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ESR characteristics of Photosystem I in deuterium oxide: further evidence that electron acceptor A_1 is a quinone

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The appearance of ESR signals from Photosystem I (PS I) electron acceptors A_1 and A_0 in water or deuterium oxide suspension was followed using a low-temperature photoaccumulation technique. In deuterated samples the A_1 signal was narrowed by a factor of 0.66 compared with the control. This effect was fully reversible upon resuspension of treated samples in H_2O. The narrow ESR signal from deuterated A_1 had similar power saturation characteristics to the normal signal; however, a signal from a second component resolved by deuteration was saturated at higher microwave powers than the control. The power saturation behaviour of A_1^- in un-modified reaction centres indicated that it is an anionic semiquinone in a 'protic' environment. Deuteration reversibly modified the relative extents of reduction of iron sulphur electron acceptors A and B such that centre B became the more stable electron acceptor. The *g*-value and line-width of iron sulphur centre X was not modified by deuteration although it appeared to become more efficiently reduced. These results are discussed in the light of current evidence from optical, electron spin polarisation and extraction experiments that suggest that A_1 is a quinone, probably vitamin K-1.

Introduction

Two low-potential electron acceptors, A_0 and A_1, have been discovered recently in PS I [1,2]. These components may be stably reduced when the terminal acceptors, iron sulphur centres X, A and B are reduced or removed (see Ref. 3), indicating that A_0 and A_1 are intermediary electron acceptors. In low-temperature photoaccumulation experiments [1,4] centre A_1 becomes reduced first, followed by centre A_0. This has been taken to indicate that A_0 has the lower potential of the two and is probably the first transient electron acceptor in PS I. Centre A_0 has an ESR spectrum which resembles that of a chlorophyll *a* monomer [1,2]. Optical difference spectra have been presented for this component which support this assignment [4–6]. However, there is not as yet a generally accepted spectrum available, indeed some reports have failed to find any signals within the expected time and spectral regions [7,8]. The latter results may be due to signal-to-noise problems and further work is required to obtain a definitive spectrum for A_0.

The steady state X-band (9 GHz) ESR spectrum of A_1 is asymmetric [1,2] and as such resembles that of a quinone. The Q-band (35 GHz) electron spin polarisation signal from PS I fragments derived from *Anacystis nidulans* (*Synechococcus* spp.) grown in deuterated medium shows characteristics of a secondary radical pair, i.e., one

Abbreviations: Chl, chlorophyll; ESR, electron spin resonance; PS I, Photosystem I, Mes, 4-morpholineethanesulphonic acid.

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in which the charge separation has been transferred from the first to a second acceptor [9]:

$$P\text{-}700^{+}A_0^{-}A_1 \rightarrow P\text{-}700^{+}A_0A_1^{-}$$

Accordingly, the Q-band spectrum contains features due to both P-700^{+} and A_1^{-}, the latter having *g*-values similar to a reduced quinone. Comparison of these signals with those from iron-depleted and deuterated photosynthetic bacterial reaction centres, where the acceptor is known to be a quinone, also supports this view [10]. Optical measurements in the ultraviolet region following low-temperature photoaccumulation of A_1^{-} clearly show absorbance changes similar to those expected of a quinone–semiquinone transition [11]. Moreover, absorbance changes in the ultraviolet region with a lifetime of 150 μs when measured at 10 K show features that resemble a P-700^{+}/P-700 difference spectrum superimposed upon a vitamin K-1^{-}/vitamin K-1 difference spectrum [12]. These experiments are supported by data showing that no absorbance changes occur in the visible region [4,13] under conditions when A_1 would be expected to become reduced, the component therefore is not a chlorophyll or pheophytin molecule.

It has been known for many years that PS I contains quinones, specifically phylloquinone or vitamin K-1 [14]. These are found in stoichiometric proportions to P-700 in PS I particles [15,16] and in PS I reaction centres [17,18] and so are presumably of some functional significance. Indeed, ultraviolet irradiation destroys one of the quinones and partially inhibits electron transport to soluble NADP [19]. It has also been shown that one of the two bound phylloquinones may be extracted without affecting PS I electron flow [20]. These results suggest that one of the bound quinones is directly involved in electron transport.

In this study we report the results of a procedure designed to substitute deuterium for hydrogen atoms in the environment of the putative quinolic PS I electron acceptor. We have used the term 'deuteration' to describe this process and stress that this method affects only freely exchangeable protons. This is in contrast to previous studies in which microorganisms have been grown in deuterium-based media including perdeuterated growth factors such that complete isotopic exchange of ^{2}H for ^{1}H in the organism was achieved. Our results suggest that the photo-reduced PS I electron acceptor A_1^{-} is an anionic semiquinone which is exposed to hydrogen bonding in its environment.

Material and Methods

PS I particles were prepared as previously described [4,11]. The final P-700/chlorophyll ratio was 1 : 32. The sample was stored at 77 K at 4.32 mg Chl per ml in 0.1% Triton X-100, 20 mM glycine (pH 10). In order to achieve deuteration the above sample was diluted to 0.1 mg Chl per ml in deuterium oxide (99.8 atom% ^{2}H, from Aldrich Chemical Company) containing 5 mM glycine and 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) to give a pH of 10. The sample was then left in the dark at 6 °C for 5 h before freeze drying overnight. It was then resuspended to 0.1 mg Chl per ml in the $^{2}H_2O$ (pH 10.0) medium. After a further 5 h at 6 °C the sample was again freeze-dried overnight and then resuspended to 0.1 mg Chl per ml in $^{2}H_2O$ buffered at pH 6.5 with 10 mM Mes and Tris. After incubation for 5 days in the dark at 6 °C the sample was once again freeze dried and then resuspended in $^{2}H_2O$ (20 mM glycine, pH 10) at about 1 mg Chl per ml. Low concentrations of buffering agents were used during the freeze-drying steps partly to prevent a build up of salts which might prevent resuspension of the sample and also to enable the pH to be easily adjusted when required. Control samples underwent the same dilution and freeze drying regime in H_2O based media, one control undergoing the pH adjustments and another being maintained at pH 7.4 with 10 mM Tris.

EPR measurements were carried out and standardised using the media and conditions previously described in [4,11]. Sodium ascorbate (100 mM) and sodium dithionite (4% w/v) stock solutions were made in either H_2O or $^{2}H_2O$ appropriate for later use. The anaerobic sodium dithionite stock solutions were buffered at pH 10 with 100 mM glycine and were added to anaerobic samples (N_2 gassed) to give 0.1% final concentration. Photoaccumulation was carried out as described in Ref. 4, measurements were taken in the

dark following the periods of low-temperature illumination stated in the figure legends. The signals disappear upon warming in the spectrometer to temperatures around 240–250 K. Subsequent illumination at 205–225 K then causes the reduced A_1 and A_0 signals to re-appear indicating that no damage to the electron-transport components is caused by the prolonged illumination at low temperatures [11]. Samples that had undergone the pH 10.0/6.3 or pH 7.4 and freeze drying regime in water-based media were found to have identical characteristics with regard to the photoaccumulation, shape and position of ESR signals from acceptors A_1 and A_0 to non-freeze dried material.

In order to check that changes due to deuteration were reversible, deuterated samples were diluted to 0.1 mg Chl per ml in distilled water, incubated in the dark at 6 °C for 5 h and freeze-dried overnight before resuspension in a water-based medium containing 0.1% Triton X-100 and 20 mM glycine pH 10 at the chlorophyll concentrations required for assay (see figure legends).

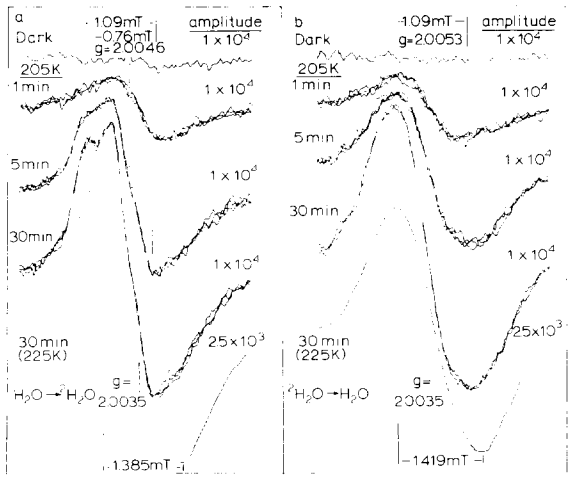

Fig. 1. The effect of deuteration on components A_1 and A_0 in pea PS I. (a) ESR spectra of a deuterated sample recorded at 205 K in the dark following illumination for the time increments shown and at the temperatures indicated. Three spectra were recorded at 4-min intervals and superimposed to demonstrate that the signals were irreversible at this temperature. (b) the sample from (a) after return to a water-based medium. The chlorophyll concentration was 300 μg/ml with 1 P-700/32 Chl. The following instrument settings were used: microwave power, 100 μW; frequency, 9.0604 GHz modulation amplitude, 0.2 mT; response, 1 s; instrument gain, expressed as amplitude in the figure. *g*-values were measured using a powdered manganese oxide sample as a standard.

Results

In Fig. 1a we show the appearance of low-temperature photoaccumulated ESR signals from a sample that had been deuterated using the procedure described earlier. ESR signals from PS I electron acceptors A_1 and A_0 in water-based media have been presented elsewhere [1,4], they show the following spectral characteristics: A_1 typically has a *g*-value of 2.004–2.005 and width of about 1.05 mT, whilst A_0 has a *g*-value of 2.0025–2.0033 and width of 1.35–1.50 mT. Following low-temperature (205 K) photoaccumulation in the presence of dithionite the A_1 signal progressively appears. Further illumination at slightly higher temperatures (225 K) allows the accumulation of reduced A_0 and hence the appearance of the characteristically broader A_0 signal with a shift in *g*-value superimposed upon the earlier signal. These characteristics may be compared with the data presented here.

Following illumination for 1 min at 205 K the photoaccumulated signal was 1.09 mT wide, this is the width expected for the A_1 signal. After a further 5 min illumination the main signal became narrowed (0.78 mT) and a shoulder which suggested the presence of a broader (1.08 mT) signal was detected. After an additional 30 min illumination at 205 K the low-field region of the signal was distinctly resolved into two separate peaks. Using the minimum of the high-field trough as a basis for measurement the outer peak was from a signal 1.09–1.15 mT wide and the inner peak was from a 0.76 mT wide signal. The outer peak is therefore similar in width to the A_1 signal in control samples, whereas the inner peak is from a signal about 0.66 times narrower. The *g*-value for the combined signal at this point was 2.0046 which is a typical value for component A_1. Further illumination at 225 K produced a signal that was indistinguishable in shape, linewidth (1.39 mT) and *g*-value (2.0035) from A_0 signals in control samples. From this we conclude that the component responsible for the A_1 ESR signal may be deuterated or is susceptible to deuteration of its environment which leads to narrowing of its linewidth. The compo-

nent responsible for the A_0 ESR signal is not similarly affected. The observation that the A_1 signal following only 1 min illumination at 205 K had the usual width may indicate that deuterated A_1 becomes reduced less efficiently than unaffected centres in the same sample. In Fig. 1b we show spectra derived from the same sample as used in Fig. 1a, except that the material had been returned to a water-based medium. It can be seen that the line narrowing entirely disappeared and the spectra of A_1 and A_0 had identical characteristics to control samples. The effect of deuteration on component A_1 was fully reversible and not, therefore, the result of unspecific denaturation.

ESR spectra were recorded at different microwave powers for a deuterated sample that had been illuminated for 30 min at 205 K (Fig. 2a). This sample was, therefore, in the condition P-700-A_0/A_1^- and showed the split signal already seen in Fig. 1a when recorded at 100 μW power. However, the two low-field peaks showed quite different power dependencies and the position of the high field minimum was seen to shift to higher g-values with increasing microwave power. At low powers (up to 100 μW) the inner peak was the dominant feature, at 1 mW and above the outer peak became prominent. Spectra were recorded under identical conditions for a control (water-based) sample (Fig. 2b). The shape of the control sample signal was unaltered at low microwave power; however, at higher powers (20 mW and above) the signal became broader and shifted to a higher g-value. The width of the signal in deuterated samples at both low (10 μW) and high (50 mW) microwave powers was less than that in the control sample at comparable powers. These findings suggest that a component measured at low powers gave a narrowed signal following deuteration as did a separate component that could be detected at higher powers.

In the power saturation profiles shown in Fig. 3 it may be seen that at lower powers the inner peak of the deuterated sample behaved identically to the control A_1 signal. At higher powers there was some deviation although this may have been due to interference in the amplitude measurements by the outer peak. It can be seen that the outer peak shows quite different power characteristics from the control A_1 signal and from that of the inner peak. The results shown in Figs. 2 and 3 indicate that the ESR signal attributed to component A_1 in

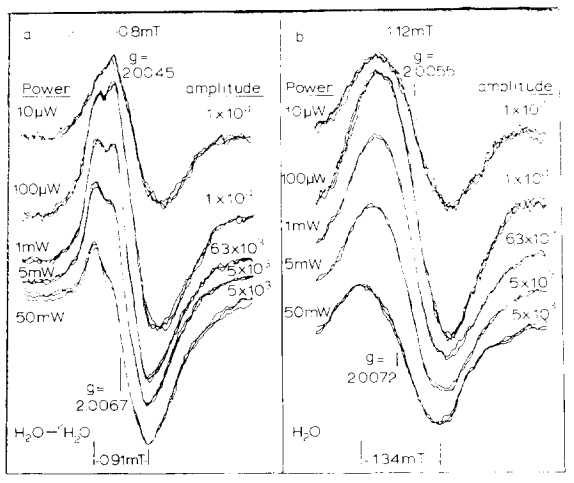

Fig. 2. The A_1-ESR signal measured at different microwave powers. (a) Deuterated PS I. (b) Control PS I which had been through the pH 6.3/10.0 and freeze drying regime used for deuteration but in a water-based medium. ESR spectra from samples poised in the state P-700/A_0/A_1^- by 30 min illumination at 205 K were recorded at the stated microwave powers. All other conditions were as in Fig. 1.

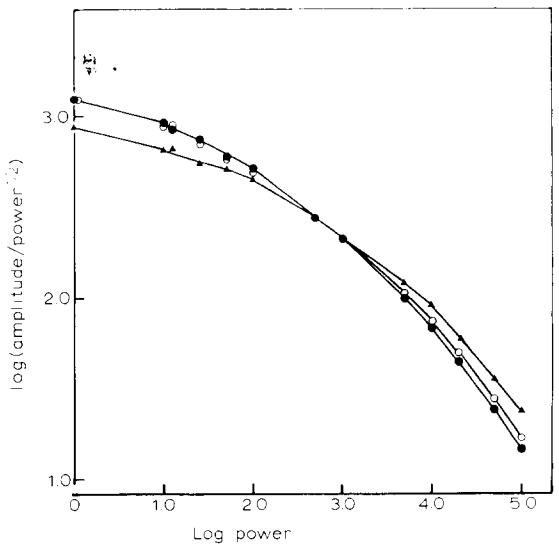

Fig. 3. Comparison of the power dependencies of the amplitudes of A_1 signals in deuterated and control samples. ●———●, control PS I; ○———○, 0.8 mT wide signal in deuterated PS I; △———△, 0.91 mT wide signal in deuterated PS I. The data were taken from the spectra shown in Fig. 2 and others in the same series.

ummodified samples conceals signals derived from two different components. These signals were resolved following deuteration (Fig. 1). From the results shown in Fig. 1 we initially assumed that the outer peak was part of an unmodified A_1 signal in reaction centres that had not become deuterated. However, since the outer peak when measured at high powers was narrower than the control and also had different power characteristics we now conclude that it was probably derived from a second component. This component was photoreducible, since we did not see the signal in non-photoaccumulated samples at high microwave powers (result not shown).

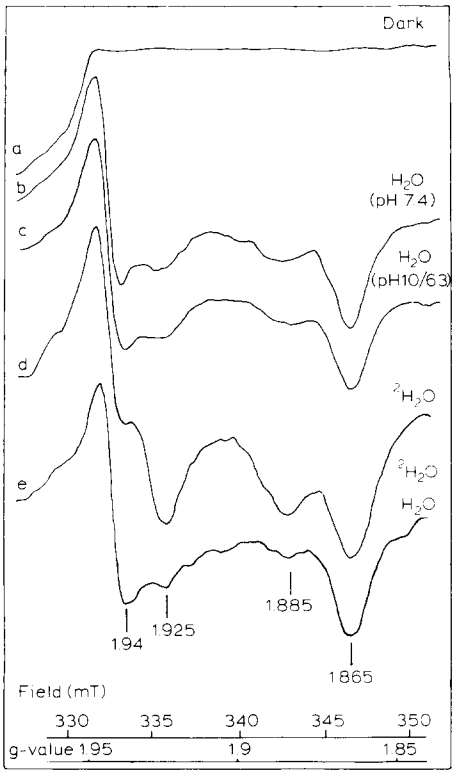

Fig. 4. The effect of PS I deuteration on the light induced reduction of centres A and B. (a) ESR spectrum of dark adapted pH 7.4 control sample; (b) and (c) are spectra of control samples having passed through the freeze drying regime at pH 7.4 and pH 10/6.3, respectively; (d) a deuterated sample; (e) a previously deuterated sample returned to water-based suspension. All samples were made to 1 mg Chl per ml in a medium containing 0.1% Triton X-100 and 20 mM glycine, pH 10.0. Following dark adaptation for 1 h 10 mM sodium ascorbate was added in the appropriate medium (^2H_2O or H_2O) and samples were then frozen in the dark. Spectra were recorded in the dark following a 30 s period of illumination at 18 K. Instrument settings were: microwave power, 5 mW; modulation, 10 G, response, 0.3 s, frequency, 9.039 GHz; gain, 630.

The results presented above show that component A_1 was modified by deuteration. We reasoned that if this component were involved in PS I activity then deuteration might affect electron flow to other components in the complex. Accordingly, we measured light-induced reduction of iron-sulphur centres A and B in control and deuterated samples (Fig. 4). Illumination at 18 K in the presence of sodium ascorbate will irreversibly reduce centres A and B. However, in otherwise untreated spinach PS I centre A is more efficiently reduced [22] as it was in the control pea samples shown here giving rise to signals with peak *g*-values of 1.94 and 1.865 (Fig. 4b and c). In contrast, in the deuterated sample the photo-induced signal shows a much greater proportion of centre B reduction giving rise to prominent signals with peak *g*-values at 1.925 and 1.885 (Fig. 4d). The overall signal from the magnetically interacting centres A and B was irreversible in the dark at the assay temperature of 18 K. The effect of deuteration on centre B reduction was fully reversible. In a deuterated sample that had been re-incubated in a water-based medium the usual ascorbate/light reduced profile was obtained (Fig. 4e). It was therefore possible to restore the usual balance of electron flow between iron-sulphur centres A and B indicating that the modification observed in Fig. 4d was not the result of destruction of centre A. Moreover, in samples in which both centres A and B were chemically reduced by addition of sodium dithionite to anaerobic samples at pH 10 the water-based and deuterated samples gave identical spectra indicating full reduction of both centres A and B (see Fig. 5a).

The more efficient reduction of centre B following deuteration may be the result of a modification of electron flow from the intermediary acceptors, A_0 and A_1, to the terminal iron-sulphur centres. This flow is mediated by component X, also an iron-sulphur centre [23], ESR signals from centre X were recorded from pre-illuminated samples that had undergone illumination at 225 K in the presence of dithionite and which were therefore in the state: P-700 A_0^- A_1^- X^- $(AB)^-$. The

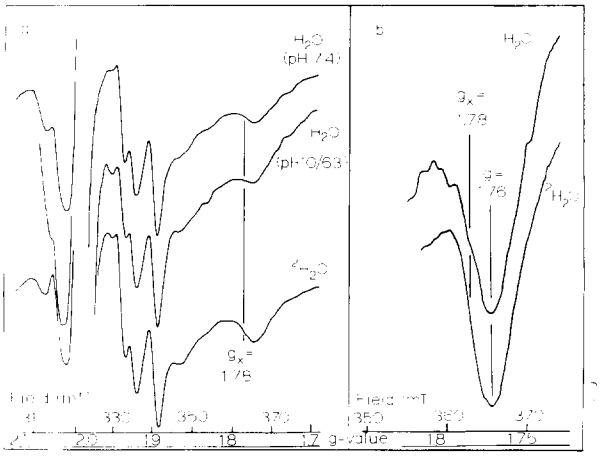

Fig. 5. EPR spectra of iron sulphur centre X in pea PS I suspended in H_2O and ^2H_2O based media following pre-illumination at room temperature in the presence of dithionite and subsequent illumination at 225 K. These samples were in the state P-700/A_0^-/A_1^-/X^-/$(A/B)^-$. (a) The upper two traces show water-based samples that were prepared and freeze dried at the pH values indicated. The lower trace was prepared in deuterated media. The instrument gains were 1000, 2000 and 6300, respectively. (b) Comparison of the ESR signal from reduced centre X in non-freeze dried PS I (upper trace) and in deuterated PS I (lower trace). The instrument gains were 200 (with 2 mg Chl per ml) and 4000 (with 0.3 mg Chl per ml), respectively. Other instrument settings were as follows: temperature, 9 K; power, 5 mW; modulation, 10 G' response, 0.3 s; frequency, 9.05 GHz.

shape, line-width and *g*-values of the centre X spectrum were unaffected although the resolution of the signal was improved following deuteration (fig. 5a). The g_X signal of centre X in deuterated samples was found to be identical to the equivalent signal in a sample that had not undergone freeze drying (Fig. 5b). Therefore, one must assume that deuteration did not significantly alter the gross environment (either proteinaceous or lipid) of centre X. The effect on centre B reduction was probably independent of any effect on centre X.

Discussion

There is abundant evidence that PS I contains stoichiometric quantities of quinone, in particular 2-methyl-3-phytyl-1,4-naphthoquinone (otherwise known as phylloquinone or vitamin K-1) [14–19]. Recent optical and ESR spectroscopy data have implicated this quinone as an early intermediate electron acceptor operating at very low potentials in PS I [9–13]. The interaction of quinones with electron-transport systems has often been investigated by the effect of deuteration on the ESR spectrum of the putative quinone [9,24,25]. Semiquinones bound to reaction centres tend to have *g*-values similar to those recorded in powder spectra or in frozen solutions [26]. Deuterated semiquinones when immobilized typically have narrower line-widths than their protonated forms [27]. Similarly, deuterated but not protonated quinones when reconstituted into extracted reaction centres present narrower ESR semiquinone signals than the endogenous component [24].

Previous studies of the effects of deuteration on electron-transport components have used microorganisms which were grown on perdeuterated growth factors [9,10,25]. However, in order to continue our study using higher plants we devised a method involving freeze drying of samples after incubation in deuterated media at different pHs. This method therefore, will influence only freely exchangeable protons and is unlikely to lead to complete replacement of all hydrogen atoms in the sample with deuterons. Using this method we have found that the line-width of the ESR signal from P-700$^+$ was unaffected (result not shown) in contrast to the significant (2–3 times) narrowing observed in cyanobacteria or algae grown in deuterated media [25]. We therefore conclude that the deuteration protocol employed here was not rigorous enough to exchange hydrogen atoms such as those on the chlorophyllous PS I donor. The freeze drying and pH regime in itself was found to destroy about 25% of the centres (as measured by electron flow to iron-sulphur centres A and B in dark-adapted ascorbate-reduced samples). The remaining centres were fully active.

Previously, it has been shown that deuteration of quinones has the following effects on their ESR spectra [26,27]. Firstly, and most obviously, the line-width of the signal becomes narrowed. Secondly, the *g*-value of the signal increases and finally, the signal becomes asymmetric. These changes are observed upon perdeuteration of protic solvents containing anionic semiquinones [27]. In the findings presented here we have demonstrated that the ESR signal from component A_1^- becomes significantly narrowed upon removal of water and

incubation in deuterated media. However, we did not observe a shift in the *g*-factor to higher values. It was also not possible to detect whether the deuterated signal was more or less asymmetric than the control. The complicated nature of the deuterated signal and insufficient resolution of the individual spectrum may well have masked the latter two changes. The effect of deuteration on the line-width of the A_1^- signal is, however, strong evidence that the component is an anionic semiquinone which is exposed to significant hydrogen bonding in vivo. These hydrogen atoms must be freely available for exchange. The same cannot be said for the binding of electron acceptor A_0, since we did not observe significant narrowing of the ESR signal from this component in deuterated samples.

The A_1 radical signal following deuteration of the PS I samples was only partially narrowed. A residual peak remained with the same spectral characteristics as the control when measured at 100 μW microwave power. However, the relative extent of this 'residual' signal was never observed to vary from one preparation to another. This suggested that there existed a constant pool of A_1 with inexchangeable hydrogen bonds or that a signal from an entirely different and previously obscured component was present. A power saturation study (see Ref. 28) revealed that indeed there were two different components responsible for the A_1^- signal in deuterated samples. One of these could be observed almost in the absence of the other at low power and was found to have the same saturation characteristics as the control A_1 signal. The signal observed at high powers was narrow (0.91 mT) relative to control A_1 signals (1.34 mT) at the same powers; it is unlikely therefore that the outer peak shown in Fig. 1 is derived from protonated A_1. The component responsible is either a previously unobserved acceptor, an oxidised donor (possibly sodium dithionite) or is a fraction of A_1 that is also affected by deuteration yet is in a different environment from that which saturates at lower powers. It is tempting to speculate that the two signals resolved by deuteration are related to the two molecules of vitamin K-1 typcially found in PS I [17,18]. Malkin has shown that one of these may be removed without affecting PS I electron flow [20] and we are now carrying out experiments to determine whether extration might remove one of the peaks observed after PS I deuteration.

By comparison of the optical difference spectrum that we obtained upon photoaccumulation of reduced A_1 with published difference spectra for a variety of quinones [29] we postulated that A_1^- might be a neutral semiquinone [11]. However, the positions of absorbance maxima and minima for vitamin K-1 anionic and neutral semiquinones in the U.V. region are similar (Land, E.J., personal communication) and do not allow such a distinction to be made. The absorbance maximum in the ultraviolet that we observed for A_1 reduction was at 290 nm. Using the extinction coefficients for the neutral and anionic semiquinones at this wavelength and assuming the presence of one active quinone per reaction centre (as seems likely [20]) the absorbance we observed in Ref. 11 was compatible with the anionic form only. This conclusion is supported by a consideration of the anisotropic saturation characteristics of the A_1 radical signal in control samples (Fig. 2b). The line-broadening and shift of the *g*-factor to higher values is typical of anionic semiquinones in protic environments, i.e., those in which hydrogen bonding to the quinone occurs. This has been demonstrated in model systems [27] and has also been implicated in the assignment of ESR Signal II from PS II as being derived from a semiquinone cation [30]. The anisotropic saturation effects are probably due to hydrogen bonding to the radical and are not seen in spectra of immobilized neutral semiquinones [27]. Furthermore, in model systems the line-widths of semiquinone anions are narrow relative to those of the neutral forms [27]. In particular for vitamin K-1 the anionic form has a width of 0.85 mT against 1.60 mT for the neutral form. Signal A_1 is typically 1.05 mT wide and is significantly narrower than all the neutral semiquinone spectra presented in Ref. 27. The component responsible for signal A_1 is likely to be exposed to a variety of line-broadening influences within the membrane and we therefore conclude that its linewidth is most compatible with it being an anionic semiquinone. The above optical and ESR considerations lead us to suggest that A_1^- is an anionic semiquinone which is hydrogen bonded to its surrounding protein. The bonding and im-

mediate environment of A_1 may resemble that of the cationic semiquinone said to be responsible for Signal II on the oxidising side of Photosystem II shown in model form in Ref. 30.

It is as important to demonstrate whether or not component A_1 is involved in PS I electron flow as to elucidate its structure. To date it has proven impossible to extract and reconstitute the component and reversibly inhibit photo-reduction of the terminal electron acceptors. We have presented data here which suggest that deuteration modifies electron flow from the intermediate acceptors such that iron sulphur centre B becomes more stably reduced upon illumination at low temperature. Under the same conditions the ESR signal from centre A_1 was markedly altered. This is not conclusive evidence that A_1 is involved in forward electron flow, since the relative rates of reduction of iron sulphur centres A and B may have been influenced independently of the effect on A_1. An alternative possibility is that hydrogen bonds in the environment of the polypeptide(s) supporting the iron sulphur groups are important. This may involve either direct involvement in electron distribution to the centres or, alternatively, maintenance of an appropriate protein configuration within the complex.

Deuteration leads to narrowing of ESR signals through removal of proton hyperfine interaction with the unpaired electron of the radical being observed. This effect is not limited to quinones, signals from other components in photosynthetic systems have been shown to become narrowed upon rigorous perdeuteration [25]. The effect seen here, however, is the result of substitution of a freely exchangeable proton which interacts with the photoaccumulated radical. Of the candidate molecules present in PS I only the phylloquinone would be likely to possess such an exchangeable group, indeed we observed no effect on the linewidth of the chlorophyllous species P-700 and A_0. The observations reported here are consistent with component A_1 being an anionic semiquinone in a protic environment and form strong evidence for this assignment. In this respect 'a protic environment' may be taken to infer hydrogen bonding from amino groups on the surrounding protein to the –O groups of the quinone which is in an otherwise aprotic region of the membrane.

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