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Evidence from time resolved studies of the $P700^{\bullet+}/A_1^{\bullet-}$ radical pair for photosynthetic electron transfer on both the PsaA and PsaB branches of the photosystem I reaction centre

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Abstract Kinetic analysis using pulsed electron paramagnetic resonance (EPR) of photosynthetic electron transfer in the photosystem I reaction centres of *Synechocystis* 6803, in wild-type *Chlamydomonas reinhardtii*, and in site directed mutants of the phylloquinone binding sites in *C. reinhardtii*, indicates 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. At low temperature reaction centres are frozen in states which allow electron transfer on one side of the reaction centre only. A fraction always donates electrons to the PsaA side quinone, the remainder to the PsaB side.

Key words: Electron transfer; Photosystem I reaction centre; *Synechocystos* 6803; *Chlamydomonas reinhardtii*

1. Introduction

The primary events of photosynthetic energy conversion occur in membrane bound protein complexes termed reaction centres. Although the basic mechanism of energy conversion is similar in all reaction centres, two types of reaction centre have been identified which differ in the redox potential range and mechanism of electron transfer from the reaction centre. Photosystem I of oxygenic organisms and the reaction centres of green sulphur bacteria and Heliobacteria (Type I) reduce a low potential ferredoxin, at −400 mV. Photosystem II of oxygenic organisms and purple bacterial reaction centres (Type II) reduce a quinone with a redox potential of around 0 mV which transfers electrons to the cytochrome complex of the electron transfer chain. High resolution structures of examples of both types of reaction centre are now available [1,2]. In both types the core of the reaction centre is a dimeric structure supporting the electron donor chlorophyll dimer, four other redox active chlorins and two quinones. The path of electron transfer in purple bacterial reaction centres is well established. Electron transfer is unidirectional using only one side of the reaction centre. The second side of the reaction centre also has a potential electron transfer pathway with the intermediary bacteriochlorophyll and bacteriopheophytin, but is not functional [3]. The mechanism which directs the electron along the active path is not known. Photosystem II functions in the same way.

Type I reaction centres are analogous to Type II centres in the reaction centre chlorin quinone part of the structure, they differ in having three iron–sulphur centres (Fe–$S_{A/B/X}$) as the terminal acceptors. Fe–S_X is suspended between the two major polypeptides (PsaA and PsaB) while Fe–$S_{A/B}$ which function as the terminal membrane bound electron acceptors are associated with a small peripheral protein (PsaC). Electrons are transferred from the quinone to Fe–S_X and then Fe–$S_{A/B}$. Although the electron transfer components of the photosystem I reaction centre are well characterised, it has been difficult to unequivocally define the route of electron transfer and the kinetic properties of the reaction centre. Experiments using either optical or electron paramagnetic resonance (EPR) measurements of $A_1^{\bullet-}$ together with biochemical or genetic modification of the PSI iron–sulphur 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 [4–6]. We have recently used site directed mutagenesis of the conserved tryptophan residue PsaAW693 to show that this rate is associated with the phylloquinone bound to PsaA [7]. It has generally been thought that by analogy with the Type II reaction centres electron transfer would be unidirectional in photosystem I, and that this route of electron transfer via PsaA would therefore be the only path of electron transfer. However it has been reported that a faster rate of electron transfer from A_1 to Fe–S_X of ≈20 ns could also be detected [8]. It was also found that mutations of 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 [7,10]. Joliot and Joliot [9] have recently developed optical techniques which allow them to measure the rate of phylloquinone 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

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Abbreviations: EPR, electron paramagnetic resonance; ESE, electron spin echo; ESP, electron spin polarisation; P700, reaction centre chlorophyll of photosystem I; A_1, phylloquinone electron carrier in photosystem I; Fe–$S_{A/B/X}$, iron–sulphur centre electron acceptors in the photosystem I reaction centre

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the A_1 to Fe–S_X rate. This suggestion is supported by analysis of the site directed mutants, PsaAW693F and PsaBW673F [10]. The fast phase of 13 ns seen in *Chlamydomonas reinhardtii* is slowed by the PsaB side mutation to 70 ns, and the 140 ns rate by the PsaA mutation to 490 ns.

At cryogenic temperatures the behaviour of photosystem I is complex. In preparations in which P700 is reduced but the acceptors oxidised, illumination results in oxidation of P700 and reduction of Fe–$S_{A/B}$. This is irreversible with $t_{1/2} = 24$ h or longer at 77 K and below. However flash illumination of samples in which this charge separation has occurred shows that electron transfer from P700 to A_1, which is reversible in the μs time scale at cryogenic temperatures, can still be observed. A detailed examination of the effect of temperature on photosystem I electron transfer by Schlodder et al. [11] showed that below about 240 K 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 [9]. 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, or 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. We have recently shown that mutation of PsaAW693 alters the electronic structure of the phylloquinone semiquinone photoaccumulated at 205 K, as monitored by proton ENDOR, indicating that the photoaccumulated phyllosemiquinone is on the PsaA side [7]. Reduction of A_1 by photoaccumulation following illumination at 205 K should affect one of the proposed routes of electron transfer to A_1, and together with analysis of mutants of the quinone binding site may therefore allow the kinetic processes to be assigned to the PsaA or PsaB side of the reaction centre.

Flash illumination of photosystem I produces the $P700^{\bullet+}/A_1^{\bullet-}$ geminate radical pair, which gives rise to an intense spin polarised EPR signal. We have used electron spin echo (ESE) kinetic measurements of the decay of this signal in samples in different redox states, in native photosystem I, and in site directed mutants of photosystem I in *C. reinhardtii*, to investigate the possibility that electron transfer from P700 to phylloquinone may occur on both sides of the reaction centre.

2. Materials and methods

C. reinhardtii strains were grown in liquid TAP medium at 25°C in dim light (< 1 E/m^2/s PAR). Cells were harvested using a Millipore Pelicon filter system. The chloroplast membrane fraction was prepared by the procedure described by Diner and Wollman [12]. The membranes were washed to remove excess sucrose and suspended in 20 mM Tris–HCl pH 8.0 containing 100 mM NaCl. Preparation and characterisation of site directed mutants of photosystem I in *C. reinhardtii* have been described elsewhere [7,13].

Synechocystis sp. 6803 was grown and membranes prepared as in [14]. EPR measurements were made on untreated thylakoid membranes at concentrations of 2–6 mg chlorophyll/ml. For kinetic measurements, samples in standard 3 mm quartz EPR tubes were reduced either with sodium ascorbate (10 mM) or sodium dithionite (0.2% w/v) for 30 min in the dark prior to freezing in the dark in liquid nitrogen. 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 205 K illumination as described previously at pH 8 or pH 10 [7].

CW EPR spectra were recorded on a JEOL RE1X spectrometer fitted with an Oxford Instruments ESR9 liquid helium cryostat. Kinetic pulsed EPR spectra were measured as described previously [4,13] using a Bruker ESP380 X-band spectrometer with a variable Q dielectric resonator (Bruker Model 1052DLQ-H 8907) fitted with an Oxford Instruments CF935 cryostat cooled with liquid nitrogen. Actinic illumination was supplied by a Nd-YAG laser (Spectra Physics DCR-11) with 10 ns pulse duration. The decay of the spin polarised 'out of phase' signal reflecting the decay of the $P700^{\bullet+}/A_1^{\bullet-}$ radical pair [7] was followed at 100 K to determine the effect of reduction of iron–sulphur centres or A_1. Forward electron flow from $A_1^{\bullet-}$ to Fe–S_X was monitored by the decay of this signal at 260 K [13]. While the decay of the electron spin polarisation (ESP) signal in less than 1 μs associated with forward electron transfer from A_1 at 260 K this clearly monitors electron transfer as it correlates with other measurement techniques. The slower rates measured at 100 K may not reflect electron transfer. The decay may reflect loss of correlation in the geminate radical pair rather than recombination of the radical pair $P700^{\bullet+}/A_1^{\bullet-}$. The relaxation ($T_1$) of $A_1^{\bullet-}$ is altered by reduction of the iron–sulphur centres [15]. However T_1 of both $P700^{\bullet+}$ and $A_1^{\bullet-}$ are in the ms time range, much slower than the rates measured here. The rates reported here do not reflect changes in T_1 or T_2. We have determined that the rates are independent of T_1 by measuring the decay using two pulse (T_2 dependent) and three pulse (T_1 dependent) electron spin echo detection of the signal with a range of tau (interpulse) times. No differences in the measured rates were observed using the different techniques. The rate is temperature dependent unlike the back reaction rates measured optically. However at temperatures below 15 K the decay rates become temperature independent and essentially the same as those measured by optical techniques [16–18]. This indicates that the two decay rates measured probably reflect properties of the two components giving rise to the two back reaction rates. These results and the parallel behaviour seen in the results presented here and similar optical experiments discussed below indicate the 100 K decay of the spin polarised signal is a useful measure of the properties of the system.

3. Results

We have shown that (at 260 K) in wild-type *C. reinhardtii* the rate of electron transfer from A_1 to Fe–S_X is $t_{1/e} \approx 350$ ns [13]. In the PsaAW693H/L mutants of *C. reinhardtii* the rate of decay of the spin polarised signal slows to 1.2–1.3 μs [7]. At low temperatures illumination of photosystem I samples re-

Table 1
Relative proportions of 27 and 2.5 μs decay phases observed in the decay of the $P700^+/A_1$ radical pair at 100 K following increasing periods of 200 K illumination

200 K illumination increment	$\%t_{1/e} = 27$ μs	$\%t_{1/e} = 2.5$ μs
Dark	100	0
1 min	80	20
5 min	70	30
10 min	55	45
20 min	44	56
40 min	30	70

See text for details.

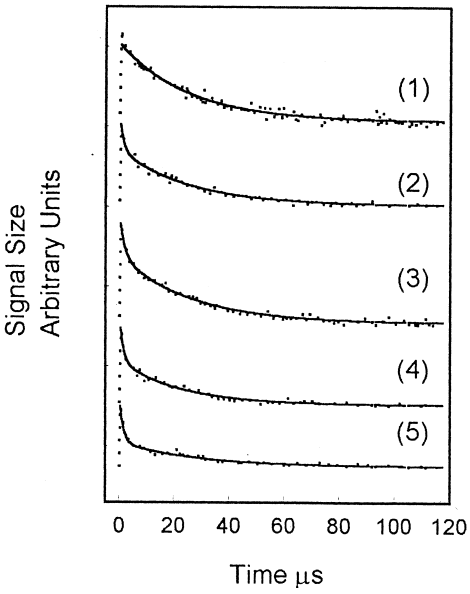

Fig. 1. The decay of the $P700^+/A_1$ radical pair at 100 K in membranes of *Synechocystis* 6803 following flash illumination. A sample reduced with sodium dithionite at pH 8.0 was illuminated for increasing time increments at 200 K before measurement. (1) Dark sample, (2) 1 min, (3) +5 min, (4) +10 min, (5) +20 min. Data were averaged for two to six experiments. Exponential fits to the data (solid lines) are single exponential $t_{1/e} = 27$ μs (1 and 2) or varying proportions (see Table 1) of a bi-exponential $t_{1/e} = 2.4$ and 27 μs (3–5).

duced with sodium ascorbate results in irreversible electron transfer from P700 to Fe–S_A in about half the centres and reversible electron transfer from P700 to A_1 in the other half. Effectively electron transfer from A_1 to Fe–S_X is blocked in half the centres. The decay of the ESP signal from the $P700^{\bullet+}/A_1^{\bullet-}$ radical pair in ascorbate reduced samples is monophasic with a rate varying slightly in preparations from different organisms of $t_{1/e} \approx 17$ μs in *C. reinhardtii* to ≈ 30 μs in *Pisum sativa* (pea). We have found that the PsaAW693H/L mutations did not significantly affect the rate of decay of the flash induced spin polarised signal seen in ascorbate reduced samples [7].

It is difficult because of the low redox potentials required and overlap of the redox potential curves to prepare samples with the iron–sulphur centres in precisely defined redox states. However by monitoring the redox state of the sample by EPR during reduction by sodium dithionite and subsequent photoaccumulation at 205 K, the effect of changes in oxidation states of the acceptor complex can be correlated with the changes in kinetics of decay of the spin polarised signal due to the $P700^{\bullet+}/A_1^{\bullet-}$ radical pair monitored by ESE. The observed redox states of the acceptor complex can be summarised as follows. Reduction with sodium dithionite at pH 8.0 partially reduces Fe–S_A/B, but not Fe–S_X. Reduction at pH 10 reduces centres Fe–$S_{A/B}$ and a small fraction of Fe–S_X. Brief (1–2 min) illumination at 205 K of dithionite reduced samples fully reduces Fe–$S_{A/B}$, a part of Fe–S_X, and a small amount of A_1. Longer periods of illumination at 205 K increase the reduction of Fe–S_X and A_1, with previous work showing that the PsaA side quinone is being reduced [7].

Fig. 1 shows the decay of the flash induced spin polarised signal in *Synechocystis* 6803 membranes reduced with sodium dithionite. In the initial measurement of the sample prepared in the dark the decay is best fitted by a single exponential with essentially the same rate as in ascorbate reduced samples with $t_{1/e} \approx 27 \pm 2.5$ μs. However following brief illumination at 205 K the decay clearly becomes biphasic and is best fit by a double exponential with $t_{1/e} \approx 2.4 \pm 0.8$ and 27 ± 2.5 μs. Initially 20–30% of the signal is in the fast phase, following

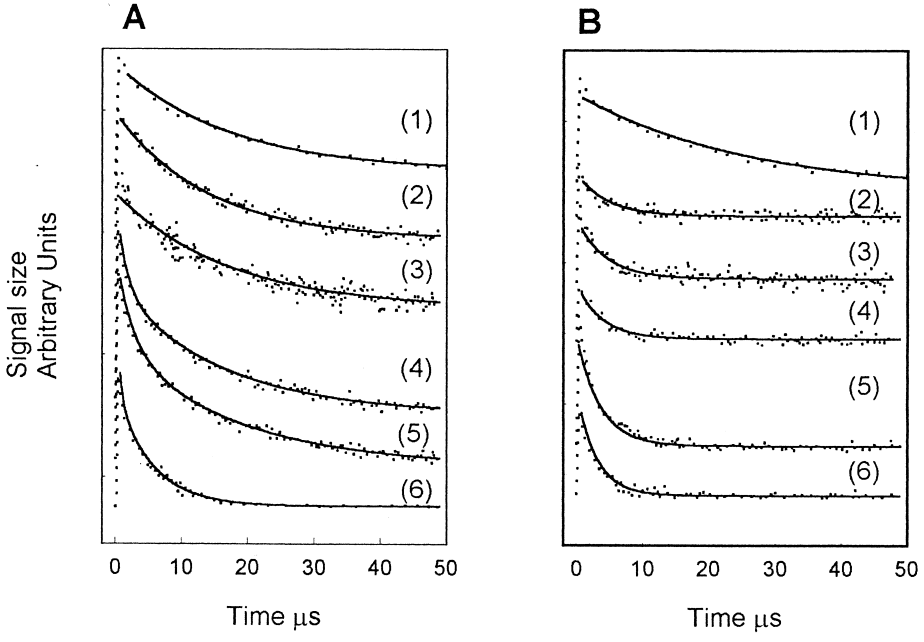

Fig. 2. The decay of the $P700^+/A_1$ radical pair at 100 K in membrane preparations of *C. reinhardtii* following flash illumination. A: Wild-type, B: PsaAW693H mutant. Samples were reduced in the dark with sodium ascorbate (1) or dithionite (2) at pH 8.0. The sodium dithionite reduced samples were then illuminated for increasing time increments at 200 K. (3) 1 min, (4) +5 min, (5) +10 min, (6) +20 min. Data were averaged from two to six experiments. Exponential fits to the data (solid lines) are A: (1–3) a single exponential $t_{1/e} = 17$ μs, (4–6) varying proportions of a bi-exponential $t_{1/e} = 17$ and 2.5 μs. **B**: (1) a single exponential $t_{1/e} = 17$ μs, (2–6) a single exponential $t_{1/e} = 4$ μs.

increasing periods of illumination at 205 K the proportions of the fast and slow phases change with the fast phase becoming dominant as the A_1 on the PsaA side is reduced by photoaccumulation (Table 1). Similar results are observed in wild-type *C. reinhardtii* samples with $t_{1/e} = 2.3 \pm 1$ and 17.7 ± 2.4 μs, and in preliminary experiments with *Synechococcus elongatus*. In samples prepared at pH 8.0 5 min illumination at 205 K was required before the fast phase was observed, with about half of the signal showing the fast decay after 35 min illumination. Samples prepared at pH 10 were more reduced initially and showed the faster phase either in the dark samples or after 1 min illumination, with the fast phase reaching 60% of the signal intensity after 15 min.

These results support the idea that two different redox chains can operate in photosystem I, one with electron transfer blocked at low temperature between A_1 and Fe–S_X, the other allowing electron transfer through to the iron–sulphur centres. When electron transfer to the iron–sulphur centres is blocked by reducing them, a spin polarised signal from $P700^{\bullet+}/A_1^{\bullet-}$ in that pathway can be detected, and the faster decay monitored.

The results obtained with the PsaAW693H mutant were quite different. Fig. 2 compares the decay of the spin polarised signal in wild-type and mutant strains of *C. reinhardtii* reduced with ascorbate and with dithionite. It is clear that while the results are the same in the mutant and wild-type in the ascorbate reduced samples with a single slow decay $t_{1/e} \approx 17$ μs, dithionite reduction of the PsaAW693H mutant results in only a fast decay being observed, $t_{1/e} \approx 4.1 \pm 0.7$ μs. The signals in dithionite reduced samples frozen in the dark are very small, brief illumination at 205 K reducing the Fe–S centres results in strong signals, but with no significant slow component. Similar results were obtained with the PsaAW693L mutant.

4. Discussion

The experiments show that in *C. reinhardtii* 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 $t_{1/e} \approx 17$ μs. Reduction of Fe–$S_{A/B}$ does not significantly affect this rate. However reduction of Fe–S_X results in the appearance of a biphasic decay with a fast component with $t_{1/e} \approx 3$ μs. As more Fe–S_X is reduced by longer periods of 205 K 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.

We have previously shown that the 'slow' forward reaction is affected by mutation of PsaAW693 to histidine or leucine [7]. When the decay of the radical pair at low temperature is investigated in mutants of PsaAW693 the H and L mutants show clear results. While in ascorbate reduced samples the slow decay is observed in PsaAW693H/L, confirming that the 'slow' quinone is present, reduction with dithionite results in loss of the slow decay, while the fast decay is still observed. In the mutants both the slow forward electron transfer and the properties of the quinone involved in the slow decay reaction are altered. This indicates that the 'slow' quinone is on the PsaA side for both reactions. We suggest that substitutions of the tryptophan are affecting the properties of the phylloquinone and making it more accessible to the environment. On addition of dithionite the PsaA side is apparently inactive in formation of a spin polarised radical pair, as shown by the lack of a significant ESP signal at 100 K until Fe–S_X is reduced by photoaccumulation. The B side phylloquinone can then be monitored as a fast decay.

The results confirm that the reduced quinone photoaccumulated by illumination at 205 K is the PsaA side quinone. This might be expected if the back reaction from A_1 to P700 is significantly slower on that side. This result is in agreement with our previous ENDOR spectroscopic data [7], and the results of Zybailov et al. [19]. The results show that the 'slow' reaction rates of A_1 both reflect the PsaA side quinone, and suggest the 'fast' rates reflect the PsaB side (Fig. 2B).

The results can be reconciled with the observation that on freezing photosystem I to cryogenic temperatures two different behaviours are observed on illumination. These observations have been interpreted as showing that the centres are frozen in two different states. In the light of our results, and work on the rate of forward electron transfer at room temperature which appears to show two rates of oxidation of $A_1^{\bullet-}$, and which are interpreted as showing electron transfer through both the PsaA and PsaB side quinones, we suggest that the two states are reaction centres in which electron transfer is locked into either the PsaA or PsaB side with two different paths of electron transfer present in the preparations.

We observe a slow and fast rate of decay of the ESP signal at 100 K 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 100 K) and one the PsaB side phylloquinone (fast decay). These results are similar to those reported by Mathis and coworkers who observed a slow back reaction with Fe–$S_{A/B}$ reduced and a faster rate with Fe–S_X also reduced. They ascribed the increased rate to electrostatic effects of the reduced iron–sulphur centres [16,17]. Brettel and Golbeck [18] however observed the two rates of decay of the radical pair in samples with the iron–sulphur centres removed. We suggest that these results should be reinterpreted as identifying different rates of back reaction on the two sides of the reaction centre. The rates reported here are six to eight 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. The qualitative similarities of the two measurements suggest that both identify two different radical pairs under similar experimental conditions.

These experiments identify the two states at low temperature as reflecting reaction centres frozen in a state which favours electron transfer on either the PsaA or PsaB sides of the reaction centre. There is no evidence to suggest what controls the conformation which is frozen in. It is unclear whether the bidirectional electron transfer observed at room temperature reflects chance distribution to equally probable processes, or shows that the reaction centres are always in one of the two conformations.

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