Biochimica et Biophysica Acta 809 (1985) 421–428
Elsevier

BBA 41849

The structure of the secondary donor of Photosystem II investigated by EPR at 9 and 35 GHz

Martin Brok, Frank C.R. Ebskamp and Arnold J. Hoff

Department of Biophysics, Huygens Laboratory of the State University, P.O. Box 9504, 2300 RA Leiden (The Netherlands)

(Received April 29th, 1985)

Key words: Signal II; EPR; Photosystem II; (*Chlorella*)

Signal II of plant photosynthesis, which is generally thought to be connected to the secondary donor complex of Photosystem II, has been investigated with EPR spectroscopy at 9 and 35 GHz. From the spectrum at 35 GHz of deuterated *Chlorella vulgaris*, the principle values of the g-tensor are determined to be $g_{xx} = 2.0074$, $g_{yy} = 2.0044$ and $g_{zz} = 2.0023$. Proton hyperfine coupling tensor elements and orientations were determined from spectral simulation of random and oriented samples, assuming that Signal II is due to a plastosemiquinone cation having its π-electrons in an antisymmetric orbital as proposed by P.J. O'Malley, G.T. Babcock and R.C. Prince (Biochim. Biophys. Acta 765 (1984) 370–379). In contrast to their work, it is found that most hyperfine interaction is due to the methylene group at ring position 5 and to both hydroxyl groups. One of the hydroxyl groups shows bond bending of 35°. We presume that this is due to hydrogen bonding and that this bond stabilizes the antisymmetric orbital of the π-electrons.

Introduction

The various forms of the so-called EPR Signal II (SII_{slow}, SII_{fast}, $\text{SII}_{\text{very fast}}$) of plant Photosystem II have been shown to be connected to the donor side of P-680, the primary donor of Photosystem II. SII_f and SII_vf arise from electron transport between the water-splitting enzyme and P-680. Although the connection between SII_s and $\text{SII}_\text{f,vf}$ is still obscure, all three signals appear to have the same shape and presumably arise from the same radical species. The origin of this species is still under debate.

Kohl and Wood [1] showed that the ratio SI : SII increased by heptane extraction of plastoquinone from chloroplasts and decreased by readdition of plastoquinone. They suggested that SII is due to a plastochromanoxyl radical. Hales and Das Gupta [2] did a computer simulation of the X-band EPR spectrum of SII assuming that it was due to a plastosemiquinone anion radical with hyperfine splittings that, according to the authors, reflected a redistribution of spin density due to a nearby metal ion. The hypothesis that SII is due to a semiquinone anion, however, cannot be easily reconciled with the high redox potential at the water-splitting side of PS II (about 1.1 V for the primary donor P-680) [3].

Recently O'Malley et al. [4] showed that the shape and the power saturation behavior of the EPR spectra of semiquinone cations are in good agreement with the characteristics of SII, even though the width of the model semiquinone anions is about 2-fold smaller than that of SII. The estimated in vitro electrode potential of the $\text{QH}_2^{+}/\text{QH}_2$ couple at pH 7 is 920 mV, close to that of P-680 [3].

Abbreviations: EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PQH_2^{+}, plastoquinone cation radical.

In order to substantiate further the idea that the various forms of SII are due to a semiquinone cation, we have undertaken an EPR study at X-band (9 GHz) and Q-band (35 GHz) on perdeuterated and fully protonated material. The experiments were focussed on the determination of the hyperfine and *g*-tensors and their relative orientation. We will show that all available spectra can be well simulated with one set of hyperfine and *g*-tensor data.

Our results do not substantiate the proposal of O'Malley et al. [5] that the structure of the SII EPR signal results from hyperfine interactions with one CH_3 group. Rather, we propose that this structure is due to hyperfine interaction with protons from a methyl, CH_2 and two hydroxyl groups in about equal proportions.

A preliminary account of this work has appeared earlier [6].

Materials and Methods

PS II particles were prepared from chloroplasts according to [7] and were concentrated to chlorophyll concentrations of about 5 mg/ml. *Chlorella vulgaris* cells were grown on a fully deuterated medium, concentrated and resuspended in 20 mM Hepes (pH 7.5)/0.1 M KCl and stored on ice in ambient light. The deuterated algae were preilluminated and pelleted in the EPR sample tube by the use of a swing-out rotor at 2000 rpm and were kept in the dark for a few minutes before freezing in a nitrogen flow cryostat.

The X-band EPR measurements were performed at −100°C with a Varian E9 spectrometer with 100 kHz field modulation equipped with a nitrogen flow cryostat. The Q-band EPR spectra were measured with a home-built spectrometer at 90 K.

Computer simulations were carried out using a program (Brok, M., De Groot, A., Babcock, G.T. and Hoff, A.J., unpublished data) on a VAX 750 computer. First-order approximations of the principal values of the *g*-tensor were taken from Q-band spectra of perdeuterated samples, those of the hyperfine tensors from ENDOR measurements on frozen solutions and from X- and Q- band EPR on unoriented and partly oriented membranes. The relative orientations of the *g*- and hyperfine tensors were to at first approximation taken from the literature and refined by variation until an acceptable fit of the experimental spectra was obtained.

Results and Discussion

The principal g values of SII

In Fig. 1A we show the Q-band EPR spectrum of perdeuterated *C. vulgaris* cells near $g = 2.00$.

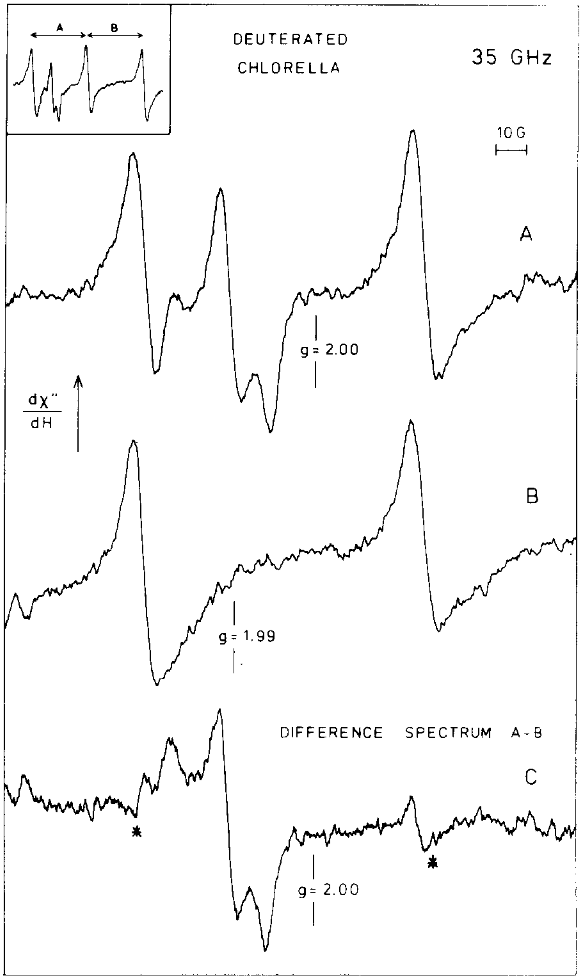

Fig. 1. Q-band spectrum of deuterated *C. vulgaris*. Microwave frequency 35 GHz; microwave power, 15 μW; modulation amplitude, 2 G; temperature 90 K. (A) Spectrum recorded around $g = 2.0$. (B) The same sample but spectrum recorded at 90 G higher magnetic field. (C) Differences spectrum of A and B. Asterisks indicate residual Mn^{2+} signals after subtracting B from A.

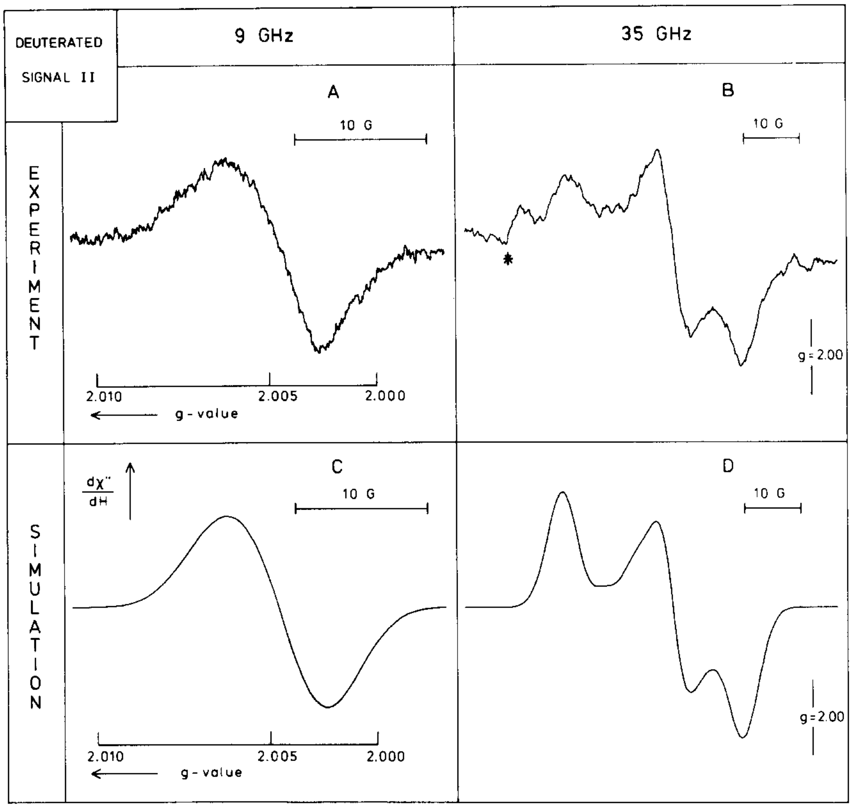

Fig. 2. The same sample as in Fig. 1. (A) Microwave frequency, 9.080 GHz; microwave power, 5μW; modulation amplitude, 0.5 G; average of 16 scans; temperature, 90 K. (B) Same as Fig. 1C (C) Simulation of A with spin packet linewidth of 2.5 G and g values as listed in Table I. (D) Simulation of B with spin packet linewidth of 2.7 G and the same g values.

The two intense peaks at low and high field are two of the six Mn^{2+} hyperfine lines which are visible at Q-band but not at X-band frequencies. These six hyperfine lines closely resemble those present in the X-band EPR spectrum of hexaquo Mn^{2+} ($S = 5/2$, $I = 5/2$), which correspond to the transition between the $m_s = +1/2$ to $m_s = -1/2$ spin states [8]. To good approximation the hyperfine interaction and g-factor of these Mn^{2+} signals are isotropic. We therefore calculated the difference spectrum of Fig. 1C by subtracting from the spectrum of Fig. 1A the neighboring Mn^{2+} hyperfine lines of the same EPR spectrum measured at an about 90 G higher magnetic field (Fig. 1B). Except for the fact that the intensities of those two Mn^{2+} lines differ slightly, which results in a small signal in the difference spectrum, this procedure yields an SII spectrum that is relatively free from distortions due to the Mn^{2+} line. As shown in Fig. 1C, the spectrum of SII, thus obtained, has the characteristic features of the spectrum of an immobilized radical with rhombic g-factor and hyperfine splittings, small compared to the g-anisotropy.

In Fig. 2 the X- and Q-band EPR spectrum of deuterated material are displayed together with their simulations. The g-values resulting from the best fit are $g_{xx} = 2.0074$, $g_{yy} = 2.0044$ and $g_{zz} = 2.0023$. The fact that the experimental spectrum of deuterated material is well fitted with a single species having a rhombic g-tensor indicates that only protons contribute to the hyperfine structure of SII in protonated materials and that SII cannot be a superposition of two or more radicals.

Orientation of SII

Fig. 3 shows two spectra of SII in orientated chloroplasts, with the magnetic field parallel (Fig. 3A) and perpendicular (Fig. 3B) to the membrane plane. The major features in both spectra (that were taken from Ref. 5) are four approximately equidistant lines. This and the observation by EN-

DOR of a large, seemingly anisotropic hyperfine coupling suggested to O'Malley et al. [5], that the main contribution to the lineshape of SII is the hyperfine coupling of the unpaired electrons with the three protons on ring position 2 (C2) of a plastosemiquinone cation. The authors' proposal was illustrated by simulations of the two spectra of oriented SII using one methyl hyperfine coupling

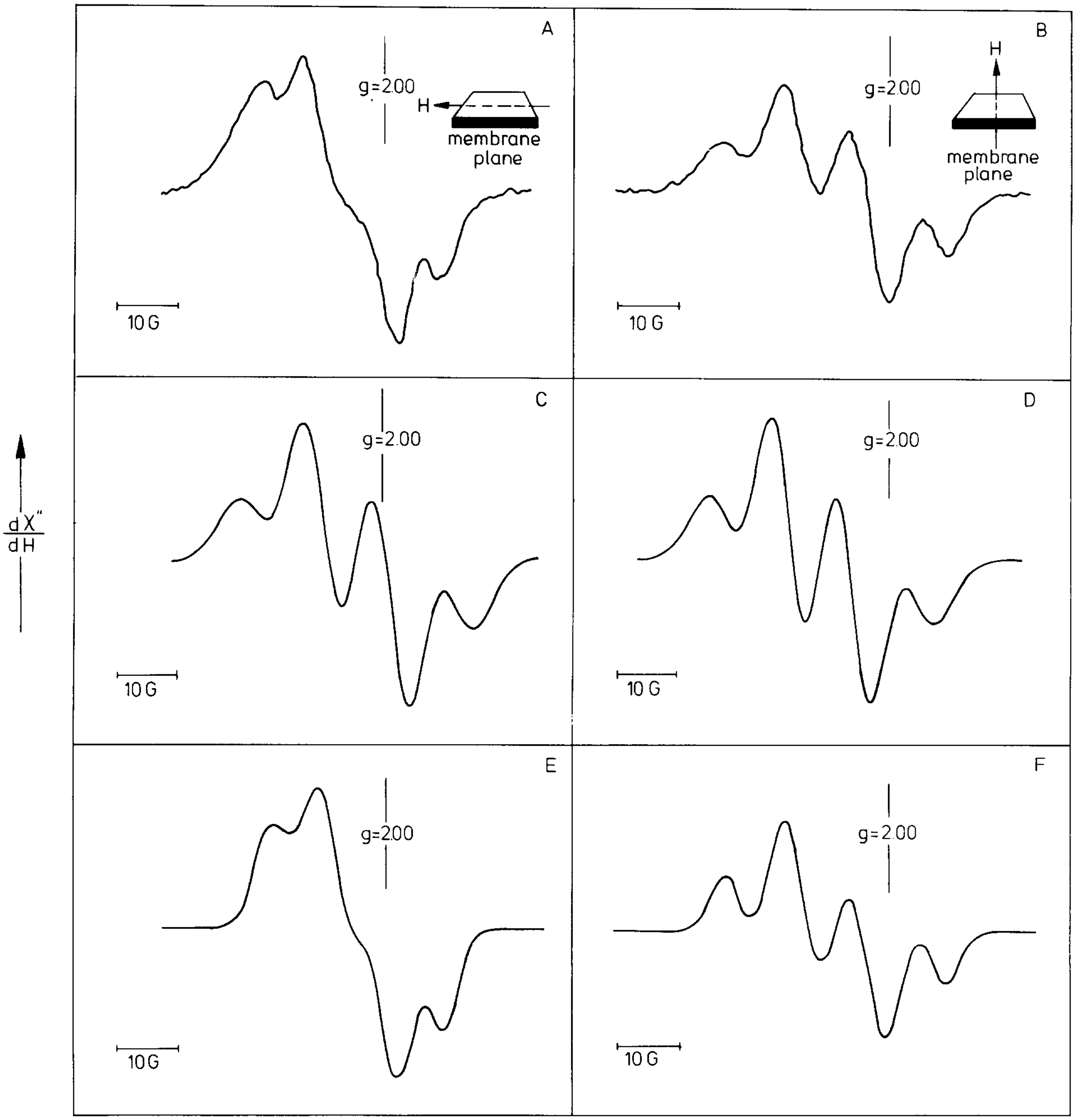

Fig. 3. (A) and (B) Oriented spectra of Signal II taken from Ref. 5. (C) and (D) Computer simulations with g values extracted from Fig. 2C and hyperfine values $A_\perp = 9.7$ G and $A_\parallel = 11.2$ G as found in Ref. 5. The spin packet linewidth is 4.0 G. (E) and (F) Computer simulation with parameters as listed in Table I. The 'wobble' parameter is 5° for all simulations. For the simulations C–F it is assumed that the X-axis of the quinone molecule (Fig. 4) is parallel to the membrane normal.

and g-values corresponding to the center fields of Fig. 3A and B. The hyperfine couplings resulting from the simulations (6.0–6.5 G and 9.10–10.5 G for Figs. 3A and 3B, respectively), however, were at variance with those found in the ENDOR experiments ($A_{\perp} = 11.2$ G, $A_{\parallel} = 9.7$ G) [5].

We have simulated the spectra of Figs 3A, B using the same hyperfine couplings and orientation with respect to the membrane (as in Ref. 5) and the g values as extracted from Fig. 2. The result is shown in Fig. 3C for the magnetic field parallel to and in Fig. 3D for the magnetic field perpendicular to, the membrane plane. It is seen that both simulations fit rather badly to the experimental spectra: they are too broad and that of Fig. 3C has a lineshape that deviates considerably from that of Fig. 3A. We note that the difference in the splittings of the two experimental spectra in Fig. 3A and B is more than a factor of 2 larger than found for the model compound, 2-methyl-5-isopropyl hydroquinone cation ($A_{\perp} = 7.1$ G, $A_{\parallel} = 7.6$ G) [5]. The latter difference corresponds well to the anisotropy expected for the hyperfine coupling of a rotating methyl group, but the difference found in the simulations of the spectra of oriented SII in Ref. 6 appears to be unacceptably large for an assignment of the hyperfine coupling to the CH_3 group. In addition, it was found previously [9], and confirmed by us (data not shown), that the lineshape of SII remains virtually unchanged in the temperature range of 1.5–300 K. As methyl groups are known to stop rotating at temperatures well above 1.5 K, this observation makes it very unlikely that the observed structure of SII is due to a predominant hyperfine coupling with the single CH_3 group.

The above observations and arguments have prompted us to reinvestigate the assignment of the hyperfine splittings.

Assignment of the hyperfine couplings

Following O'Malley et al. [5], we start with the assumption that SII is due to a plastosemiquinone cation whose π-electrons are in an orbital which is completely anti-symmetric. This means that practically all the electron spin density is located on carbon positions 1, 2, 4 and 5 [4]. The observation that the shape of SII is only slightly temperature-dependent down to 1.5 K indicates that the coupling to the CH_3 group, and therefore the spin density on C2, is low.

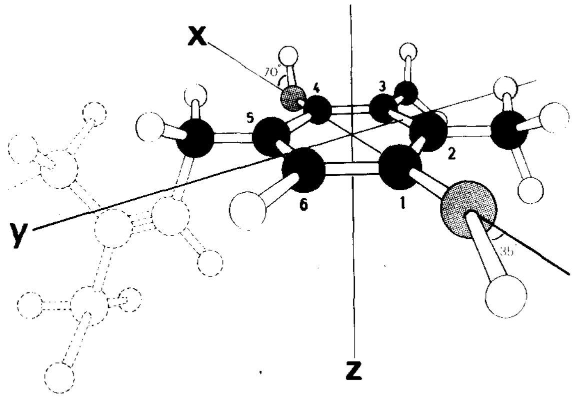

Fig. 4. Plastosemiquinone cation.

The two β-protons at position 5 are not able to rotate, so that even a large spin density on C5 will not lead to a temperature-dependent change in the shape of SII. The same is true for all other protons. The hyperfine couplings to the hydroxyl protons were estimated on the basis of the following arguments. Muto et al. [10] found that the anisotropic component of the hyperfine tensor of the hydroxyl groups in the electron-repelling anions of L-valine, L-analine and succinic acid is independent of the isotropic component and that the isotropic value is related to the dihedral angle, such that the isotropic value increases with larger angles. In contrast, for our radical cation the OH groups are strongly electron-donating substituents, so we expect that the isotropic hyperfine coupling will decrease when the proton is rotated out of the plane of the radical.

The angles that the protons make with the plane of the quinone ring were estimated from a space-filling model of the plastosemiquinone cation. They are depicted in Fig. 4, from which it is seen that the proton of the C4-OH group PQH_2 is lifted by a dihedral angle of 70° and consequently has a small isotropic hyperfine coupling. In contrast, the isotropic hyperfine splitting of the proton on the C1-OH group will be fairly large, because it lies close to the plane of the benzene ring. We now make use of the information contained in the SII spectrum in oriented material. As suggested in Ref. 5, it is likely that the apparent four-line structure of Fig. 3B results from three protons with about equal hyperfine splittings. If we accept that the PQH_2 plane is perpendicular to the thylakoid membrane surface. then these splittings must represent the components of the hyperfine tensors which lie perpendicular to the PQH_2 plane.

The considerable change in the shape of SII for a 90° rotation of the oriented sample (Fig. 3A, B) indicates that at least one of these protons must have a strongly anisotropic hyperfine tensor. Since the hyperfine tensors of the two β-protons at C5 have only about 10% anisotropy, the most likely candidate is one of the hydroxyl protons. On the basis of Muto's results [10], we take the C1-hydroxyl and assign to its principal A_{xx} element a value close to the isotropic coupling of the two β-protons at C5. These protons will have the same hyperfine tensor because they make the same dihedral angle of about 45° with respect to the benzene plane. Their hyperfine interaction is given [11] by $a_H = B_0 + B_2 \cos^2\theta$, where θ is the angle of twist between the α-carbon $2p_z$ orbital and the plane containing the β-proton CH-bond.

The proton of the C4-OH group must have a small isotropic hyperfine coupling and a small anisotropic value in the plane of the radical by virtue of its being rotated out of the plane by 70°, as judged from the space filling model. We have taken an isotropic coupling of about zero and an anisotropy obeying the relation given by Muto et al. [10].

For our simulation we took for the starting values of the most important couplings the hyperfine couplings found by ENDOR, 11.2 G and 9.7 G, of which the former one was assigned to the C1-OH and the latter to the two β-protons at C5. The anisotropies and angles were taken as discussed above.

A 'best fit' of the oriented spectrum of Fig. 3 was sought by varying three parameters: the 'wobble' parameter in the gaussian distribution representing the misorientation of the membranes, the spin packet linewidth and the in-plane rotation of the C1-OH proton. The result is displayed in Fig. 3E, F; the various parameters used for the fit are listed in Table I.

The simulations are quite satisfactory and certainly considerably improved over those given in Ref. [5]. The fit of Fig. 3A, B was obtained for an angle of 35° between the C1-OH proton and the X-axis of the molecule (see Fig. 4). As mentioned above, this angle is about 70° for the space-filling in vitro model. The difference is likely caused by hydrogen bonding of the C1-OH proton with the proton matrix.

Of course, the parameters used for the best fit of the oriented spectra shown in Fig. 3E, F must also give acceptable fits of the X- and Q-band spectra of randomly oriented samples. In Fig. 5 we present the X- and Q-band spectra of randomly oriented PS II particles together with their simulation using the parameters of Table I. Again, the

TABLE I

COMPONENTS IN GAUSS OF THE PROTON HYPERFINE TENSORS AND THEIR ORIENTATION, AND THE PRINCIPAL g-VALUES OF PQH_2^{+}

The principle values of the hyperfine tensors and their orientation are derived from spectral simulation as described in the text, using a 'wobble angle' of 5°. θ is the angle between the z-axis hyperfine tensor and the molecular z-axis, ϕ is the angle between the respective y-axes. These angles represent two of the three Euler angles giving the rotational transformation between the two axis systems, the third Euler angle being set to zero.

Signal II	X (G)	Y (G)	Z (G)	ISO (G)	θ	ϕ
A(C5-H)	9.7	8.2	8.2	8.7	45°	10°
A(C5-H)	9.7	8.2	8.2	8.7	45°	50°
A(C2-H_3)	1.7	1.5	1.5	1.6	0°	60°
A(C1-OH)	11.2	2.5	2.0	5.2	0°	35°
A(C4-OH)	6.0	−2.5	−3.5	0°	70°	0°
g	2.0074	2.0044	2.0023	2.0048		

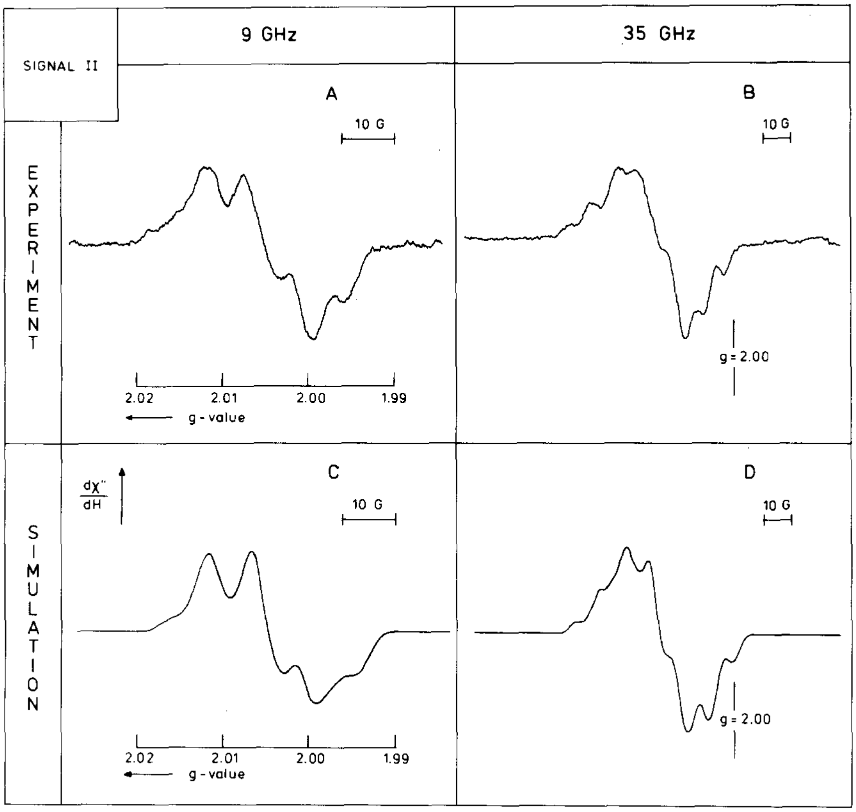

Fig. 5. Powder spectra of SII at 90 K. (A) Microwave frequency, 9.0 GHz; microwave power, 2μW; modulation amplitude, 4G. (B) Microwave frequency, 35.0 GHz; microwave power, 15 μW; modulation amplitude, 5 G. (C) and (D) Computer simulations with parameters as listed in Table I.

fits are quite good. We stress that we have not done any parameter variations for this simulations. In separate simulations we have found that the Q-band spectrum especially is quite sensitive to such variations. Thus, the good agreement between experimental and simulated spectra in Figs. 3 and 5 provides strong support for the correctness of our assignments as collected in Table I.

Our hyperfine splitting assignments are based on extensive spectral simulations. Although the fits achieved are quite good, simulation alone can never be proof. In principle, such proof can be provided by ENDOR spectroscopy. O'Malley et al. [5] have published part of the proton ENDOR spectrum of SII, in the frequency range 27–31 MHz. Two broad lines of opposite sign were detected, which were ascribed to the parallel and perpendicular components of the axial hyperfine tensor of a rotating CH_3 group. In view of the arguments given above, this assignment is unlikely to be correct. We think it more likely that the broad feature at 31.1 MHz is due to the hydroxyl proton of C1. Our values for the axial hyperfine tensor of the methylene protons at C5 would give positive ENDOR intensity at 26.0 MHz and negative intensity at 28.1 MHz. Obviously, this does not agree with the spectrum presented in Ref. 5. We are unable, however, to fit our SII spectrum with a parallel CH_2 hyperfine component giving ENDOR intensity at 27.7 MHz, as observed in Ref. 5, because then the splittings in the randomly oriented spectra become much too large. This is even more severe when the ENDOR line is assigned to a methyl group as is suggested in Ref. 5. More extensive ENDOR experiments are now in progress in order to resolve this question.

In conclusion, our work shows that although the basic idea of O'Malley et al. that SII is due to a plastosemiquinone cation is likely to be correct, their interpretation of the lineshape of SII is at variance with a number of observations. We have presented a detailed quantitative picture of the g-tensor, the relevant hyperfine tensors and the hydrogen bond angles of SII that is in full agreement with the available EPR data. One of the hydroxyl protons shows a bond bending, which is probably caused by hydrogen bonding to the proton matrix. Presumably, it is this bond that stabilizes the antisymmetric orbital of the unpaired electron of PQH_2^{+}.

Acknowledgements

We are indebted to Dr. G.T. Babcock for many enjoyable and stimulating discussions, to A.H.M. de Wit for culturing the deuterated algae, to Lenneke Nan for the preparation of the PS II particles and to Hans Horikx for drawing Fig. 4. This work was supported by the Netherlands Foundation for Chemical Research (SON) and financed by the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

1 Kohl, D.H. and Wood, P.M. (1969) Plant Physiol. 44, 1439–1445
2 Hales, B.J. and Das Gupta, A. (1981) Biochim. Biophys. Acta 637, 303–311
3 Bouges-Bocquet, B. (1980) Biochim. Biophys. Acta 594, 85–103
4 O'Malley, P.J. and Babcock, G.T. (1984) Biochim. Biophys. Acta 765, 370–379
5 O'Malley, P.J., Babcock, G.T. and Prince. R. (1984) Biochim. Biophys. Acta 766, 283–288
6 Brok, M., De Groot, A. and Hoff, A.J. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 677–680. M. Nijhoff & W. Junk, Dordrecht
7 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234
8 Siderer, Y., Malkin, S., Poupko, R. and Luz, Z. (1977) Arch. Biochem. Biophys. 179, 174–182
9 Nishi, N., Hoff, A.J. and Van der Waals, J.H. (1980) Biochim. Biophys. Acta 590, 74–88
10 Muto, H., Nunome, K. and Iwasaki, M. (1974) J. Chem. Phys. 61, 5311–5314
11 Carrington, A. and McLachlan, A.D. (1979) Introduction to Magnetic Resonance, Chapman and Hall, London