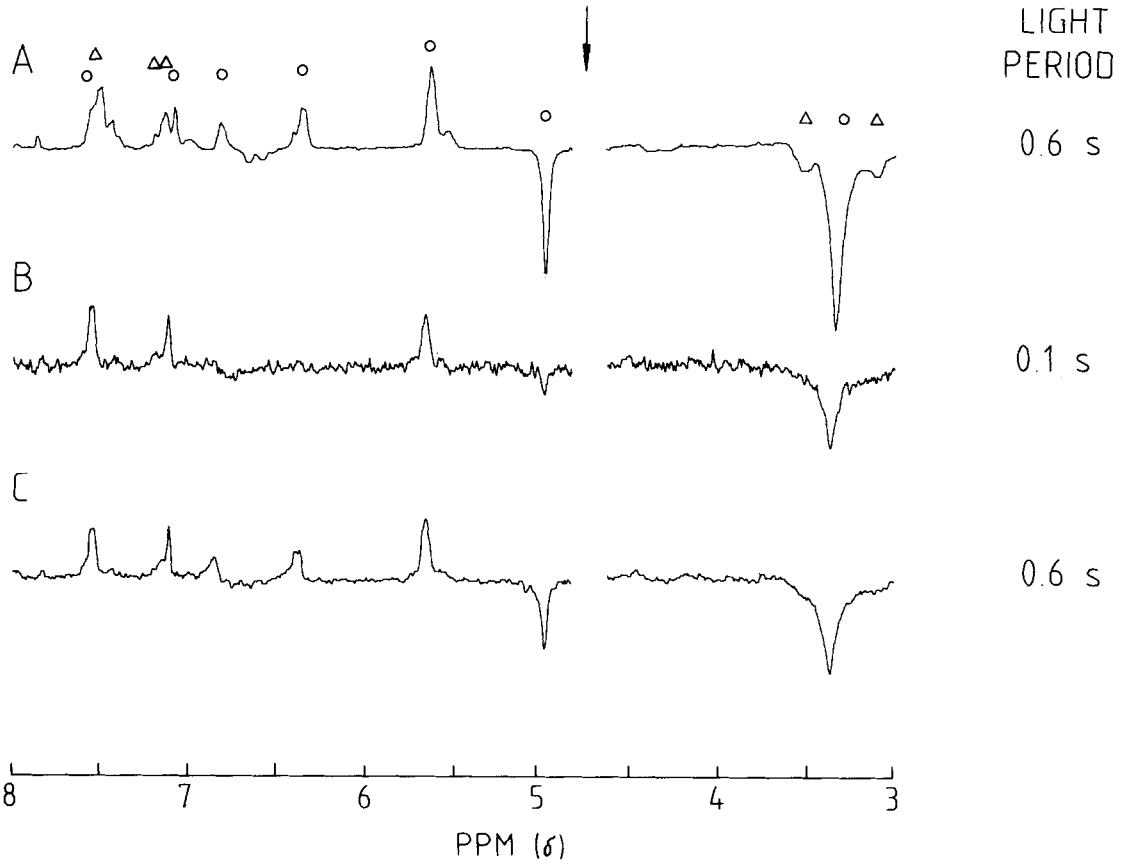

Fig. 2. Photo-CIDNP spectra of *Clostridium MP* flavodoxin with 0.5 mM flavin II (A) and 0.5 mM flavin I (B,C). A delay of 0.05 s was used with light periods as indicated. Notation as in fig. 1.

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

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

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

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

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

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

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

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

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

ACKNOWLEDGEMENTS

We thank Miss C.M. Verstege for typing the manuscript, Mr M.M. Bouwmans for the preparation of the figures, Mr W.A.M. van den Berg for the isolation of *M.elsdenii* flavodoxin and Mr K. Dijkstra for excellent technical assistance. 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). P.J.H. is grateful to the Royal Society of London for the award of a research fellowship.

REFERENCES

[1] 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.
[2] McCormick, D.B. (1970) Experimentia 26, 243–244.
[3] Mayhew, S.G. (1971) Biochim. Biophys. Acta 235, 289–301.
[4] 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, 4383–4392.
[5] Kaptein, R. (1978) in: NMR Spectroscopy in Molecular Biology (Williams, G.D. ed) vol. 5, pp. 211–229, D. Reidel, Dordrecht.
[6] Kaptein, R. (1982) in: Biological Magnetic Resonance (Berliner, L.J. and Reuben, J. eds) vol. 4, pp. 145–191, Plenum, New York.
[7] Kaptein, R., Dijkstra, K., Müller, F., Van Schagen, C.G. and Visser, A.J.W.G. (1978) J. Magn. Res. 31, 171–176.
[8] van Schagen, C.G., Müller, F. and Kaptein, R. (1982) Biochemistry 21, 402–407.
[9] Mayhew, S.G. and Massey, V. (1969) J. Biol. Chem. 244, 794–802.
[10] Tanaka, M., Haniu, M., Yasunobi, K.T., Mayhew, S.G. and Massey, V., J. Biol. Chem. (1973) 248, 4354–4366; (1974) 249, 4397.
[11] Hore, P.J., Egmond, M.R., Edzes, H.T. and Kaptein, R. (1982) J. Magn. Res. in press.
[12] IUPAC–IUB Commission on Biochemical Nomenclature, Biochemistry (1970) 9, 3471–3479.

[13] Moonen, C.T.W. and Müller, F. (1982) Biochemistry 21, 408–414.
[14] Ferrige, A.G. and Lindon, J.C. (1978) J. Magn. Res. 31, 337–340.
[15] Tanaka, M., Haniu, M., Yasunobu, K.T. and Mayhew, S.G. (1974) J. Biol. Chem. 249, 4393–4396.
[16] Perkins, S.J. and Wüthrich, K. (1979) Biochim. Biophys. Acta 576, 409–423.
[17] van Schagen, C.G. and Müller, F. (1981) FEBS Lett. 136, 75–79.
[18] Moonen, C.T.W. and Müller, F. (1983) submitted.