Flavoproteins involved in photosynthetic electron transport in the cyanobacterium *Anabaena* sp PCC 7119. Electron spin-echo envelope modulation spectroscopic studies

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The pulsed EPR technique of electron spin echo modulation (ESEEM) has been utilized to examine the flavoproteins ferredoxin-NADP$^+$ reductase (FNR)† and flavodoxin from the cyanobacterium *Anabaena* PCC 7119 in their semiquinone states. Fourier transforms of the three-pulse ESEEM showed the presence of prominent nuclear modulation frequencies around 3.08 MHz for both flavoproteins. Moreover, another prominent component was also observed at 3.47 MHz in FNR. Broader components were observed for the flavosemiquinones in the 4.7 MHz region. These modulations are consistent with the presence of at least one ^{14}N magnetically coupled to the paramagnet and can be interpreted as arising from nitrogens at positions 1 and/or 3 of the flavin ring system. In FNR, distortion of the ESEEM spectrum was observed in the presence of NADP$^+$, while 2′,5′-ADP, an NADP$^+$ analogue which lacks the nicotinamide ring, did not show major differences from the spectrum of the native enzyme. This indicates a specific interaction of the flavosemiquinone with the nicotinamide ring, which produces a change in the electron distribution of the flavin ring system. Exchange in deuteriated buffer gave rise to deuteron modulations in ferredoxin-NADP$^+$ reductase semiquinone, indicating the presence of exchangeable protons or water molecules in the flavin ring neighbourhood. The results, together with previous ENDOR studies, provide structural information which is complementary to the reported X-ray crystallographic structures.

Introduction

The photochemical reduction of NADP$^+$ to NADPH has been shown to proceed *via* ferredoxin and the flavoenzyme ferredoxin-NADP$^+$ reductase (FNR).[1,2] When cultures of cyanobacteria, and certain other autotrophic and heterotrophic organisms, are deprived of iron, they synthesise a low-M_r FMN-containing protein, flavodoxin, which replaces ferredoxin in most reactions, including electron transfer from photosynthetic membranes to FNR, and electron transfer to nitrogenase in nitrogen fixation.[3,4]

Flavodoxin and FNR from the cyanobacterium *Anabaena* PCC 7119 have been extensively characterized.[5–8] *Anabaena* PCC 7119 FNR contains one mole of non-covalently bound FAD cofactor/mol enzyme. A molecular model of *Anabaena* PCC 7119 FNR has been determined at 1.8 Å resolution,[9] indicating that the protein folds in a three-dimensional structure similar to that reported for the spinach enzyme.[10] The three-dimensional structures of both complexes of *Anabaena* and spinach FNR with NADP$^+$ and its analogues have been studied.[9,10] Nevertheless, in the electron density map of spinach FNR, only the 2′,5′-ADP portion of NADP$^+$ can be seen, indicating that the NADP$^+$ nicotinamide moiety might have multiple conformations within the crystal.[10,11] NADP$^+$ has been reported to bind *Anabaena* FNR with a dissociation constant of 13.25 μM and a binding energy of −27.7 kJ mol^{-1}.[12] Similar values were described for the binding of the substrate analogue 2′,5′-ADP, suggesting that the nicotinamide nucleotide moiety within NADP$^+$ does not contribute to the stability of the enzyme-NADP$^+$ complex. Flavodoxin contains 1 mol FMN/mol protein, and forms a 1 : 1 complex with FNR.[4,7] The semiquinone of FMN in *Anabaena* PCC 7119 flavodoxin is highly stable, so that close to 100% of the flavin is in the form of the semiquinone after addition of one electron.[6,13] The maximum proportion of the *Anabaena* FNR semiquinone at equilibrium, in the pH range 6–8 was 29–12%.[13]

Flavin semiquinones are important intermediates in electron transfer between one-electron donors such as ferredoxin or cytochrome, and two-electron carriers such as NAD(P)$^+$.[14] In FNR, the FAD semiquinone state almost certainly plays a role in the enzyme-catalysed reduction of NADP$^+$.[2] The neutral semiquinones of FNR and flavodoxin from *Anabaena* PCC 7119 have been characterized by EPR and ENDOR spectroscopies.[15] ENDOR spectroscopy has shown that upon binding NADP$^+$ a change in the electron density distribution on the flavin ring takes place, concomitant with electron withdrawal by interaction with the nicotinamide ring, while binding of ferredoxin has no significant effect.

Structural data on paramagnetic centres in biological systems have been obtained by analysing electron spin-echo envelope modulations (ESEEM) from various types of nuclei.[16] ESEEM is a pulsed EPR method which is particularly sensitive to weak hyperfine interactions between paramagnetic metal centres and quadrupolar nuclei such as ^{14}N or ^{2}H; such nuclei may be detected in the second coordination sphere of a metal centre.[17] Electron spin echoes are formed by the application of two or more resonant microwave pulses at measured time intervals, τ. Fourier analysis of the resulting electron modulation pattern reveals the frequencies of the various nuclear transitions. These frequencies can then be interpreted to determine superhyperfine and electric quadrupolar couplings. Coordination to nitrogens in proteins containing paramagnetic metal centres has been detected by pulsed EPR techniques.[17–23] ESEEM has also been used to provide evidence of the presence of nitrogen nucleus magnetically coupled to radical intermediate states in amine oxidases.[24]

In the present paper we have applied the ESEEM technique to the study of FNR and flavodoxin from *Anabaena* PCC 7119

† *Enzymes:* Cholesterol oxidase (3β-hydroxysteroid oxidase, EC 1.1.3.6); Ferredoxin-NADP$^+$ reductase (FNR, EC 1.18.1.2); Nitrogenase (EC 1.18.6.1).

in their semiquinone states to further characterize these systems. Changes in distribution of the unpaired electron on the FNR flavin ring on bonding to the substrate were studied by comparing the ESEEM spectra collected for FNR samples in the presence of either $NADP^+$ or 2′,5′-ADP.

Experimental

Flavodoxin and FNR were isolated from *Anabaena* PCC 7119 as described by Pueyo and Gomez-Moreno.[5] Samples were transferred into the desired buffer (10 mM HEPES, in H_2O or ^2H_2O, pH 7) by dilution and ultrafiltration through Centricon 30 microconcentrators (Amicon), at 4 °C.

Flavodoxin and FNR were reduced anaerobically at 4 °C by light irradiation with a 150 W Barr & Stroud light source in the presence of 20 mM EDTA and 2.5 μM 5-deazariboflavin. Samples were prepared in a sealed glass vessel under argon and transferred anaerobically using a gas-tight microsyringe into the EPR tubes as previously described.[15] $NADP^+$ or 2′,5′-ADP were added under anaerobic conditions to a semiquinone sample of FNR previously photoreduced, to a final concentration of 1.5 mM. All samples used for ESEEM experiments were previously characterized by EPR and ENDOR spectroscopies.[15]

Pulsed EPR measurements were recorded at X-band on a Bruker ESP380 spectrometer, with a dielectric variable-Q resonator in an Oxford Instruments CF 935 liquid helium immersion cryostat. ESEEM data were collected at 3.8 K by the three-pulse, stimulated echo (90°–τ–90°–T–90°) procedure with values of τ that ranged from 112 to 480 ns, so that the τ suppression behaviour of the resolved modulation frequency components could be examined.[18] The ESEEM data collected were linear phase corrected before Fourier transformation using the Bruker pulsed spectra manipulation routines or the Bruker program WinEPR. Magnitude calculated spectra are shown.

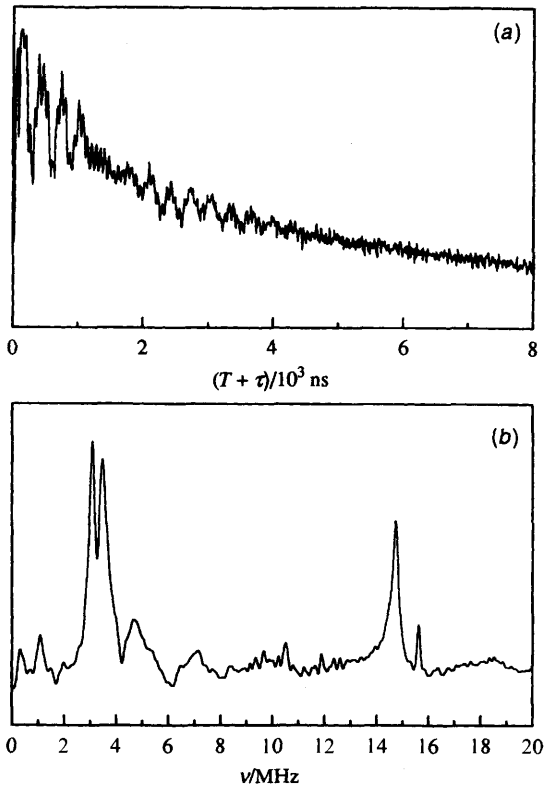

Fig. 1 Three-pulse ESEEM spectra of ferredoxin-$NADP^+$ reductase from *Anabaena* PCC 7119 in its semiquinone state at 346 mT magnetic field (corresponding to $g_x = g_y = g_z = 2.005$). (*a*) Echo decay envelope, (*b*) Fourier transform. Measurement conditions: T, 3.7 K; τ, 112 ns; pulse width (for a 90° pulse) 16 ns; bandwidth, 100 MHz; microwave frequency, 9.71 GHz; number of shots, 2; video amplifier gain, 58 dB; travelling wave tube attenuation, 5 dB; shot repetition time, 30.72 ms.

Results

Electron spin-echo envelope modulation of ferredoxin-$NADP^+$ reductase in its semiquinone state

The ESEEM technique was applied to the ferredoxin-$NADP^+$ reductase semiquinone from *Anabaena* PCC 7119 in H_2O, ^2H_2O and in H_2O in the presence of the two substrates, $NADP^+$ and 2′5′-ADP, with identical instrumental parameters. The ESEEM data presented below were obtained by the three-pulse (90°–τ–90°–T–90°) or 'stimulated echo' sequence. In all the cases the spin-echo absorption envelope of the semiquinone was observed. Measurements on the enzyme in H_2O, at a magnetic field corresponding to $g = 2.005$, showed modulations due to the matrix protons (14.75 MHz) (not shown). These 'matrix' protons weakly coupled to the semiquinone have been extensively characterized by ENDOR.[15] Modulations due to the more strongly coupled 8-CH_3 protons (8.12 MHz) were not observed, presumably because the anisotropy is small. Different τ-values were searched. The best data were obtained at τ-values of 112 and 136 ns, where maxima were obtained for the low-frequency (less than 10 MHz) components. Suppression of most of the resonances was observed at higher τ-values, indicating fast relaxation properties of the paramagnet. Fig. 1(*a*) shows the time-domain spectrum of FNR semiquinone recorded at a τ value of 112 ns. The corresponding Fourier transforms for τ values of 112 and 136 ns are shown in Figs. 1(*b*) and 2(*a*), respectively. The three-pulse spectra revealed low-frequency modulations between 0.6 and 4.8 MHz, with two prominent components at 3.08 and 3.47 MHz [Figs. 1(*b*) and 2(*a*)]. Broad peaks were also detected at 4.7 and 7.1 MHz. At the different τ-values the relative amplitudes changed, but the positions of the lines on the frequency scale did not vary. These modulation frequencies are consistent with coupling to at least one ^{14}N nucleus.

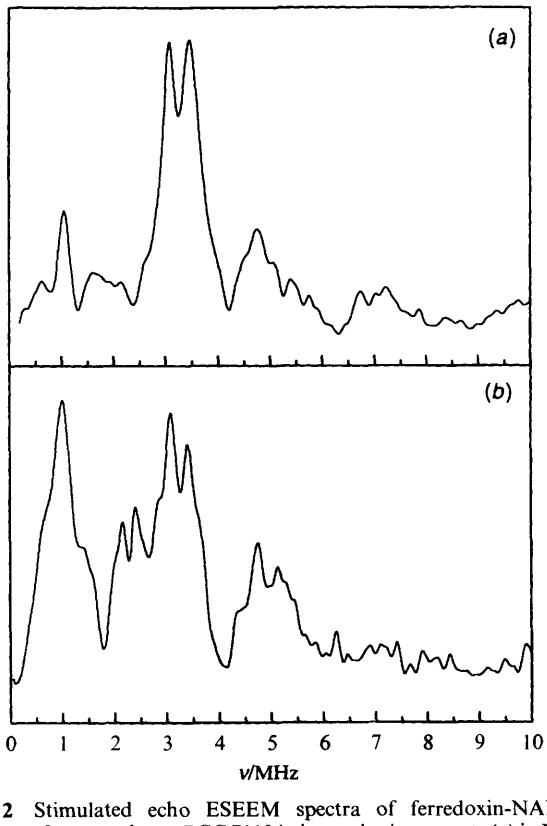

Fig. 2 Stimulated echo ESEEM spectra of ferredoxin-$NADP^+$ reductase from *Anabaena* PCC 7119 in its semiquinone state (*a*) in H_2O, (*b*) in ^2H_2O. Measurement conditions as in Fig. 1. τ-value was 136 ns.

The spectrum of a frozen solution is obtained by averaging the spectrum for a range of molecular orientations relative to the applied magnetic field. The most intense and well defined ESEEM spectra arise at the condition of exact cancellation, when $\nu_i = |A/2|$, where ν_i is the nuclear Zeeman frequency at the observing magnetic field and A the hyperfine coupling constant.[25-27] Typically, the salient spectral features for ^{14}N are the three pure quadrupole peaks (NQR, ν_o, ν_-, ν_+) in the manifold where the Zeeman and hyperfine interactions cancel. The two lower components add to give the frequency of the third[19,28] and a broader double-quantum peak occurs in the opposite manifold (4–6 MHz).[25] In this situation, the frequencies of the pure quadrupolar peaks permit a direct determination of the asymmetry parameter (η) and the quadrupole coupling constant (e^2qQ), while the double-quantum peak permits the calculation of the hyperfine coupling, A_{iso}.[25] Model calculations of ESEEM spectra from nucleus $I = 1$ have shown that this analysis can be applied if $|\nu_i - A/2|$ does not exceed 25% of ν_i, and the quadrupole frequencies can be obtained within 10% accuracy.[29]

^{14}N ESEEM spectra observed when $0.25\ \nu_i < |\nu_i - A/2| < 2/3(e^2qQ/4)$ are similar in appearance to those observed for exact cancellation conditions in that generally four frequency components can be resolved.[21] For this case the ESEEM is weaker, and superhyperfine couplings are dominated by ^{14}N nuclear quadrupole interactions. For cases where $|\nu_i - A/2| > 2/3(e^2qQ/4)$, typical ^{14}N ESEEM spectra consist of just two broad peaks corresponding to the '$\Delta m_I = 2$' transitions from each electron spin manifold. This case has been reported most often when the isotropic component of the hyperfine coupling tensor is large.[30]

Two prominent lines are observed in the FNR ESEEM spectra at 3.08 and 3.47 MHz, where the ν_+ frequency component could be expected for a nitrogen nucleus interaction with a spin at exact cancellation conditions. A prominent component at 3.03 MHz has also been found for the anionic semiquinone of cholesterol oxidase when study under the same instrumental conditions, the neutral semiquinone of flavodoxin also shows this prominent modulation (see below). This fact indicates that this feature might arise from a nitrogen which seems to be coupled in a similar way to the paramagnet in different flavoproteins, suggesting one of the nitrogens of the flavin ring system as candidate. The prominent component at 3.47 MHz has not been detected so far in the other flavoproteins studied. The shapes of both of them suggest they arise from NQR transitions. Nevertheless, it is difficult to detect the corresponding ν_o and ν_- NQR transitions, since the amplitude of the lines under 2.5 MHz (0.7, 1.05, 1.7, 2.0, 2.16) is very low as compared with the peaks at 3.08 and 3.47 MHz. Model calculations of ESEEM spectra from a nucleus with $I = 1$ have demonstrated that upon going away from the exact cancellation conditions for cases where $\nu_i > |A/2|$, broadening becomes more pronounced for the lines corresponding to ν_o and ν_-, resulting in a decrease in amplitude, while the broadening is less pronounced for the ν_+.[29] Our experimental data are consistent with this case. Exact cancellation conditions do not apply at X-band frequencies for FNR semiquinone. Nevertheless, the prominent components at 3.08 and 3.47 MHz kept sharp and constant at the different τ-values searched, and taking also into account the values observed for the double quantum transitions, an approximate calculation of the hyperfine and quadrupole coupling constants can be done, assuming near cancellation conditions. Since the intensity of ν_- frequencies was hard to detect, a range of values were determined for the hyperfine and quadrupolar coupling constants by varying the asymmetry parameter between 0 and 1. It is expected that this assumption makes only a minor uncertainty (*ca.* ± 15%) in the estimates of A and e^2qQ.[23] Using this approximation a value in the range 1.2–1.4 MHz would be expected for the hyperfine coupling constant of the ^{14}N magnetically coupled to the paramagnet, while the value of the quadrupole coupling constant would be in the range 3.4–4.3 MHz. Since the modulations at 3.08 and 3.47 MHz might arise from two ν_+ NQR transitions, the ESEEM spectra are also consistent with the presence of two nitrogen nuclei, with similar but not identical coupling constants.

The broad peak at 7.1 MHz could be explained as arising from another nitrogen nucleus coupled to the paramagnet for which $|\nu_i - A/2| > 2/3(e^2qQ/4)$. In this case, typical ^{14}N ESEEM spectra consist of just two peaks corresponding to the '$\Delta m_I = 2$' transitions from each electron spin manifold; these are broader than the lines observed under cancellation conditions. The second double-quantum transition would be expected in the 3–4 MHz region, and probably cannot be detected due to its superposition on the prominent features at 3.08 and 3.47 MHz. The hyperfine coupling constant expected for such nitrogen nucleus would be at least 4 MHz.

Deuterium effect of the electron spin-echo envelope modulation of ferredoxin-NADP$^+$ reductase in its semiquinone state

A three-pulse ESEEM spectrum of FNR semiquinone which had been exchanged into ^2H_2O showed deep modulations, additional to those seen in unexchanged samples, that gave rise to two lines at 2.20 and 2.40 MHz in the Fourier transform [Fig. 2(*b*)] attributed to interaction of the semiquinone radical with exchangeable deuterium. This result is in agreement with previous ENDOR studies[15] and demonstrates the presence of exchangeable protons on protein groups, position 5 of neutral flavin cofactor and probably also water molecules, surrounding the FAD. These are the exchangeable protons which are responsible for the increased linewidth in the EPR spectrum in H_2O of neutral flavoprotein semiquinones. The FAD cofactor of *Anabaena* FNR is bound to the protein, either directly or through well-ordered water molecules, by hydrogen bonds and stacking forces.[9] The isoalloxazine, which constitutes the FAD-reactive part and carries the unpaired electron in the semiquinone state, is maintained within the protein by stacking interactions with two tyrosines and by hydrogen bonds with one serine, one cysteine, one arginine residues and several water molecules. The exchangeable protons of these amino acid residues, nitrogen on position five and water molecules are probably those for which modulations appear in the ESEEM spectrum when the semiquinone is studied in ^2H_2O.

Although the deuterium modulations occupy a major part of the spectra below 2.5 MHz, modulations due to the nitrogen were still seen between 3 and 5.5 MHz in the Fourier transform spectra. No changes in the position of the lines at 3.08 and 3.47 MHz were detected. The broad peak around 4.7 MHz seemed slightly distorted and shifted to higher values and the peak found at 7.1 MHz for the FNR semiquinone was not resolved, possibly because of greater noise in the spectrum.

Electron spin echo envelope modulation of ferredoxin-NADP$^+$ reductase semiquinone in the presence of its substrates, NADP$^+$ and 2′,5′-ADP

The ESEEM spectrum of FNR semiquinone was also studied in its complex with NADP$^+$, its substrate, and an analogue 2′,5′-ADP, which lacks the nicotinamide ring but shows a similar dissociation constant[12] (Fig. 3). It has been shown that upon anaerobic addition of NADP$^+$ to the FNR semiquinone a significant decrease of the relative amount of FNR semiquinone is observed.[15] Such a decrease was not observed when 2′,5′-ADP was added to the FNR semiquinone, retaining almost the same amount of semiquinone after addition. Fig. 3(*a*) shows the Fourier transform ESEEM spectrum obtained for FNR semiquinone in the presence of 2′,5′-ADP. No major changes were observed for the position and shape of the principal modulations under 4 MHz. The broad peaks around 4.7 and 7.1 MHz seemed slightly distorted, the latter shifting slightly to higher values (7.3 MHz). When the three-pulse ESEEM was

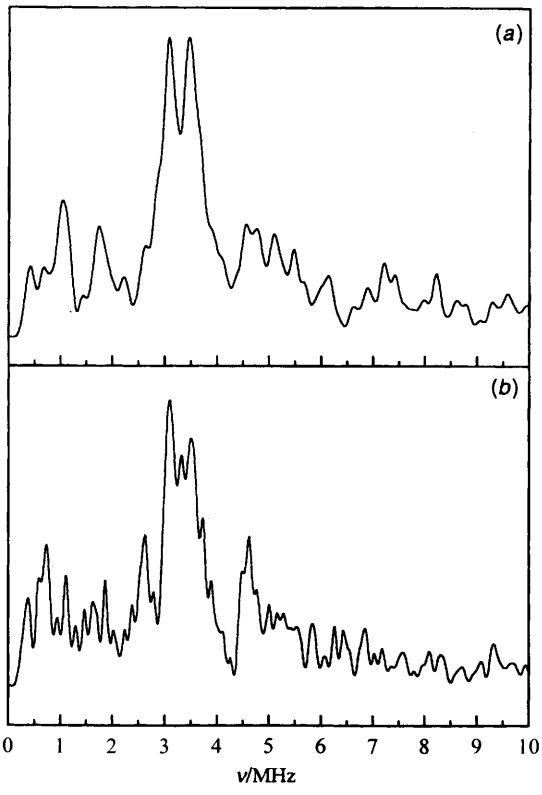

Fig. 3 Stimulated echo ESEEM spectra of ferredoxin-NADP$^+$ reductase from *Anabaena* PCC 7119 in its semiquinone state in the presence (*a*) of 2′5′-ADP and (*b*) of NADP$^+$. Measurement conditions as in Fig. 1. τ-value was 136 ns.

carried on a sample of FNR semiquinone complexed with its substrate, NADP$^+$, Fourier transform spectra as shown in Fig. 3(*b*) were obtained. Although a decrease in the signal : noise ratio was evident as a consequence of the decrease in semiquinone state amount due to NADP$^+$ reduction by reduced FNR, changes in the Fourier transform were clear. An intense modulation was still observed at 3.09 MHz. Meanwhile, the peak at 3.47 MHz seemed to split in two, 3.31 and 3.51 MHz. Changes at lower frequencies, between 0 and 3 MHz were difficult to distinguish due to the noise. The broad line at 4.62 MHz remained while the broad peak around 7.1 MHz was not detected. As with the free enzyme, the best-resolved modulations were obtained at short τ-values, between 112 and 136 MHz.

Electron spin echo envelope modulation of *Anabaena* PCC 7119 flavodoxin semiquinone

Although the semiquinone of FMN in *Anabaena* PCC 7119 flavodoxin is highly stable, and almost all of the flavin is in the form of the semiquinone after addition of one electron, not very informative three-pulse Fourier transform ESEEM spectra were obtained at X-band frequencies. Fig. 4 shows the Fourier transform spectrum of flavodoxin semiquinone. An intense component is observed at 3.08 MHz. Two other peaks can be detected at 3.78 and 4.07 MHz. A broad feature also appears at 4.75 MHz with maxima at 4.55 and 4.9 MHz. These modulation frequencies are consistent with coupling to one ^{14}N nucleus. Exact cancellation conditions did not apply under the experimental conditions for flavodoxin semiquinone. Nevertheless, using the procedure above described for FNR semiquinone, a value around 1.2 MHz can be estimated for the hyperfine coupling constant of the ^{14}N nucleus coupled to the unpaired electron on the flavin system, while the expected value for the quadrupole coupling constant ranged between 3.1 and 4.0 MHz.

Three-pulse ESEEM EPR studies of flavodoxin semiquinone

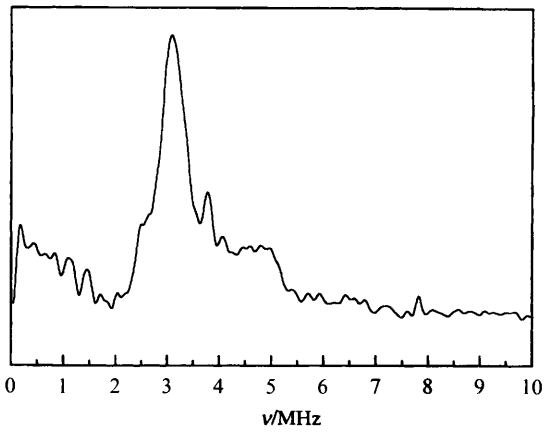

Fig. 4 Three-pulse ESEEM spectra of flavodoxin from *Anabaena* PCC 7119 in its semiquinone state at 346 mT magnetic field (corresponding to $g_x = g_y = g_z = 2.005$). Measurement conditions: T, 3.7 K; τ, 112 ns; pulse width (for a 90° pulse) 16 ns; bandwidth, 100 MHz; microwave frequency, 9.71 GHz; number of shots, 2; video amplifier gain, 58 dB; travelling wave tube attenuation, 5 dB; shot repetition time, 30.72 ms.

were also carried out in ^2H_2O with identical instrumental settings (not shown). No major changes were detected in the position of the lines and a low-intensity signal, due to deuteron modulations, appeared at 2.3 MHz. At τ-values where proton modulations were not suppressed, modulations due to the matrix protons (14.7 MHz) were still detected with high intensity. The matrix proton signals remaining in ^2H_2O are due to non-exchangeable protons. All the three-dimensional structures reported for different flavodoxins show the FMN group buried inside the protein core, with just the 8-CH_3 and 7-CH_3 groups accessible to the solvent.[31] The residues in direct contact with the FMN are mainly hydrophobic residues, with the isoalloxazine ring sandwiched between two aromatic residues. Our results are in agreement with a concentration of hydrophobic residues in the direct neighbourhood of the FMN isoalloxazine ring, and with previous ENDOR studies.[15]

Discussion

Electron spin-echo envelope modulation is a very sensitive technique for the detection of nitrogen nuclei that are weakly coupled to a paramagnetic centre. In the case of flavoproteins, such a contact interaction could result either from ligation of a nitrogen directly to the flavin ring, hydrogen bonding or close proximity to the paramagnet. The observed resonances due to nitrogen nucleus for FNR and flavodoxin semiquinones were not consistent with the couplings expected for nitrogens on the position 5 or 10 on the flavin ring. These ^{14}N are expected to show hyperfine couplings of at least 22 and 10 MHz respectively for a neutral flavin semiquinone,[32–34] which are values greater than can be observed with conventional ESEEM.[28] The high value of the ^{14}N ESEEM frequency (4.7 MHz at 346 mT) indicates that it is due to interactions with specific nitrogen nuclei in the immediate vicinity of the paramagnet, the semiquinone ring system. This is the maximum frequency attributable to a ^{14}N transition from a peptide nitrogen at the frequencies at which we are working.[21,35] Nevertheless, the isotropic hyperfine coupling through a hydrogen bond would be expected to change upon exchange in ^2H_2O, as ^2H in general forms weaker hydrogen bonds. The ^{14}N ESEEM frequencies in the FNR or flavodoxin semiquinones remained unchanged upon exchange in ^2H_2O, although the ENDOR spectrum has shown that a significant number of couplings due to protons are replaced by deuterons in FNR.[15] Because acidic protons are expected to exchange readily, this would also indicate that the nitrogen modulation does not arise from an amide or amino

group which is hydrogen bonded to the flavin radical. A more likely source of the nitrogen interaction detected by ESEEM is through superhyperfine coupling between a nitrogen ligand from an amino acid and the unpaired spin on the flavin ring or one of the nitrogens of the flavin ring, *i.e.* N1 and/or N3.

When studied under the same experimental conditions, we have observed that similar ESEEM patterns are obtained for all the flavoprotein semiquinones studied so far, FNR, flavodoxin and cholesterol oxidase, presenting a strong modulation around 3.03–3.08 MHz as well as a broad peak around 4.7 MHz, the latter being consistent with a double-quantum transition frequency peak. The ESEEM pattern observed for cholesterol oxidase semiquinone was more informative, near cancellation conditions applied and a good estimate could be made of the hyperfine and quadrupole coupling constants. The data obtained are in close agreement with the ones presented here for FNR and flavodoxin semiquinones. These results suggest that the nuclei we are observing by pulsed EPR are the same in all of them, indicating that the presence of such modulations could rather be due to a nucleus of the flavin ring system than to any other nitrogen in the environment. These results suggest nitrogens at the positions 1 and/or 3 of the flavin ring as good candidates to produce the observed modulations. The observed differences are expected, since the apoprotein determines the (protonation) state of the prosthetic group and thereby regulates the reaction mode, producing slightly different environments of the flavin ring. This regulation mechanism could be brought about by positive charges or hydrogen bonds from the protein to the nitrogens in position 1 and/or 5 of the flavin moiety, resulting in the blocking or deblocking of these positions. This could stabilize or destabilize the radical state and determine the function of the enzyme as one-electron or two-electron mediator.[36] So far, no values of the hyperfine couplings for nitrogens N1 and or N3 of the flavin ring in model compounds have been reported. Small values are expected due to the almost non-existent spin-density on these positions estimated for flavin semiquinone models.[37,38] Unpublished electron spin-echo data on flavodoxin show that the N1 and/or N3 nitrogens exhibit a weak coupling on the order of 1 MHz.[34] To our knowledge there are no other reports of ESEEM studies on flavoprotein semiquinones.

Although the presence of 2′,5′-ADP did not produce major changes in the ENDOR or ESEEM patterns of FNR semiquinone, indicating no alterations in the flavin ring electron spin density distribution, the ESEEM spectra of FNR semiquinone in the presence of $NADP^+$ showed a slightly different pattern and the appearance of new resonances between 2.5 and 4 MHz. These results indicate that the nicotinamide ring is either producing a change in the electron spin density of the flavin ring or that one of its nitrogen atoms is also coupled to the paramagnet. However, since multiple conformations could be expected for the $NADP^+$ nicotinamide ring[11] it is unlikely that the nitrogen coupling of the nicotinamide nitrogen to the unpaired spin would be detected as sharp lines. ENDOR studies on these samples have demonstrated that binding of the nicotinamide ring of $NADP^+$ influences the electron spin density distribution of the flavin by polarization effects towards the pyrimidine ring, decreasing the electron spin density on the benzene ring.[15] A decrease in the electron density on the benzene ring has also been observed in other flavoproteins upon substrate binding.[33,39]

^{14}N ESEEM spectra, where only a single well-resolved spectral feature is found, have been reported in other systems.[24,40] Computer simulations of the ^{14}N ESEEM spectra of the semiquinone state of amino oxidase showed that such results are expected when large anisotropic hyperfine interactions dominate the electron–nucleus coupling and a perpendicular 'edge', in at least one of the powder pattern line shapes, occurs at low frequency.

We have characterized by ESEEM spectroscopy the neutral semiquinone forms of ferredoxin-$NADP^+$ reductase and flavodoxin from *Anabaena* PCC 7119 in H_2O, ^2H_2O and FNR complexed with its substrate, $NADP^+$, and a fragment of it, 2′,5′-ADP, to further understand these flavoproteins and the mechanism of the process they catalyse. Although we cannot conclusively establish which one is the nitrogen nucleus we detect magnetically coordinated to the unpaired electron spin, all the data point to N1 and/or N3 of the flavin ring system. ESEEM studies on these flavoprotein semiquinones have provided additional information on the differences in environment and electron-density distribution in the free enzyme and the substrate-bound semiquinone. To the best of our knowledge, this is the first report of an ESEEM characterization of neutral flavin semiquinones in a protein molecule. Further work must be done in the ESEEM characterization of flavoproteins, involving other frequencies in order to get a full understanding.

Acknowledgements

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