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Photoaccumulation of the PsaB phyllosemiquinone in Photosystem I of *Chlamydomonas reinhardtii*

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Abstract

Photoaccumulation of membrane preparations of *Chlamydomonas reinhardtii* at pH 8 and 220 K reduces the primary and secondary electron acceptors in the Photosystem I (PSI) reaction centre, and produces a maximum of two spins per $P700^{\bullet+}$. Proton electron nuclear double resonance (ENDOR) spectra demonstrate that the phyllosemiquinone produced is that attributed to the PsaA branch of electron transfer. Photoaccumulation at pH 10 and 220 K produces a maximum of four spins per $P700^{\bullet+}$, and proton ENDOR spectra indicate that a second phyllosemiquinone is being photoaccumulated, with markedly different proton hyperfine couplings (hfcs). This phyllosemiquinone is unaffected by mutation of PsaAW693, confirming that it does not arise from the PsaA branch of electron transfer, and we therefore attribute it to the PsaB phyllosemiquinone.

Keywords: Photosystem I; Photoaccumulation; Magnetic resonance; ENDOR; Phyllosemiquinone; PsaB; *Chlamydomonas reinhardtii*

1. Introduction

The Photosystem I (PSI) reaction centre is a plastocyanin–ferredoxin oxidoreductase (see Ref. [1] and subsequent articles in the same edition for recent reviews). Light energy initiates electron transfer from a primary electron donor P700, which is a dimer of chlorophyll *a* molecules. The primary electron acceptor A_o is a chlorophyll *a* monomer, which then donates electrons to a phylloquinone (vitamin K_1) secondary electron acceptor A_1. Subsequently, electrons are transferred to a bound [4Fe–4S] centre, F_X, and then on to two further bound [4Fe–4S] centres called F_B and F_A. The PSI reaction centre contains two major proteins PsaA and PsaB [2], which show extensive sequence homology, and form a heterodimeric complex binding P700, A_o, A_1, and F_X. During the last 10 years, the groups of Witt and Saenger in Berlin have worked to resolve the X-ray crystal structure of the PSI complex from *Synechococcus elongatus*, and the two research groups have recently published a new structure of a trimeric form of this complex at 2.5 Å resolution [3].

One region of the PSI reaction centre that has been the focus of recent research is the bound phylloquinone electron acceptor(s) A_1 and the binding pockets for these redox cofactors on the PsaA and PsaB core polypeptides. The phylloquinone/phyllosemiquinone redox couple is estimated to be functioning at a somewhat lower redox potential ($E_M \sim -820$ mV) than is observed for this redox couple in vitro [4]. The polypeptide binding pocket is thought to have a role in engineering the electronic structure and thus redox potential of the phylloquinone. A number of sug-

Abbreviations: ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; ESP, electron spin polarised; PSI, Photosystem I; P700, primary electron donor of Photosystem I; A_o, chlorophyll primary electron acceptor in Photosystem I; A_1, phylloquinone secondary electron acceptor in Photosystem I; F_A, F_B and F_X, [4Fe–4S] centres of Photosystem I; PhQ, phylloquinone, hfcs, hyperfine couplings; RC, reaction centre

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gested mechanisms have been put forward, including the lack of an extensive H-bonding network [3], π−π stacking of the phylloquinone ring with a conserved tryptophan residue [5–7], and asymmetric H-bonding to the carbonyl oxygens of the phyllosemiquinone [3,8].

Experiments using either optical or EPR measurements of $A_1^{\bullet -}$ together with biochemical or genetic modification of the PSI iron–sulfur centres established an electron transfer path from A_1 to $Fe-S_X$ with an electron transfer rate at room temperature of $t_{1/e} \approx 200$ ns [9–11]. We have recently used [12] site-directed mutagenesis of the conserved tryptophan PsaAW693 of *Chlamydomonas reinhardtii* to show that this rate is associated with the phylloquinone bound to PsaA. Substitution of histidine or leucine for the tryptophan slows down forward electron transfer from A_1 to F_X by a factor of 2 to 3.

It has generally been thought that by analogy with the Type II reaction centres that electron transfer would be unidirectional in PSI, and that this route of electron transfer via PsaA would therefore be the only path of electron transfer. However, there have been reports in the literature that a faster rate of electron transfer from A_1 to F_X of ≈20 ns at room temperature could also be detected [13]. It was also found that mutations of the conserved tryptophans in the phylloquinone binding sites on either PsaB(PsaBW673F) or PsaA(PsaAW693H/L) did not prevent photoautotrophic growth, although the PsaA mutations did make the cultures oxygen sensitive [12,14]. Joliot and Joliot [15] have recently developed optical techniques that allow them to measure transients, which they attribute to phyllosemiquinone oxidation, in whole cells of the green alga *Chlorella pyrenoidosa*. They observe two rates of oxidation, $t_{1/e}$=13 and 140 ns at room temperature. The two phases are of approximately equal intensity. They suggest that electron transfer is in fact bidirectional, initial electron transfer is randomly directed to either side of the reaction centre with the overall rate limited on each side by the A_1 to F_X rate [15]. This suggestion is supported by analysis of site-directed mutants of *C. reinhardtii*, PsaAW693F and PsaBW673F [14]. The fast phase of 13 ns seen in *C. reinhardtii* is slowed by the PsaB side mutation to 70 ns, and the 140 ns rate by the PsaA mutation to 490 ns. In a recent experiment, we have measured the decay of the electron spin polarised (ESP) signal at 100 K, where the rates recorded reflect the influence of two different protein environments (PsaA and PsaB) on the decay of the correlation of the $P700^{\bullet +}/A_1^{\bullet -}$–geminate radical pair [16]. We interpret the results as indicating that electron transfer from the reaction centre primary electron donor, $P700^{\bullet +}$, to the iron sulfur centres, $Fe-S_{X/A/B}$, can occur through either the PsaA or PsaB side phylloquinone. However, the extent to which electron transfer occurs on the PsaB branch at room temperature is still a controversial topic mainly because it has not proved possible to measure an ESP transient corresponding to the fast phase of phyllosemiquinone oxidation reported by Joliot et al. [14,15].

There are two principal means of monitoring the phyllosemiquinone $A_1^{\bullet -}$ using paramagnetic resonance spectroscopy. These are:

(a) The ESP transient EPR signal shown to arise from the germinate radical pair $P700^{\bullet +}/A_1^{\bullet -}$ [17–19].

(b) An asymmetric electron paramagnetic resonance (EPR) signal at g=2.00 photoaccumulated at low temperatures (200 K) under reducing conditions [20], or produced by illumination whilst freezing in the presence of reductants [21]. The assignment of this signal (g=2.0048 and ΔH_{ptp} of 0.875 mT) to the phyllosemiquinone $A_1^{\bullet -}$ was conclusively demonstrated by experiments in which cyanobacteria were grown under conditions that resulted in biosynthetic deuteration of the phylloquinone [22].

We have recently shown [12] that mutation of PsaAW693 in *C. reinhardtii* alters the electronic structure of the phyllosemiquinone photoaccumulated at 205 K, as monitored by ENDOR spectrometry, indicating that the photoaccumulated phyllosemiquinone is on the PsaA side. The ESP signal arising from the radical pair $P700^{\bullet +}/A_1^{\bullet -}$ when monitored at room temperature [9,10] or 260 K [12,23] decays as electrons are transferred forward to F_X. Mutagenesis of PsaAW693 significantly slows down the forward rate of electron transfer [12], demonstrating that this technique is monitoring forward electron transfer and $P700^{\bullet +}/A_1^{\bullet -}$ on the PsaA branch of electron transfer in PSI.

Normally, photoaccumulation at pH 8 and 205 K reduces one phyllosemiquinone per $P700^{\bullet +}$, and photoaccumulation at pH 8 and 220 or 230 K reduces one phyllosemiquinone and one chlorophyll anion (attributed to $A_0^{\bullet -}$) [24,25]. We have previously reported [24] that it is possible under some conditions (220 K and pH 10) to photoaccumulate four spins per $P700^{\bullet +}$ in PSI preparations from spinach, and interpreted these results as indicating that under these conditions, the phyllosemiquinone $A_1^{\bullet -}$ and the chlorophyll anion $A_0^{\bullet -}$ were being formed by photoaccumulation on the PsaB branch of electron transfer. However, in the cyanobacterium *Synechococcus*, inhibition of the photoaccumulation of one phyllosemiquinone by deletion of PsaE or PsaF from PSI did not result in photoaccumulation of a second phyllosemiquinone [26], and it was concluded that only one branch of electron transfer is functional in PSI reaction centres.

Since we were now able to identify the PsaA phyllosemiquinone because of the effect of the PsaAW693H/L substitutions [12], we decided to reinvestigate the photoaccumulation of additional spins at pH 10 and 220 K using wild type and mutant *C. reinhardtii*. Photoaccumulation at pH 8 produces a maximum of two spins per $P700^{\bullet +}$, and proton ENDOR spectra demonstrate that the phyllosemiquinone produced is that attributed to the PsaA branch of electron transfer. Photoaccumulation at pH 10 and 220 K produces a maximum of four spins per $P700^{\bullet +}$, and proton ENDOR spectra indicate that a second phyllosemiquinone is being photoaccumulated, with markedly different proton hyperfine couplings (hfcs). This phyllosemiquinone is unaf-

fected by mutation of PsaAW693, confirming that it does not arise from the PsaA branch of electron transfer, and we therefore attribute it to the PsaB phyllosemiquinone.

2. Materials and methods

The plasmid construction, chloroplast transformation and characterisation of the mutants (PsaAW693H/L) has been described previously [12]. Wild type and mutant *C. reinhardtii* were grown, and chloroplast membrane preparations obtained, as detailed in Ref. [12].

CW EPR and ENDOR measurements were made on chloroplast membrane preparations at concentrations of 2–6 mg chlorophyll/ml. In order to generate $P700^{\bullet+}$ samples in standard 3 mm quartz EPR tubes were reduced with sodium ascorbate (10 mM) for 30 min in the dark prior to freezing in the dark in liquid nitrogen. These samples were then illuminated in the EPR cavity at 75 K for several minutes in order to generate maximal $P700^{\bullet+}$ and spectra immediately recorded. In order to photoaccumulate $A_1^{\bullet-}$ the samples in standard EPR tubes were reduced for 30 min in the dark with sodium dithionite prior to freezing in liquid nitrogen. The $A_1^{\bullet-}$ was then photoaccumulated by illumination at 205 and 220 K as described previously at pH 8 [23,24] and pH 10 [24].

CW EPR spectra were recorded on a JEOL RE1X spectrometer or a Bruker ESP 300 EPR spectrometer fitted with an Oxford Instruments ESR9 liquid helium cryostat. Double integration of spectra was carried out using software written in the laboratory. ENDOR spectra were obtained at X-band using a Bruker ESP300 spectrometer as described by Rigby et al. [27,28].

3. Results

Whilst photoaccumulation at pH 8 and 205 K produces an EPR spectrum characteristic of phyllosemiquinone (g=2.0048 and ΔH_{ptp} of 0.875 mT), photoaccumulation at 220 K produces an additional signal [24,25] with a line width of approximately 17.5 G that was previously attributed to a chlorophyll anion [24]. Fig. 1b shows the EPR spectrum of a membrane sample from *C. reinhardtii* photoaccumulated at pH 8 and 220 K, and Fig. 1a the spectrum of a different sample of the same membranes photoaccumulated at pH 10 and 220 K. If the spectrum photoaccumulated at pH 8 and 220 K is subtracted from the pH 10/220 K spectrum, the difference spectrum representing the additional signals obtained at pH 10 (Fig. 1c) includes contributions from both a narrow (possibly phyllosemiquinone) and broad (possibly chlorophyll anion) components.

The EPR spectra of WT membrane samples at pH 8 and pH 10 were recorded at intervals during photoaccumulation, at nonsaturating microwave powers and temperatures, and the intensity of spectra relative to those of $P700^{\bullet+}$ in the same preparation quantified by double integration. Fig. 2a indicates that photoaccumulation at pH 8 and 205 K produces a maximum of approximately one spin per $P700^{\bullet+}$, as previously reported and attributed to photoaccumulation of the phyllosemiquinone from of one of the two phylloquinones in PSI [22,24,25], now shown to be the PsaA branch phyllosemiquinone [12]. In fact, the maximum spins accumulated are slightly in excess of one per $P700^{\bullet+}$ in preparations from *C. reinhardtii* (Fig. 2a); as in our experience, it is difficult to avoid some photoaccumulation of chlorophyll anion in PSI from this organism during a prolonged period of photoaccumulation. Fig. 2a also indicates that photoaccumulation at pH 8 and 220 K produces a maximum approaching two spins per $P700^{\bullet+}$, as previously reported and attributed to photoaccumulation of both a phyllosemiquinone and a chlorophyll anion [24,25]. It is possible to acquire proton ENDOR spectra from this sample photoaccumulated at pH 8 and 220 K at conditions (low temperature and high microwave power) that allow for the detection of the phyllosemiquinone spectrum, but not that of the chlorophyll anion radical. Fig. 3b shows such a spectrum, and the hyperfine couplings listed in Table 1

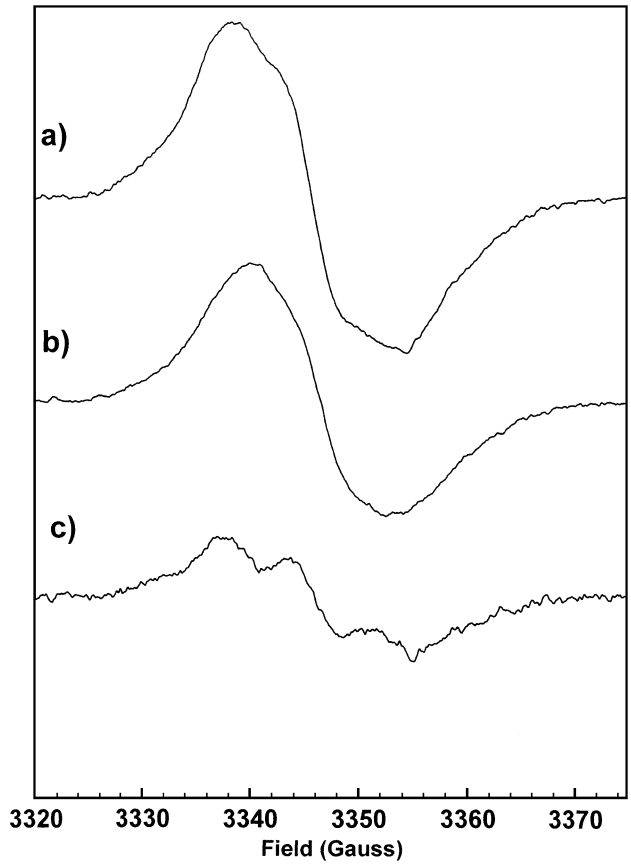

Fig. 1. EPR spectra of wild-type *C. reinhardtii* membranes following photoaccumulation in the presence of sodium dithionite at (a) pH 10 and 220 K, (b) pH 8 and 220 K. Spectrum (c) is the difference spectrum (a) minus (b). Experimental conditions: microwave power 40 μW; modulation amplitude 0.12 mT; modulation frequency 12.5 kHz; temperature 70 K; each spectrum is the sum of four scans; measured using the ENDOR cavity.

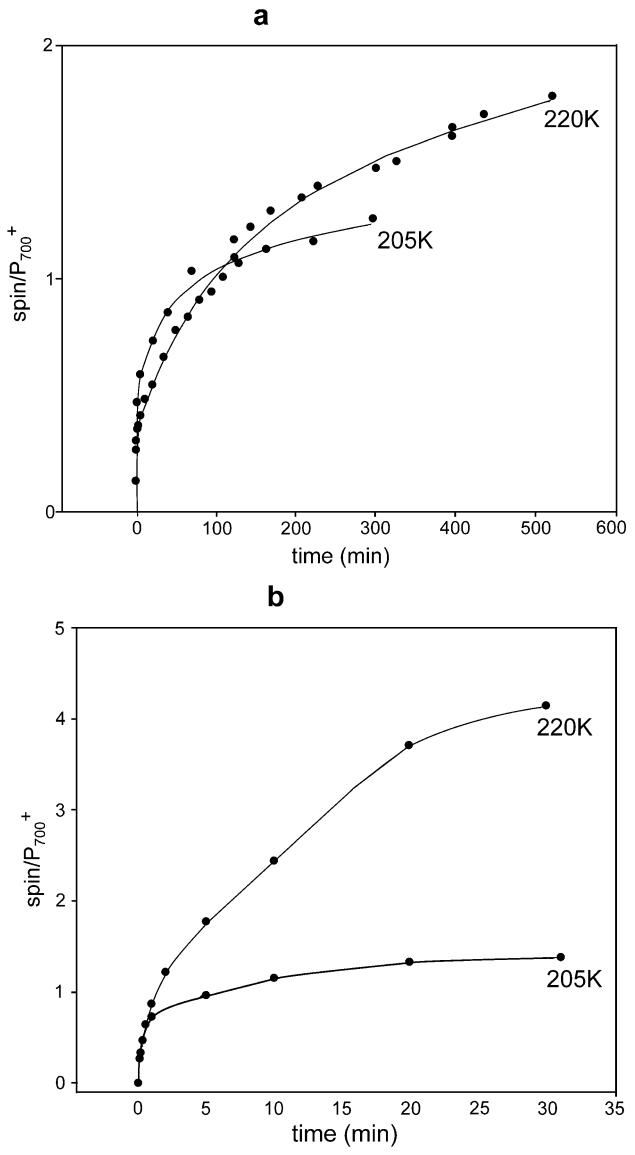

Fig. 2. The quantification of the number of spins relative to $P700^+$ photoaccumulated in PSI of *C. reinhardtii* during the course of illumination at 205/220 K in the presence of dithionite for prolonged periods. The results in (a) were obtained with samples at pH 8, and those in (b) at pH 10. The EPR spectra of photoaccumulated samples were recorded at the following experimental conditions: temperature 75 K, microwave power 10 μW, modulation amplitude 0.2 mT. The number of spins photoaccumulated was quantified by double integration and comparison with the number of spins from $P700^{\bullet+}$ measured in identical samples under the same experimental conditions (see Materials and methods).

demonstrate that this is the phyllosemiquinone [22,29] previously attributed to the PsaA branch [12].

A maximum of one spin per $P700^{\bullet+}$ can be photoaccumulated at pH 10 and 205 K (Fig. 2b) and this signal has been previously attributed to photoaccumulation of the PsaA branch phyllosemiquinone [24,25]. However, approaching a maximum of four spins per P700 can be photoaccumulated at pH 10 and 220 K (Fig. 2b) as previously reported for PSI preparations from spinach [24]. Fig. 3a shows the proton ENDOR spectrum from the sample photoaccumulated at pH 10 and 220 K obtained under conditions that suppress the chlorophyll contribution. It is clear that in comparison to the proton ENDOR spectrum obtained at pH 8 and 220 K (Fig. 3b), this spectrum (Fig. 3a) contains additional contributions. Subtraction of the pH 8/220 K spectrum from the pH 10/220 K spectrum yields the proton ENDOR difference spectrum shown in Fig. 3c. This is a spectrum not previously reported in the literature, and the proton hfcs listed in Table 1 are different from those attributed to the PsaA phyllosemiquinone.

This new radical signal can be assigned as a semiquinone radical through the anisotropy of the methyl group hfcs. Semiquinones are the only radical species formed under reducing conditions (i.e. in the presence of sodium dithionite) that have methyl group hfcs with axial anisotropies of 3–4 MHz ($A_{\|}-A_{\perp}$). This arises from dipolar interactions

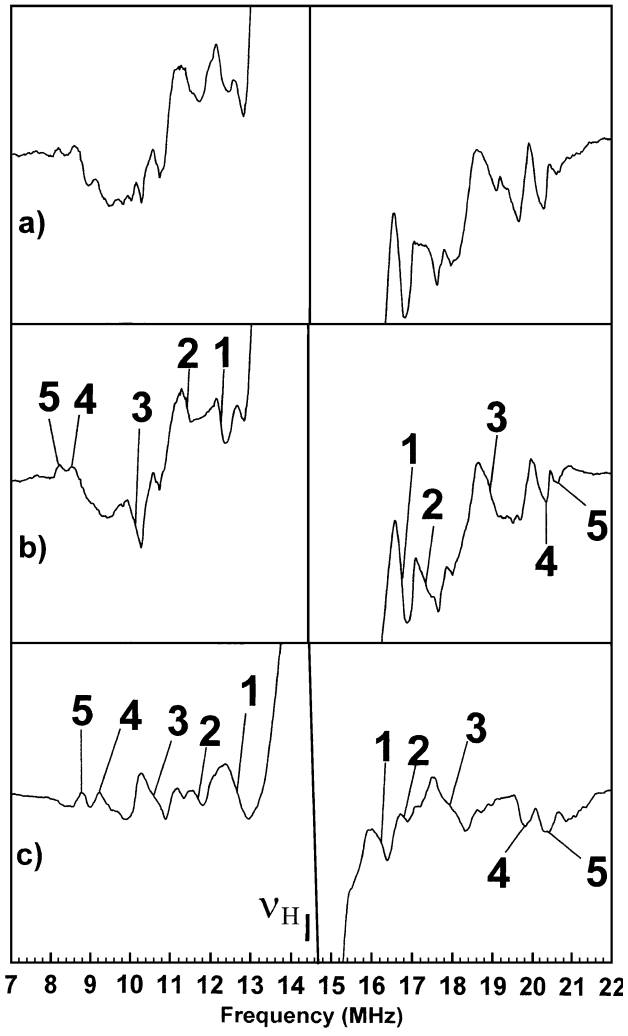

Fig. 3. ENDOR spectra of semiquinone radicals photoaccumulated in wild-type *C. reinhardtii* membranes in the presence of sodium dithionite at (a) pH 10 and 220 K, (b) pH 8 and 220 K. Spectrum (c) is the difference spectrum (a) minus (b). Experimental conditions: microwave power 7.9 mW; r.f. power 100 W; r.f. modulation depth 177 MHz; temperature 75 K; each spectrum is the sum of 200 scans.

Table 1
Hyperfine coupling constants (hfcs) and assignments for the two phyllosemiquinone radicals photoaccumulated in *C. reinhardtii* and for the phyllosemiquinone radical in isopropanol

Feature	hfc (MHz)			Assignment
	pH 8, 220 K	Difference between pH 10 and pH 8, 220 K	Isopropanol	
	("A" side)	("B" side)		
1	(−)4.5	(−)3.6	(−) 2.2	H-bond $A_{\perp}$
2	(−)6.2	(−)5.0		H-bond $A_{\perp}$
3	8.9	7.1	6.8	Methyl $A_{\perp}$
4	12.0	10.5	10.0	Methyl $A_{\parallel}$
5	12.6	11.6	5.2	H-bond $A_{\parallel}$

with the high unpaired electron spin densities on the oxygen atoms of semiquinones and is diagnostic of such radicals. Stable anion radicals of amino acid residues have not been reported in the literature, and chlorophyll anion radicals exhibit methyl group hfc axial anisotropies of less than 10% (i.e. around 1 MHz). The hfcs determined for this new radical are therefore assigned to hydrogen bonds and methyl group protons by analogy with the assignments already determined for the PsaA phyllosemiquinone (Table 1). The major difference between the PsaA branch phyllosemiquinone hfcs and those reported here for this new phyllosemiquinone radical is a reduction in both hydrogen bond and methyl hfcs in the new signal, and the methyl group hfcs are approaching but not identical to those reported for phyllosemiquinone in vitro (Table 1).

In order to confirm that the new signal is arising from the second phyllosemiquinone in PSI (the PsaB branch phyllosemiquinone), we repeated photoaccumulation at pH 8/220 K and pH 10/220 K in identical samples of chloroplast membrane preparations prepared from the PsaA W693H mutant of *C. reinhardtii*. The results presented in Fig. 4 and Table 2 indicate that the substitution of histidine for the conserved tryptophan on PsaA has altered the proton ENDOR spectrum of the phyllosemiquinone photoaccumulated at pH 8 and pH 10 (Fig. 4b), as previously reported [12], which attributes this EPR signal to the PsaA phyllosemiquinone. However, the new phyllosemiquinone ENDOR spectrum (i.e. that only photoaccumulated at pH 10 and 220 K) is not altered by this substitution (Fig. 4c and Table 2), confirming that it does not arise from the PsaA branch phyllosemiquinone and indicating that it must therefore arise from the PsaB branch phyllosemiquinone.

Table 2
Hyperfine coupling constants (hfcs) and assignments for phyllosemiquinone radicals in wild type and an A-side mutant of *C. reinhardtii*

Feature	hfc (MHz)				Assignment
	WT		PsaA W693H		
	'A side'	'B side'	'A side'	'B side'	
1	(−)4.5	(−)3.6	(−)4.5	(−)3.6	H-bond $A_{\perp}$
2	(−)6.2	(−)5.0	(−)5.2 (−)6.0	(−)5.0	H-bond $A_{\perp}$
3	8.9	7.1	8.3	7.1	Methyl $A_{\perp}$
4	12.0	10.5	11.6	10.5	Methyl $A_{\parallel}$
5	12.6	11.6	10.8 12.5	11.6	H-bond $A_{\parallel}$

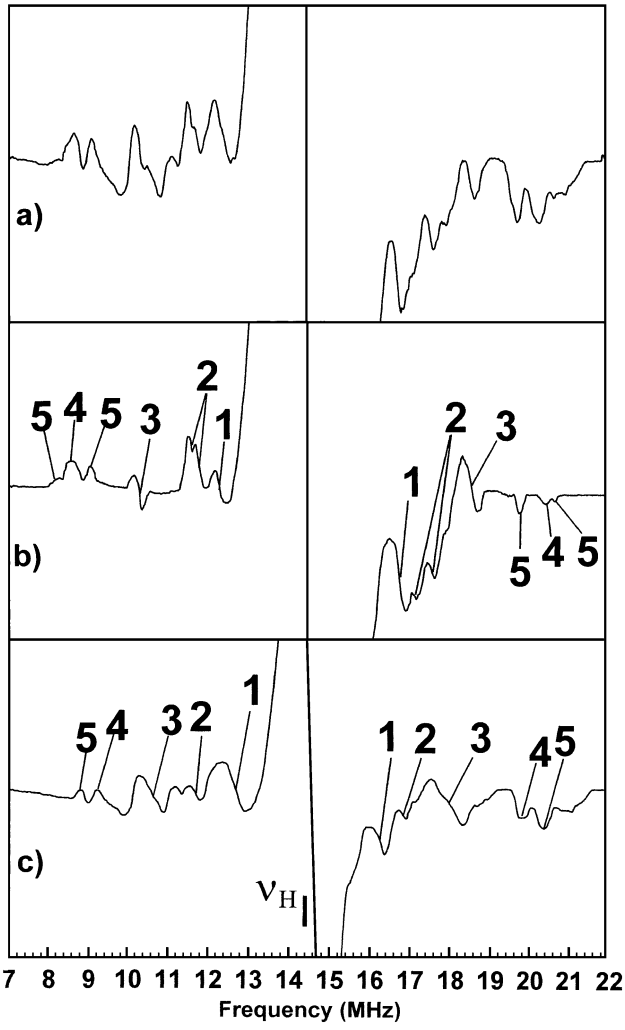

Fig. 4. ENDOR spectra of semiquinone radicals photoaccumulated in membranes of the *C. reinhardtii* PsaA W693H mutant in the presence of sodium dithionite at (a) pH 10 and 220 K, (b) pH 8 and 220 K. Spectrum (c) is the difference spectrum (a) minus (b). Experimental conditions: microwave power 7.9 mW; r.f. power 100 W; r.f. modulation depth 177 MHz; temperature 75 K; each spectrum is the sum of 200 scans.

4. Discussion

We have previously reported that if photoaccumulation was carried out with spinach PSI samples prepared at pH 10 and at 220 K, then it was possible to accumulate four spins per P700 [24]. At the time, we attributed the four spins photoaccumulated at pH 10 to photoaccumulation of both the PsaA and PsaB branch primary and secondary electron acceptors A_0 and A_1. Photoaccumulation at pH 8 and 205 K results in the photoaccumulation of one spin, attributed to a phyllosemiquinone by biosynthetic labelling of PSI in the

cynaobacterium *Anabaena variabilis* [22]. Mutagenesis of PsaA W693H/L in *C. reinhardtii* demonstrated that the phyllosemiquinone photoaccumulated at pH 8 was that on the PsaA branch [12], and also demonstrated that the ESP signal detected in PSI arose from the germinate radical pair $P700^{\bullet+}/A_1^{\bullet-}$ on the PsaA branch of electron transfer, as observation subsequently confirmed by analysis of mutants of the conserved tryptophans on both the PsaA and PsaB binding pockets for phylloquinone in *C. reinhardtii* [30]. The results presented in Fig. 2 demonstrate that as previously reported for spinach [24], photoaccumulation at pH 10 and 220 K produces two spins additional to those photoaccumulated at pH 8 and 220 K. The proton ENDOR spectra presented in Fig. 3 and Table 1 indicate that whereas a phyllosemiquinone attributed to the PsaA branch [12] is photoaccumulated at pH 8 and 220 K, a new and additional set of hfcs are produced by photoaccumulation at pH 10, which are attributed to a second phyllosemiquinone. Fig. 4 and Table 2 indicate that this new phyllosemiquinone is unaffected by mutation of PsaA W693, supporting the assignment of this phyllosemiquinone to the PsaB branch.

These results are in contrast to some reports in the literature. A previous study of a PSI preparation obtained using the detergent Triton X-100 from a $psaE^{-}psaF^{-}$ deletion mutant of *Synechoccoccus* [26] reported that this PSI preparation was unable to photoaccumulate a phyllosemiquinone. The authors concluded that the PsaA branch of electron transfer was affected by this mutation, such that although the PsaA phylloquinone was present it was not able to form a stable phyllosemiquinone radical (perhaps being reduced to the quinol due to exposure of the binding site to the solvent). Since the PsaB phyllosemiquinone was also not being reduced by photoaccumulation, the authors concluded that electrons could not be transferred up the PsaB branch by photoaccumulation techniques. We, however, believe that photoaccumulation on the PsaB branch may be a property seen in PSI from eukaryotic sources (as we have reported for spinach [24] and here *C. reinhardtii*), but that it may not be possible to photoaccumulate on the PsaB branch in PSI from cyanobacteria. We have also recently reported results [16] that indicate that electron transfer to the PsaB phyllosemiquinone does occur at cryogenic temperatures, based upon measurements of the decay of the ESP signal at 100 K.

Whereas we have reported [12] that mutation of the conserved tryptophan on the PsaA branch (W693H/L) in *C. reinhardtii* affects both the photoaccumulated phyllosemiquinone on the PsaA branch and the ESP signal arising from the germinate radical pair $P700^{\bullet+}/A_1^{\bullet-}$, Boudreaux et al. [30] have more recently reported that although substitution of PsaA W693F in *C. reinhardtii* similarly affects the ESP signal that they were unable to photoaccumulate a phyllosemiquinone in these preparations. Although this may seem at first sight surprising since the substitution of phenylalanine for tryptophan could be viewed as a more conservative replacement than the substitution of histidine or leucine, we believe it is difficult to draw any firm conclusion since the effect of substitutions is difficult to predict without high-resolution structures of the mutant PSI. Again, the authors [30] attributed the failure to photoaccumulate a phyllosemiquinone on the PsaA branch to reduction of the quinone to the quinol, basing this conclusion on a number of observations that suggested that the A branch phylloquinone was present including the determination of PSI phylloquinone content in the PsaA W693F mutant as 80% of wild type [30].

We have looked at the differences in the proton ENDOR spectra of the two phyllosemiquinones bound on the PsaA and PsaB polypeptides (Fig. 3 and Table 1), and attempted to correlate these differences with differences in the two binding pockets revealed by the recent 2.5 Å structure of PSI from *S. elongatus* [3]. The hfcs measured for the B side phyllosemiquinone suggest that the H-bonds may be slightly longer in the B side quinone binding pocket and the unpaired electron spin density at C(2) is lower. To quantify this we can use the relationship

$$A_{\mathrm{iso}} = Q\rho$$

where Q is a constant (81 MHz) and ρ is the unpaired electron spin density at C(2) and A_{iso} is the isotropic hfc of the methyl group and

$$\mathrm{A}_{\perp} = -\rho_{\mathrm{O}}(79/r^3),\ \mathrm{A}_{\parallel} = 2\rho_{\mathrm{O}}(79/r^3)$$

where r is the O–H distance for a hydrogen bond and ρ_{O} is the unpaired electron spin density at the oxygen atom forming the H-bond. This shows that the spin density at C(2) is decreased by 17% relative to the A side $A_1^{\bullet-}$, but is still 4.7% greater than that exhibited by phyllosemiquinone in isopropanol. The H-bond distances (assuming no change in ρ_{O}) are increased by 7.5% for the B side phylloquinone. This would amount to only ~0.15 Å, probably too small to be detected in the X-ray crystal structure at its current resolution, although curiously the O–Donor distances (since one cannot observe H in the current X-ray structure) on the B side are longer by 0.07 Å in the modelled X-ray structure [3] (as compared to the environment of the A side phylloquinone). The ρ_{O} at each oxygen would have to decrease by 20% at both oxygens if the smaller H-bond hfcs were attributed solely to spin density changes. With a decreased 2-methyl hfc, there is no evidence for this amount of spin being relocated from the carbonyl oxygens on to the semiquinone ring.

Therefore, the conclusion is that there do not seem to be sufficient differences between the structures of the A side and B side phylloquinone binding pockets to account for the differences in the methyl hfcs of the A side and B side phyllosemiquinones we are observing. However, the X-ray crystal structure includes the phylloquinones in the oxidised state. Therefore, it does not allow for the effect of the negative charge on the phyllosemiquinone anion on the structures of the binding pockets. The proximity of a

conserved phenylalanine to the phylloquinone may also be a contributing factor to the observed differences in the C-2 methyl hfcs between the A and B phyllosemiquinones, since PsaB F669 in *S. elongatus* is modelled as being 0.5 Å further away from the PsaB phylloquinone 2-methyl group than PsaA F689 on the A side [3]. It is also important to note that we are reporting two H-bond hfcs for the PsaA and PsaB phyllosemiquinones in *C. reinhardtii* (Refs. [12,23] and in this paper), and two H-bond hfcs for the PsaA phyllosemiquinone in *A. variabilis* and spinach [29]. In contrast, the recent structural model at 2.5 Å [3] suggests that there is only one H-bond to the phyllosemiquinone from a leucine backbone nitrogen. We do not believe that the similarity in C-2 methyl and C-3 methylene hfcs originally reported for the PsaA phyllosemiquinone [29] could be explained if there was asymmetric H-bonding to the carbonyl oxygens of the phyllosemiquinone. In addition, the C-2 methyl hfc we are reporting here for the PsaB phyllosemiquinone is too close to that seen in vitro to be easily explained by asymmetric H-bonding to only one oxygen, or indeed two H-bonds to one carbonyl oxygen. At the moment, one possible means of reconciling these differences is to suggest that there is indeed a structural reorganisation in the pocket upon reduction of the phylloquinone, as only a small rearrangement would be needed in order to allow a serine residue (A692/B672 in *S. elongatus*) OH sidechain or the backbone NH of the phenylalanine (A689/B669) to act as H-bond donors. Another is to suggest that a water molecule not visible in the current structure is available to act as an H-bond donor.

The results reported here only indicate that in eukaryotic PSI preparations, it is possible at pH 10 and 220 K to use multiple turnovers to drive electrons up the PsaB and PsaA side of the reaction centre as far as the phylloquinone, and thus photoaccumulate the phyllosemiquinones on both branches. Although it might at first be argued, by analogy with the reaction centres of purple photosynthetic bacteria, that photoaccumulation of the PsaB phyllosemiquinone could be occurring via the PsaA branch and PsaA phyllosemiquinone, we do not believe this is the case. The electron transfer route between the quinones via the nonhaem iron and ligands found in purple photosynthetic bacteria is not present in PSI. We have preliminary evidence [31] from a PsaA M684H mutant of *C. reinhardtii* that this replacement of the axial ligand for A_o blocks electron transfer to phylloquinone on the PsaA branch. This mutation blocks photoaccumulation of the PsaA phyllosemiquinone, but not that of the phyllosemiquinone reported here. This supports the assignment of this EPR signal to the PsaB phyllosemiquinone, and indicates that it is not being photoaccumulated via the PsaA branch of electron transfer.

Since the PsaB phyllosemiquinone can only be photoaccumulated at pH 10 and 220 K, it is tempting to speculate that a pH-dependent (pH 8–10) or temperature-dependent (205–220 K) structural transition is controlling electron transfer between P700 and the A_0 on PsaB side during photoaccumulation. However, recent unpublished results in our laboratory indicate that it is possible to photoaccumulate the PsaB phyllosemiquinone either at pH 8, 220 K or at pH 10, 205 K suggesting that neither the pH 10 or 220 K requirement is absolute. Photoaccumulation depends on the competition between the light-driven reduction of the acceptor, the back reaction from the reduced acceptor, and the donation of an electron from dithionite to $P700^+$. This interplay, together with the competition between the PsaA and PsaB branches for the electron transfer from P700, means that normally the PsaB phyllosemiquinone can only be significantly photoaccumulated at pH 10 and 220 K. However, if electron transfer on the PsaA branch is significantly perturbed by site-directed mutagenesis, then it becomes possible to photoaccumulate the PsaB phyllsemiquinone either at pH 8, 220 K or at pH 10, 205 K.

The observation that it is possible to photoaccumulate the phyllosemiquinones on both putative branches of electron transfer in PSI does not have any immediate bearing on the current controversy over whether at room temperature there are two possible branches of electron transfer in the PSI reaction centre. However, the ability to monitor the PsaB phyllosemiquinone, and electron transfer to it under the photoaccumulation conditions, should prove a useful tool to investigate this controversy.

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