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Evidence for two functional phylloquinones in Photosystem I from *Chlamydomonas reinhardtii.*

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Introduction

We have recently used (Purton *et al* 2001) site directed mutagenesis of the conserved tryptophan PsaAW693 of *Chlamydomonas reinhardtii* to show that the electron transfer rate at room temperature of $t_{1/e}$ ~ 200ns for A_1 to Fe-S_X is associated with the phylloquinone bound to PsaA, and that it is the phyllosemiquinone on the A branch that is photoaccumulated by illumination at 205K in the presence of dithionite. Substitution of H/L for the tryptophan slows down the reoxidation of A_1 as monitored by the decay of the Electron Spin Polarised (ESP) signal arising from the $P700^{\bullet+}/A_1\bullet-$ geminate radical pair to ~1300ns at 260K.

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 Fe-S_X of ≈ 20ns could also be detected. 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 (Purton *et al* 2001, Guergova-Kuras *et al* 2001). Joliot and Joliot (1999) have recently developed optical techniques which allow them to measure the rate of phylloquinone oxidation in whole cells of the green alga *Chlorella sorokiniana*. They observe two rates of oxidation, $t_{1/e}$= 18 and 160 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 Fe-S_X rate. This suggestion is supported by analysis of site directed mutants of *C. reinhardtii*, PsaAW693F and PsaBW673F (Guergova-Kuras *et al* 2001). 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.

We report here the following advances in study of the phylloquinone binding sites in PSI.

1] using preparations of PSI from *C. reinhardtii* it has been possible to photoaccumulate ~ 4 spins per $P700^+$ at pH10 and 220K in the presence of dithionite, as previously reported for other preparations (Heathcote *et al* 1993). The proton Electron Nuclear Double Resonance (ENDOR) spectra of the samples reveal that photoaccumulation of a additional

phyllosemiquinone with an electronic structure different to that of the PsaA phyllosemiquinone is taking place, and analysis of site-directed mutants indicates that this is the PsaB phyllosemiquinone.

2] the reoxidation of phyllosemiquinone as monitored by the decay of the ESP signal arising from the $P700^{\bullet+}/A_1\bullet-$ geminate radical pair has been measured in site directed mutants of *C. reinhardtii*, PsaAW693F and PsaBW673F. The rates measured in the PsaA side mutants at 260K are comparable to those measured by optical techniques (Guergova-Kuras *et al* 2001). The mutations in the PsaB side do not affect the decay of the ESP signal or introduce a new kinetic phase.

3] we have measured the decay of the ESP signal at 100K, 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 (Muhuiddin *et al* 2001). We interpret the results as indicating that electron transfer from the reaction centre primary electron donor, P700, to the iron sulphur centres, Fe-$S_{X/A/B}$, can occur through either the PsaA or PsaB side phylloquinone.

Materials and methods

Site-directed mutants of *C. reinhardtii* and digitonin PSI preparations were obtained as previously described (Purton *et al* 2001, Guergova-Kuras *et al* 2001). Photoaccumulation of PSI was carried out using the method of Heathcote *et al* (1993). Proton ENDOR spectra and pulsed EPR kinetics were recorded as detailed in Evans *et al* (1999), with the kinetic measurements of forward electron transfer carried out at 260K.

Results

Photoaccumulation of a second phyllosemiquinone

Digitonin PSI preparations from wild type *C. reinhardtii* were photoaccumulated in the presence of dithionite, and the number of spins photoaccumulated quantified by double integration of the Electron Paramagnetic Resonance (EPR) signal relative to that of $P700^{\bullet+}$ in the same preparations. A maximum of one spin per $P700^{\bullet+}$ can be photoaccumulated at 205K with samples prepared at pH8, whereas two spins per $P700^{\bullet+}$ can be photoaccumulated at 220K with samples prepared at pH8, or at 205K with samples prepared at pH10. However photoaccumulation of samples prepared at pH10 at 220K yields a maximum approaching four spins as previously reported (Heathcote *et al* 1993). We had previously suggested that the photoaccumulation of 4 spins suggested that both branches of electron transfer might be operative and photoaccumulating redox components. In order to investigate this further we compared the proton ENDOR spectra of samples with two spins (pH8, 220K) and four spins (pH10, 220K), under temperature and microwave power conditions that suppress any contribution from the A_o chlorophyll anion that is photoaccumulated.

The sample with two spins yields the ENDOR spectrum (hyperfine couplings listed in Table 1) of phyllosemiquinone previously obtained from *C.reinhardtii* (Evans *et al* 1999), and attributed to the PsaA branch phylloquinone since it is altered by site-directed mutagenesis of PsaAW693H/L (Purton *et al* 2001). Subtraction of this ENDOR spectrum from that obtained from the sample with four spins gives the spectrum of a second phyllosemiquinone with different proton hyperfine couplings from the PsaA branch phyllosemiquinone (Table 1) suggesting it arises from a phyllosemiquinone in a different protein environment. If the same subtraction is carried out for PsaAW693H/L mutants then the ENDOR spectrum of the PsaA branch phyllosemiquinone is altered as previously reported (Purton *et al* 2001), but the new phyllosemquinone spectrum is unaffected.

Table 1. Hyperfine coupling constants (MHz) and assignments for the two A_1•- phyllosemiquinone radicals photoaccumulated in *C. reinhardtii.*

pH8, 220K	(-)4.5	(-)6.5	8.9	12.0	12.6
pH10-pH8 220K	(-)3.6	(-)5.0	7.1	10.5	11.6
Assignments	H-bond $A_\perp$	H-bond $A_\perp$	Methyl $A_\perp$	Methyl $A_{\|\|}$	H-bond $A_{\|\|}$

The reoxidation of phyllosemiquinone in site directed mutants of C. reinhardtii, PsaAW693F and PsaBW673F.

We have shown that at 260K in wild type *C. reinhardtii* the rate of electron transfer from A_1 to Fe-S_X is $t_{1/e}$ 350ns (Evans *et al* 1999). In the PsaAW693H/L mutants of *C. reinhardtii* the rate of decay of the spin polarised signal slows to 1.2-1.3 µs (Purton *et al* 2001). We have now made preliminary measurements of the rates in PsaAW963F, $t_{1/e}$ ~ 640ns, PsaBW673F, $t_{1/e}$ ~ 410ns, and the double mutant PsaAW693F-PsaBW673F, $t_{1/e}$ ~ 710ns. These results show that the more conservative phenylalanine substitution slows the measured rate on the PsaA branch less than the H or L substitutions, suggesting it slows rather than stops electron transfer on that branch. The PsaB mutation has no effect on the rate of transfer on the PsaA branch, and does not slow electron transfer on the PsaB branch sufficiently to allow detection by this technique (time resolution ~50ns).

Measurements of the decay of the ESP signal at 100K with F_x and A_1 reduced

A detailed examination of the effect of temperature on photosystem I electron transfer by Schlodder *et al* (1998) showed that below about 240K illumination produces irreversible charge separation in about half the reaction centres, while in the other half electron transfer from A_1 to Fe-S_X is blocked and the reversible charge transfer is observed. They concluded that two different states of the reaction centre were frozen in. If electron transfer can occur through both sides of the reaction centre and there is a large rate difference between the two sides it seems possible that the two states seen in low temperature experiments reflect centres "locked in" to electron flow on one side of the reaction centre only. In one state electrons flow irreversibly from P700 through to Fe-$S_{A/B}$ on one side, while in the other "state" electron flow from A_1 to Fe-S_X is blocked resulting in reversible reduction of A_1 on the other side. It seems possible that this might reflect the fast and slow sides of the reaction centre observed by Joliot and Joliot (1999) and Guernova-Kuras *et al* (2001). This idea can be tested by preparing samples in which the electron acceptors for irreversible charge separation, Fe-$S_{A/B}$, or the reversibly reduced A_1, Fe-S_X, are reduced. If the iron-sulphur centres are fully reduced, but the phylloquinones oxidised, both states of the reaction centre should show reversible electron transfer from P700 to A_1, possibly at different rates. Reduction of the PsaA branch phylloquinone by photoaccumulation following illumination at 205K should affect one of the proposed routes of electron transfer to A_1, and together with analysis of mutants of that quinone binding site may therefore allow the kinetic processes to be assigned to the PsaA or PsaB side of the reaction centre.

The experiments were carried out with PSI from *Synechocystis* str 6803*,* and used kinetic measurements of the decay of ESP signal arising from the $P700^{\bullet+}/A_1$•- geminate radical pair (see Table 2). In samples with the iron-sulphur centres oxidised a single rate of decay of the ESE signal from $P700^{\bullet+}/A_1^{\bullet-}$ is observed with $t_{1/e} \approx 27\mu s$. Reduction of Fe-$S_{A/B}$ does not significantly affect that rate. However reduction of Fe-S_X results in the appearance of a biphasic decay with a fast component with $t_{1/e} \approx 2.4$ µs. As more Fe-S_X is reduced by longer periods of 205K illumination the extent of the fast phase of decay increases. Equally as the extent of reduction of the PsaA side phylloquinone increases, defined by an increase in the

$A_1^{\bullet-}$ EPR signal, the extent of the slow phase of decay decreases. We interpret these results as showing that two different spin polarised radical pairs can be detected in these experiments. When the decay of the radical pair at low temperature is investigated in *C. reinhardtii* PsaAW693H/L these are affected in both the slow forward and slow decay reactions. This indicates that the "slow" quinone is on the PsaA side for both reactions, and suggest the "fast" rates reflect the PsaB side.

Table 2. Measurement of two kinetic phases of ESP decay at 100K during photoaccumulation of F_X and A_1•- at 205K in *Synechocystis* str 6803.

205K *hv* (mins)	Dark	1	5	10	20	40
$\%t_{1/e}$= 27μs	100	80	70	55	44	30
$\%t_{1/e}$= 2.4μs	0	20	30	45	56	70

Discussion

The photoaccumulation of a second phyllosemiquinone with different proton hfc's indicates that the environment of the two phyllosemiquinones on the PsaA/B polypeptides is different. This agrees with the recent report indicating that two different FTIR spectra for A_1/ A_1•- can be generated in PSI depleted of FeS centres (Hastings and Sivakumar 2001). The proton ENDOR spectra of phyllosemiquinone clearly show two H bonds to carbonyl oxygens, which taken with the close correspondence between the hfc's for methyl and β-methylene protons previously reported (Rigby et al 1996) argues against asymmetric hydrogen bonding to one carbonyl oxygen.

The rates observed for reoxidation of phyllosemiquinone in the PsaAW963F mutant are equivalent to those previously reported using optical techniques, once allowance is made for the different temperatures at which the rates were measured. We observe a slow and fast rate of decay of the ESP signal at 100K which we attribute to two different populations of the $P700^{\bullet+}$ /$A_1^{\bullet-}$ radical pairs, one involving the PsaA side phylloquinone (slow decay at 100K) and one the PsaB side phylloquinone (fast decay). We suggest these results should be reinterpreted as identifying different rates of back reaction on the two sides of the reaction center. The rates reported here are 6-8 times faster than the optically measured reoxidation of the quinone. That is probably because our experiments measure the loss of correlation of the radical pair rather than electron transport.

Results obtained with a PsaAM684H mutant of *C. reinhardtii* (Fairclough *et al*, this volume) which blocks electron transfer on the PsaA branch, support the assignment of the second phyllosemiquinone and the fast phase of decay to the PsaB branch.

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