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Replacement of the methionine axial ligand to the primary electron acceptor A_0 slows the A_0^- reoxidation dynamics in Photosystem I

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Abstract

The recent crystal structure of photosystem I (PSI) from *Thermosynechococcus elongatus* shows two nearly symmetric branches of electron transfer cofactors including the primary electron donor, P_{700}, and a sequence of electron acceptors, A, A_0 and A_1, bound to the PsaA and PsaB heterodimer. The central magnesium atoms of each of the putative primary electron acceptor chlorophylls, A_0, are unusually coordinated by the sulfur atom of methionine 688 of PsaA and 668 of PsaB, respectively. We [Ramesh et al. (2004a) *Biochemistry* 43:1369–1375] have shown that the replacement of either methionine with histidine in the PSI of the unicellular green alga *Chlamydomonas reinhardtii* resulted in accumulation of A_0^- (in 300-ps time scale), suggesting that both the PsaA and PsaB branches are active. This is in contrast to cyanobacterial PSI where studies with methionine-to-leucine mutants show that electron transfer occurs predominantly along the PsaA branch. In this contribution we report that the change of methionine to either leucine or serine leads to a similar accumulation of A_0^- on both the PsaA and the PsaB branch of PSI from *C. reinhardtii,* as we reported earlier for histidine mutants. More importantly, we further demonstrate that for all the mutants under study, accumulation of A_0^- is transient, and that reoxidation of A_0^- occurs within 1–2 ns, two orders of magnitude slower than in wild type PSI, most likely via slow electron transfer to A_1. This illustrates an indispensable role of methionine as an axial ligand to the primary acceptor A_0 in optimizing the rate of charge stabilization in PSI. A simple energetic model for this reaction is proposed. Our findings support the model of equivalent electron transfer along both cofactor branches in Photosystem I.

Keywords: A_0 mutant; Femtosecond spectroscopy; Energy transfer; Electron transfer; Photosystem I; *Chlamydomonas reinhardtii*

1. Introduction

Photosystem I (PSI) is a multi-subunit pigment–protein complex embedded in the thylakoid membranes of photosynthetic cyanobacteria, algae and higher plants. It acts as a light-driven oxidoreductase that catalyzes the light-induced electron transfer from plastocyanin/cytochrome c_6 on the lumenal side of the membrane, to ferredoxin or flavodoxin on the stromal side of the membrane [1,2]. The reaction center of PSI is a heterodimer of the PsaA and PsaB proteins which contain the redox components involved in the first steps of photoinduced electron transfer. In PSI, photoexcitation of P700 initiates sequential electron transfer (ET) through two spectroscopically identified electron acceptors, A_0, a Chl *a* molecule unusually coordinated by a methionine residue and A_1, a phylloquinone. A recent work by Holzwarth et al. [3] has suggested that the initial charge separation originates at the accessory chlorophylls, instead of P700. From A_1, the electron is transferred to the [4Fe–4Fs] cluster F_X, and further to F_A and F_B, two iron–sulfur clusters which then transfers the electrons to the ferredoxin. The X-ray crystal structure of PSI from *Thermosynechococcus elongatus* at 2.5-Å resolution [4] shows that the

Abbreviations: A, accessory chlorophyll in Photosystem I; A_0, primary electron acceptor in photosystem I (chlorophyll *a* monomer); A_1, phylloquinone secondary electron acceptor in photosystem I; Chls, chlorophylls; DAS, decay associated spectra; ETC, electron transport chain; F_A, F_B and F_X, iron sulfur centers of photosystem I; ND, non-decaying; PMS, phenazine methosulphate; PSI, photosystem I; P_{700}, primary electron donor of photosystem I

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electron transfer chain is symmetrically arranged with respect to the axis perpendicular to the membrane plane and has allowed identification of amino acid residues interacting with the electron transfer cofactors, permitting the use of site-directed mutagenesis to explore the electron transfer pathway in PSI. Structural and functional evidence has pointed toward the possibility that the ET takes place in both branches (PsaA and PsaB) of cofactors. Even though mounting evidence for bi-directional electron transfer in PSI is available [5–11], there is a general agreement that the major fraction of electron transfer occurs in the PsaA branch [5–18]. The involvement of PsaB branch in some organisms, particularly cyanobacteria is still under debate [14–18]. Most of the evidence for one or two active branches has primarily come from studies involving specific mutations around the respective phylloquinone binding sites on the PsaA and PsaB polypeptides [5,10,12,13,15,23]. Attention has now been focused to the cofactors immediately upstream of the phylloquinones, i.e., the A_0 Chls (ec3 chlorophylls) that are the primary electron acceptors of PSI (Fig. 1). The axial ligands to eC-A3 and eC-B3 are Met 688 of PsaA and Met 668 of PsaB, respectively [4]. In *C. reinhardtii*, the respective axial ligands are Met 684 and Met 664. Recently, we constructed site-directed mutants of *C. reinhardtii* in which the axial ligands to chlorophylls eC-A3 and eC-B3 were changed to His, and our ultrafast pump-probe data showed that both mutations block or slow electron transfer from A_0^- to A_1 [11]. Pulsed EPR data from the similar mutants of *C. reinhardtii* [7] also implied the use of both branches. Similarly, in the same His mutants, Santabarbara and co-workers [8] showed evidence for bidirectional electron transfer using ESEEM. In contrast, EPR [16] and optical ultrafast spectroscopy [19] data from site-directed mutants of the cyanobacterium *Synechocystis* sp. PCC 6803, in which the axial methionine ligands to chlorophylls eC-A3 and eC-B3 were changed to Leu, showed that the electron transfer occurred primarily along the PsaA-side branch. Without concomitant structural evidence, kinetic data is open to multiple interpretations. It may be possible that some of the controversy concerning the directionality of electron transfer in PSI originates from the use of different spectroscopic techniques, different organisms, and, in some cases different choices of axial ligand substitution [5–11,14–18].

Therefore, to further address the question of whether different choices of axial ligand substitution show different proportions of electron transfer on the two branches, we have probed the electron transfer activity and A_0 reoxidation dynamics in six different mutants of *C. reinhardtii* in which methionine axial ligand to Chl A_0 has been changed to His, Leu and Ser on both the PsaA and PsaB branches. Using fast optical spectroscopy we show that the mutation of either Met axial ligand to any of the alternate amino acids, always significantly slows reoxidation of A_0^-.

2. Materials and methods

2.1. Site-directed mutagenesis and transformation of chloroplasts

Oligonucleotide-mediated site-directed mutagenesis was used to introduce mutations into the *psaA* or *psaB* gene. The Met A684 and Met B664 axial ligands to primary electron acceptor A_0 (in *C. reinhardtii*) were replaced with leucine and serine using protocols essentially as described previously [20–22]. Chloroplast transformation was performed by the bio-ballistics technique [23,24]. Bombarded cells were transferred to plates containing CC media [21] supplemented with 100 μg mL^{-1} spectinomycin and 1.2% agar and placed under dim light for 7–10 days until colonies appeared. Single transformant colonies were re-streaked onto solid medium. Total DNA was isolated from cells taken from confluent regions of the plates as previously described and resuspended at a final volume of 100 μL. One microliter of this DNA isolation was then used as a template for PCR to confirm the introduction or loss of a specific restriction enzyme site. To confirm the presence of the desired mutation the amplified DNA from the homoplasmic strains was sequenced.

2.2. Strains and media

The *Chlamydomonas reinhardtii* recipient strains for transformation were CC125 (wild-type mt^+) and CC2696 obtained from the *Chlamydomonas* Culture Collection at Duke University. CC125, CC2696 strains and their transformants were maintained on agar plates containing CC medium [21]. For

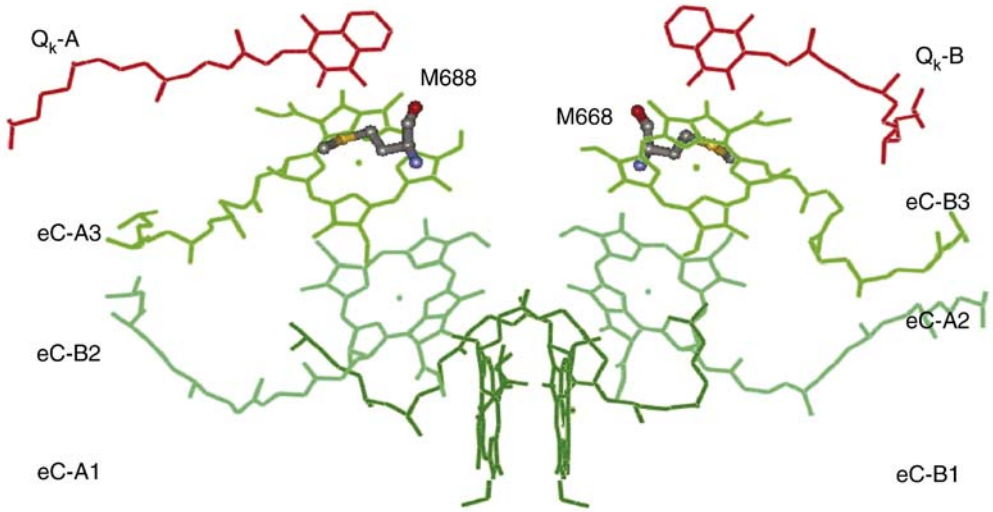

Fig. 1. The chlorophyll cofactors eC-A1, eC-B2, eC-A3, and eC-B1, eC-A2, and ec-B3 of the A and B-branch of the ETC in *Thermosynechococcus elongatus*. The primary electron acceptors eC-A3 and eC-B3 are axially liganded by Met A688 and Met 668, respectively.

thylakoid membrane preparation, cells were grown in CC liquid medium to 1–1.5×10^{-6} cells mL^{-1} at a light intensity of 25–40 μmol m^{-2} s^{-1}.

2.3. Isolation of PSI complex

The isolation of PSI complexes was carried out according to the procedure of Ramesh et al. [11] and Krabben et al. [25].

2.4. Femtosecond transient absorption measurements

Femtosecond transient absorption measurements were performed as described earlier [26–28]. For experiments at room temperature, the primary donor was kept neutral during repetitive excitation by addition of 20 mM sodium ascorbate and 10 μM phenazine methosulfate (PMS). Then, the sample was placed in a glass wheel rotating with a rotation frequency of ~2 Hz so that each laser flash excited a fresh sample. The OD of the sample was typically between 1 and 1.2 at 676 nm. Spectrally narrow (fwhm of ~5 nm) laser pulses with a ~150 fs duration at 695 nm were used to excite the sample with repetition rate of 1 kHz. The polarization of the pump and pulsed femtosecond white probe beams was set at the magic angle with respect to each other. Transient absorbance spectra in the region between 630 and 750 nm were collected on two time scales: from −10 to 400 ps (with a step size of 5 and 100 ps from −10 to 15 ps and from 15 to 400 ps, respectively) and from −400 to 4000 ps (with a step size of 80 ps). The excitation intensities were kept on a level of <1 and 2–3 photons per RC for the former and latter time scale, respectively.

Reducing conditions, aiming at reduction of phylloquinones, were obtained by combination of illumination and by suspending the sample in 200 mM glycine buffer, pH=11, containing 20 mM sodium ascorbate, 10 μM phenazine methosulfate (PMS), and 30 mM sodium dithionite added to the sample after degassing [29]. Chemical oxidation of the primary donor was realized by addition of 300 mM potassium ferricyanide to the sample.

Decay-associated spectra (DAS) were calculated from global fitting, accounting for deconvolution of the recorded signals with instrument response function using locally written software (ASUFIT, http://www.publicasu.edu/laserweb/asufit/asufit.html).

At 20 K, the concentrations of reducing chemicals (sodium ascorbate and PMS) were doubled. Then, the sample was mixed with glycerol (1: 2 v/v), centrifuged in a vacuum to remove any dissolved air, which disturbs homogenous freezing of the sample, and placed in a thin cuvette (~1 mm thick). The final OD of the sample was between 1 and 1.2 at 676 nm. The cuvette was placed in the cryostat and frozen to 20 K in darkness. Excitation energies were kept on the low level of <1 photon per RC. For excitation, 5-nm wide (fwhm) ~150-fs pulses centered at 700 nm were applied. The transient spectra were measured between 630 and 750 nm with a step size of 54 fs from 1 ps before to 2 ps after the excitation pulse.

3. Results

3.1. Detection of A_0^- in different mutants of C. reinhardtii under neutral conditions

Fig. 2 shows absorbance difference spectra recorded under neutral conditions at 200–300 ps after excitation at 695 nm, when virtually all excited states of the antenna are already trapped by the RC, for PSI preparations from wild type and four mutants (ML(A684) (methionine (M) 684 of PsaA replaced to

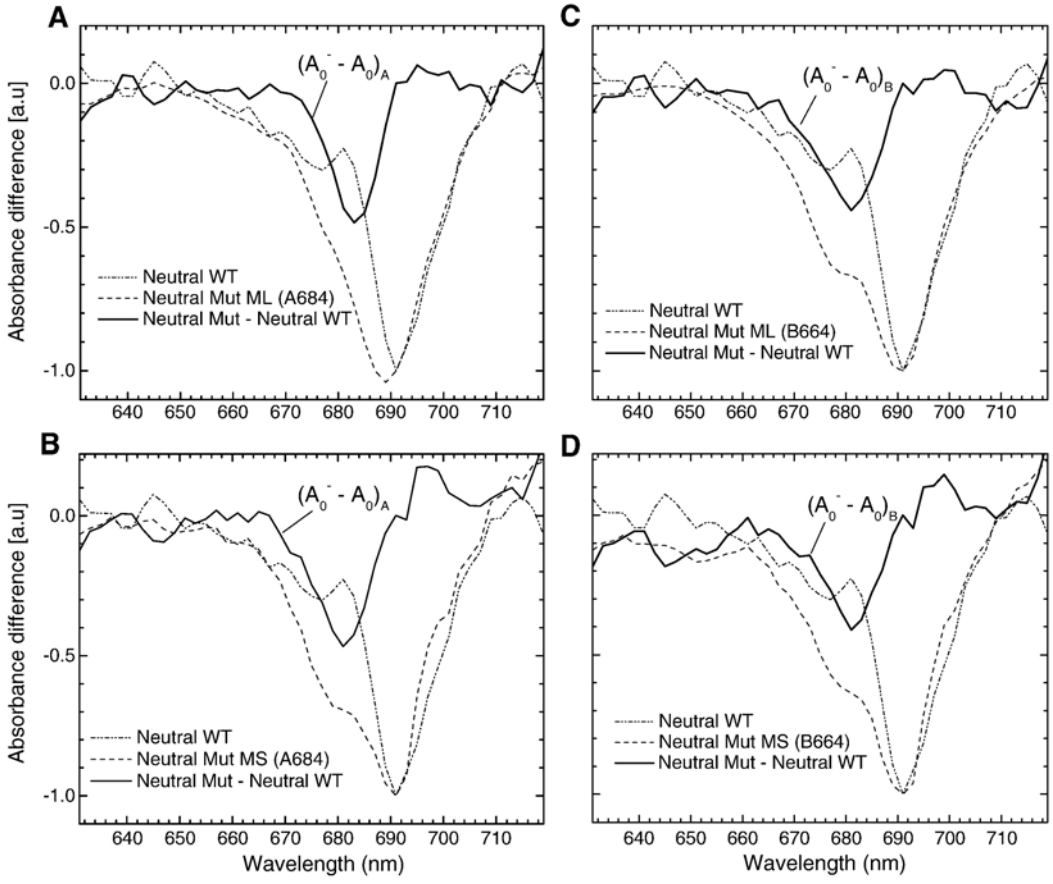

Fig. 2. Accumulation of ($A_0^- - A_0$) absorbance difference signal in the ML(A684) (A), MS(A684) (B), ML(B664) (C), and MS(B664) (D) mutant PSI from *C. reinhardtii* at room temperature under neutral conditions. (The spectra presented were recorded 200–300 ps after ~150 fs excitation at 695 nm.)

leucine (L), MS(A684) (methionine (M) 684 of PsaA replaced to serine (S), ML(B664) (methionine (M) 664 of PsaB replaced to leucine (L) and MS(B664) (methionine (M) 664 of PsaB replaced to serine (S)). Interestingly, the shapes of the spectra from all the four mutants are different from wild type in the ~682 nm region, a result also found for the MH(A684) (methionine (M) 684 of PsaA replaced to histidine (H) and MH (B664) (methionine (M) 664 of PsaB replaced to histidine (H) mutants (11). The $\Delta\Delta A$ signals resulting from subtraction of the wild-type trace from the mutant traces (after normalization of both traces as described in [11]) are shown as solid lines and represent the $(A_0^- - A_0)$ signal.

The PSI preparations from the ML(A684) and ML(B664) mutants were treated with sodium dithionite under anaerobic conditions at pH=11 in combination with illumination to completely reduce A_1 and block electron transfer from A_0^- to A_1 [11,29]. The ΔA signals recorded under these reducing conditions 200–300 ps after excitation (Fig. 3) are significantly different from the corresponding signals recorded under neutral conditions (Fig. 2C, D). As seen from subtraction of the wild type neutral signal from the mutants reduced signals (Fig. 3) the resulting $\Delta\Delta A$ spectra are similar in shape to the corresponding signals obtained under neutral conditions (Fig. 2C, D) but their amplitudes are roughly twice bigger, which demonstrates accumulation of A_0^- in both branches.

3.2. Decay of accumulated A_0^- under neutral conditions

To determine whether the electron transfer is completely blocked at the level of A_0 or significantly slowed down, transient absorption spectra were recorded for wild type and all the six mutants under neutral conditions, on a longer time scale of 4000 ps after excitation at 695 nm and the data fitted by a global analysis. In wild type, the best fit was obtained for a one exponential component with a lifetime of ~30 ps and a nondecaying component with maximum bleaching at 691 (693) nm that represents the (P_{700^+}–P_{700}) spectrum [11,26,29,30]. The DAS of the fast component with a lifetime of 30 ps has a negative peak at 683 (685) nm and represents the decay of excited PSI core antenna Chls (data not shown). In the mutants, three components were found: a fast ~30-ps DAS (decay of excited state; not shown), the nanosecond component which varies between 1100 and 1700 ps depending on the specific axial ligand substitution (Table 1),and a non-decaying component (P_{700^+}–P_{700}) (Fig. 4). The slow component that decays in ~1–2 ns is attributed to A_0^- reoxidation in the mutated branch, since it has a spectrum identical to $(A_0^- - A_0)$ with a maximum bleaching at 681 nm (Figs. 2 and 3) [11,29,30]. In the His and Leu mutants (MH(A684), MH(B664), ML(A684) and ML(B664)), the $(A_0^- - A_0)$ spectrum decays in approximately 1700±150 ps. In the Ser mutants (MS(A684) and (MS(B664)) this component decays in ~1100±150 ps, somewhat faster than the other mutants. Fig. 5 compares the kinetics at 681 nm observed in the wild type and one of the mutants, (MS(A684)), to demonstrate the signal to noise ratio of our kinetic results.

Table 1
The lifetimes of nanosecond component in all the mutants under study

Mutants	Life time of nanosecond component
MH(A684)	1760±150 ps
MH(B664)	1780±150 ps
ML(A684)	1770±150 ps
ML(B664)	1700±150 ps
MS(A684)	1100±150 ps
MS(B664)	1150±150 ps

To exclude the possibility that the 1–2 ns component and double difference spectra in Figs. 2 and 3 come from excited Chls which are not quenched by photochemical reactions of electron transfer, we performed two types of control measurements. First, we measured transient ΔA spectra on a time scale of 4000 ps under reducing conditions (A_1 pre-reduced). The global analysis of these data for ML(A684) and ML(B664) resolved only one exponential component with a lifetime of ~30 ps and a non-decaying component due to accumulation of

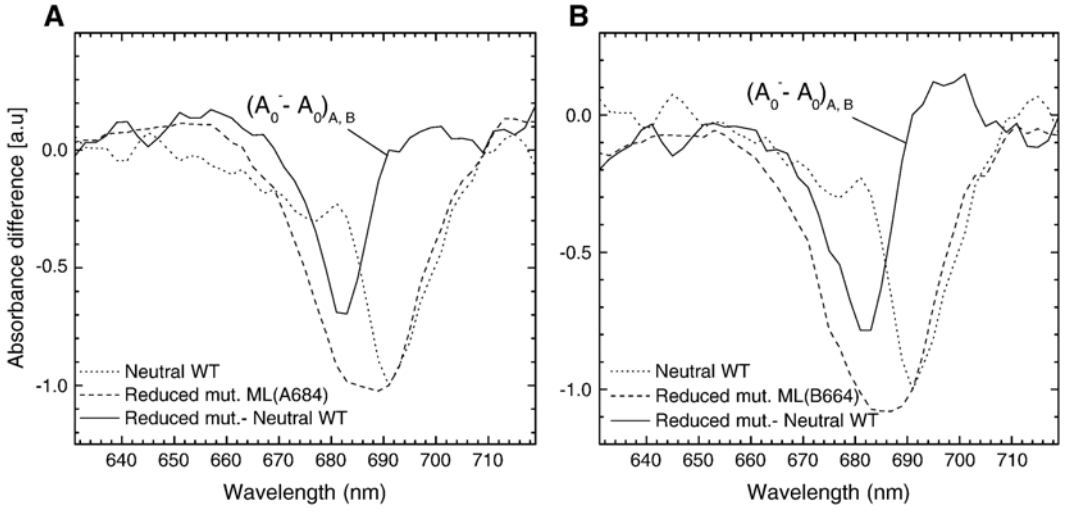

Fig. 3. Absorption difference spectrum of ML (A684) (A), and ML(B664) (B) under reducing conditions, recorded 200–300 ps after ~150-fs excitation at 695 nm at room temperature.

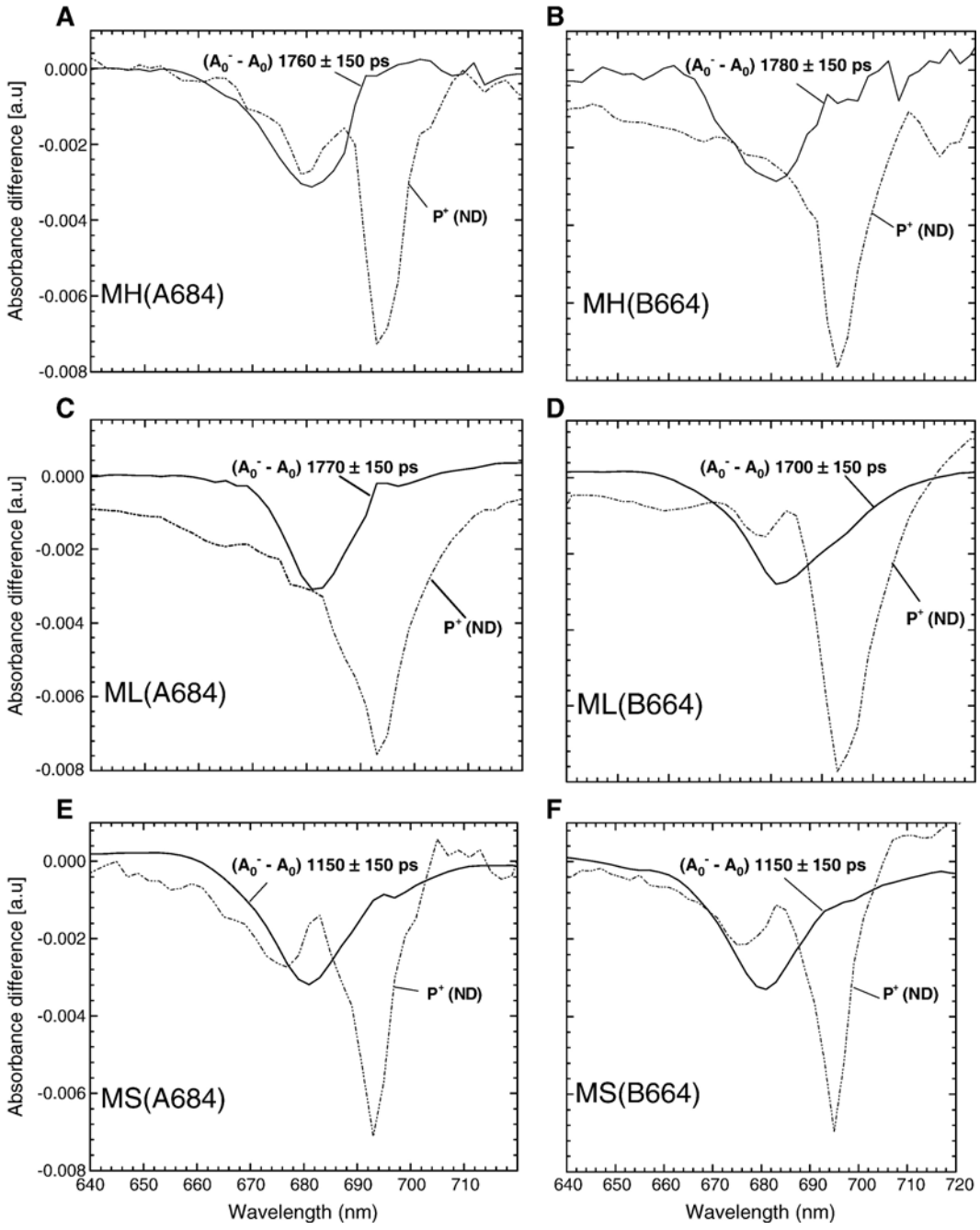

Fig. 4. Decay associated spectra (1100–1780 ps, solid) and nondecaying (dashed) components obtained by global analysis of data measured under neutral condition in PSI from six mutants of *C. reinhardtii*. Data were collected at room temperature on a 4000 ps time scale, using high intensity excitation pulses at 695 nm.

$P_{700}{}^{+}A_0{}^{-}$ (data not shown), very similar in shape to the signals obtained from reduced mutant samples measured at 300 ps after excitation (Fig. 3). No significant 1–2 ns component that could possibly be attributable to Chls uncoupled from the ETC was observed under these conditions. Similar experiments were also performed under oxidizing conditions where the primary donor was chemically preoxidized. Under these conditions, in addition to the ~30-ps trapping component, there was only a minor contribution (~10%) of a ND component (on the time scale of few hundreds of ps) possibly originating from uncoupled Chls, in both wild type and mutants (Fig. 6) and, therefore, virtually not represented in the double difference spectra shown in Figs. 2 and 3.

3.3. Excitonic coupling

Transient absorption measurements on a very early time scale (up to 2 ps after excitation) were performed at 20 K for PSI from wild type and leucine and serine mutants as described by Gibasiewicz et al. [27,31].

In the transient spectrum recorded ~300 fs after excitation at 700 nm, there are four negative bands (peaking at 667, 675,

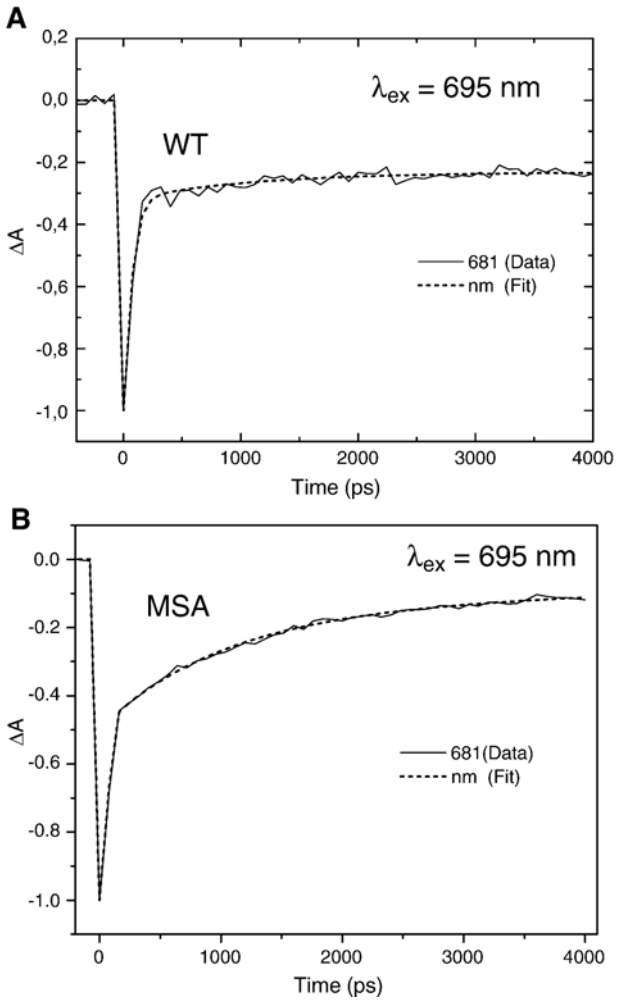

Fig. 5. Kinetics of the absorption changes observed at 681 nm following 695-nm excitation of PSI from wild type (A) and MS (A684) mutant (B) of *C. reinhardtii* at room temperature. The wild type trace was globally fitted with a 30-ps and a non-decaying component, and the mutant's trace was fitted with a 30-ps, 1100-ps and a non-decaying component.

683–685, and 695 nm) and four positive bands (peaking at 635, 645, 653 and 661 nm) in the wild type PSI. These bands have been reported to arise from excitonic interaction between at least 8 Chls: the 6 ETC Chls and two connecting chlorophylls [31]. Absorbance changes for all the four mutants are similar to each other, but significantly different from the wild type (Fig. 7). For the mutants, the amplitude of all the shorter-wavelengths bands drops about twice relative to the band at ~695 nm, similar to results reported previously for the His and B-side Ser and Leu mutants.

4. Discussion

As we reported earlier, substitution of the methionine with leucine on both PsaA and PsaB branches severely impaired the photoautotrophic growth of *C. reinhardtii* [11]. In contrast, replacement of methionine to leucine affects the photoautotrophic growth and forward electron transfer only on the PsaA branch in cyanobacterium *Synechocystis sp*. [16,19]. Because of the conflicting results from different species, we have carried out a more detailed spectroscopic characterization of leucine and serine mutants of PSI from *C. reinhardtii*.

To determine whether the PsaA or PsaB branch is active in forward electron transfer in PSI from leucine and serine mutants, we have measured absorbance difference spectrum a few hundreds of picoseconds after excitation, when excitation energy is already trapped by the RC. At this delay time, the ΔA signal for the wild type contains contribution exclusively from (P_{700^+}–P_{700}) [26,29,30]. Fig. 2 demonstrates a significant difference between the ΔA spectrum from wild type and the

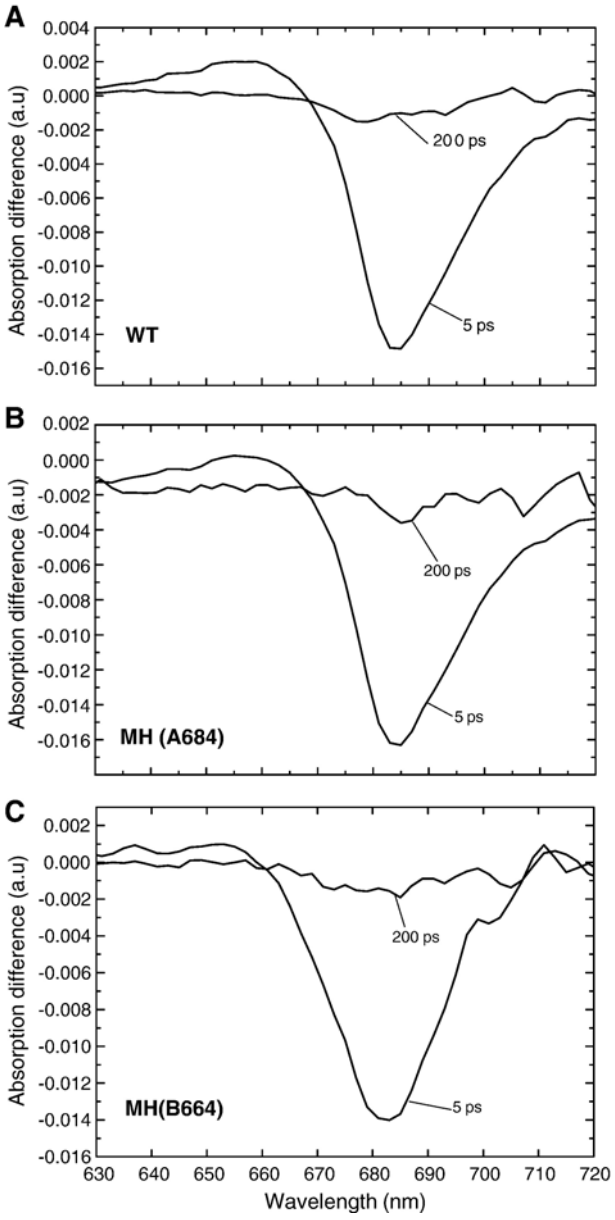

Fig. 6. Absorption difference spectrum of wild type (A), MH (A684) (B), and MH(B664) (C) under oxidized conditions, recorded 200–300 ps after ~150-fs excitation at 695 nm at room temperature. The 5 ps component is due to antenna excitation. Note that the amplitude of the 200 ps component from the mutants is same as in wild type (wild type) indicating that the subtracted traces "$A_0^- - A_0$" (Fig. 2) do not contain contribution from uncoupled Chls.

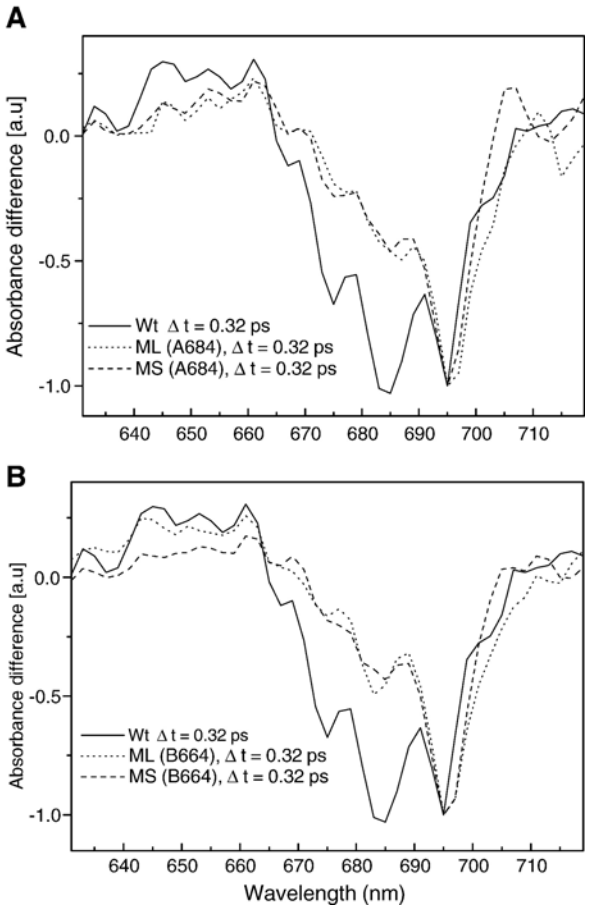

Fig. 7. 20-K absorbance difference spectra of wild type and four different A_0 (branch A and B) PSI mutants measured 0.32 ps after excitation at 700 nm and normalized to the same amplitude at 695 (697) nm. The difference between mutants and wild type demonstrates the loss of excitonic coupling between Chls in the respective mutated branches A and B.

mutants. The shape of the ΔA signal for the wild type agrees well with the shape of (P_{700^+}–P_{700}) from the literature [26,29,30]. The signals for the mutants are significantly different. As in our previous work [11], we have assumed that the (P_{700^+}–P_{700}) ΔA spectrum was the same for the mutant and wild type and subtracted the latter one from the ΔA spectrum measured for the mutants. We are aware that the real (P_{700^+}–P_{700}) ΔA spectrum may be somewhat different in WT and mutants but to a first approximation we neglect this effect. The extra photobleaching at ~682 nm in the mutants was attributed to A_0^- accumulated in the mutated branch in analogy to His mutants (Fig. 3 in [11]). Apparently, under neutral conditions, all four mutants accumulate A_0^- in the mutated branch to a similar extent.

As a control experiment, for each mutant we have found the ($A_0^- - A_0$) ΔA spectrum as a difference between ΔA measured under reducing conditions (A_1 is chemically pre-reduced and electron is trapped on A_0) and ΔA measured from wild type under neutral conditions. Under neutral conditions the wild type spectrum contains a contribution exclusively from the (P_{700^+}–P_{700}) signal, whereas the spectra from the ML(A684) and ML (B664) mutants under reducing conditions contain an additional contribution from the ($A_0^- - A_0$) signal (Fig. 3). Thus, the difference between these two traces is ascribed to the ($A_0^- - A_0$) difference spectrum. The similarity in the shape of the $\Delta\Delta A$ signal obtained from all four mutants under both neutral and reducing conditions confirms that under neutral conditions A_0^- is accumulated in the mutant. The results strongly support a model for PSI in which electron transfer can occur down either the PsaA or the PsaB branch. It should be emphasized that the shape of the $\Delta\Delta A$ spectrum assigned to ($A_0^- - A_0$) in the mutants agrees well with those measured for wild type PSI from *C. reinhardtii* [11,29,30].

In order to determine whether the electron transfer from A_0^- is blocked or slowed down, the transient absorption experiment was repeated for all the preparations on a longer time scale of 4000 ps. Global analysis of data from mutants enabled us to resolve a component that decays with a lifetime between 1100±150 ps and 1700±150 ps, and which has a spectrum identical to ($A_0^- - A_0$) (Fig. 4; compare to Fig. 2) [11,26,30]. This indicates that electron transfer from A_0^- to A_1 is dramatically slowed from approximately 20 ps (wild type) to ~1100±150 ps in the Ser mutants and 1700±150 ps in the His and Leu mutants. Salikhov and co-workers [17] also reported that the lifetime of the $P_{700^+}A_0(A)^-$ radical pair was lengthened to about 1 ns in a site directed mutant of *Synchocystis* sp. in which the natural methionine axial ligand to A_0 was substituted with an alanine. It is interesting to note that independently of the character of the amino acid replacing the methionine (basic histidine, non-polar leucine or polar serine), the effect of the mutation on the reoxidation of A_0 is large. This demonstrates an exceptional role of methionine in maintaining the proper energetics and kinetics of the primary electron transfer reactions in PSI. Finally, it was excluded that the 1–2 ns component originates from excited states of Chls energetically uncoupled from the RC. This conclusion was based on the observation that the 1–2 ns phase is absent under reducing conditions in both leucine mutants when the lifetime of the $P_{700^+}A_0^-$ state is extended to a few tens of ns due to a block in forward electron transfer. The 1–2 ns phase should have been observed independently of the redox states of A_1, if it originated from uncoupled Chls. It was also excluded that the double difference spectra in Figs. 2 and 3 originated from uncoupled Chls based on the experiment under oxidizing conditions, which demonstrated a negligible contribution from long-lived excited states of Chls.

In the wild type PSI, the initial ΔA bands peaking at ~675 and ~685 nm measured ~300 fs after excitation (Fig. 7) were proposed to be associated with excitonic interaction between the A and A_0 Chls and between the A_0 Chls and connecting Chls, respectively, in both branches [31]. On the other hand, the 695-nm band is contributed largely by a species that is independent of the excitonically coupled system of Chls [31]. In the case of A_0 mutants, the amplitudes of the 675 and 683 nm bands drops about twice relative to the 695 nm bands and compared to the wild type PSI (Fig. 7). This effect was previously observed and interpreted for the His and B-side Ser and Leu mutants [11,31]: a mutation-induced change in the position/orientation of the primary acceptor in either of two branches weakens interactions of the A_0 Chls with their closest neighbors, the accessory Chls,

A, and connecting Chls. Apparently, the same situation occurs for all the other mutants under study. Thus, the observed change in the shape of the early transient spectra is indicative for modification of A_0 position in the mutants.

4.1. Kinetics and energetics of A_0^- reoxidation

The experimentally observed reoxidation of A_0^- in the mutants, within 1–2 ns, is two orders of magnitude slower than in wild type (~20 ps) [32–34]. The most straightforward explanation for this effect is a large drop of the electron transfer rate from A_0^- to A_1. Moser and Dutton proposed a simple relation between the electron transfer rate, k_{et}, the distance between the electron donor and acceptor, R, the standard free energy difference between the final and initial redox states, ΔG^0, and the reorganization energy of the medium, λ, (log $k_{et}=15-0.6R-3.1(\Delta G^0+\lambda)^2/\lambda$, with k_{et} — in s^{-1}, R — in Å, ΔG^0 and λ — in eV [35]). From this expression one can see that electron transfer rate may be lowered both by change in ΔG^0, λ, and by increase of the distance R. In first approximation we assumed that the mutations of the A_0 ligands change exclusively ΔG^0 between $P^+A_0^-$ and $P^+A_1^-$ by shifting redox midpoint potential (E_m) of A_0^-/A_0. Calculations based on the Moser–Dutton expression show that ΔG^0 of $P^+A_0^-$ shifts ±430 meV in the mutants relative to WT (to account for 1 ns electron transfer rate). Since the methionine ligand was proposed to be responsible for the very negative E_m value for A_0^-/A_0 [36], any mutation of this amino acid is expected to increase this value and therefore we will limit our discussion to this case, although the opposite case cannot be completely ruled out. In consequence, the energy level of $P^+A_0^-$ is ~80 meV below that of $P^+A_1^-$ (Fig. 8B). But such a big shift of ΔG^0 of $P^+A_0^-$ is very unlikely, because the Boltzman distribution would then strongly

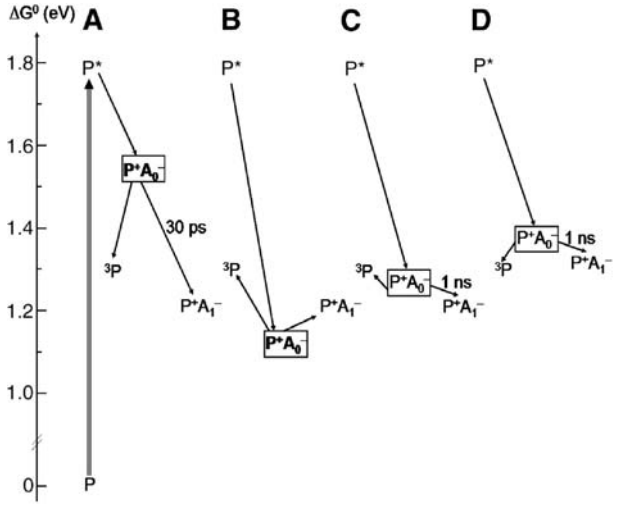

Fig. 8. Standard free energy levels for the PSI reaction center in the ground, excited, and two charge separated states in the wild type (A) and mutants (B–D). The energy level of $P^+A_0^-$ in the mutants was calculated assuming that mutation affects exclusively $\Delta G^0(P^+A_0^-)$ (B), both $\Delta G^0(P^+A_0^-)$ and distance between A_0 and A_1 (C), $\Delta G^0(P^+A_0^-)$, $\Delta G^0(P^+A_1^-)$, and distance between A_0 and A_1 (D). Calculation were done for $\Delta G^0(P^+A_1^- - P^+A_0^-)=-350$ meV for the wild type, $\lambda=350$ meV (1), $R=7.3$ Å for A–B, and $R=8.8$ Å for C–D. See text for details.

favor population of the state $P^+A_0^-$ over the state $P^+A_1^-$ and, in consequence, further electron transfer from A_1^- to F_x (~20–200 ns) [10] should limit the observed decay of A_0^-. Apparently, this is not the case. The same energetic argument could be used to exclude a possibility of very similar energy levels for the two charge-separated states and significant back reaction from A_1^- to A_0.

Therefore, we think that the change in the E_m of A_0^-/A_0 is not the only effect of the mutation. If, in addition, the distance between A_0 and A_1 increases from 7.3 (wild type) to e.g. 8.8 Å as a result of mutations, the energy level of $P^+A_0^-$ would remain (~65 meV) above that for $P^+A_1^-$ and the downhill electron transfer from A_0^- to A_1 would easily occur (Fig. 8C). The possibility of the change in the position of A_0, including a change in the distance from A_1, in the mutants is supported by the observation of a loss of the excitonic coupling between A_0 and neighboring Chls in the mutated branch (see Results, Fig. 7; see also [11,31]).

The scheme presented in Fig. 8C is still not fully satisfying because the level of $P^+A_1^-$ lies, in the mutants, below the level reported for the primary donor triplet state, ^3P [37]. One of the experimental observations is that the growth of the mutants is severely impaired by the presence of oxygen ([11]; see also [12,38]). This suggests an efficient formation of ^3P, an intermediate species in production of destructive singlet oxygen. To ensure this efficient formation, two prerequisites should be satisfied: a long lifetime of $P^+A_0^-$ (this is satisfied) and favorable energetics for its recombination to the ^3P state. Therefore, the level of $P^+A_0^-$ is expected to lye above that of ^3P. This is easy to introduce to the scheme in Fig. 8, if one assumes that the mutation of the methionine ligand affects to some extent also the ΔG^0 of $P^+A_1^-$ (via E_m of A_1^-/A_1). This assumption is reasonable since the methionine molecules occupy the sites between A_0 and A_1. If E_m of A_1^-/A_1 is decreased by e.g. 100 meV in the mutants, the energy levels for $P^+A_0^-$ and $P^+A_1^-$ goes up by 100 meV (Fig. 8D).

The presented modifications in the energetic scheme well explain the observed slowdown of electron transfer between A_0^- and A_1, and account for loss of excitonic coupling in the mutated branches and increased vulnerability of mutants to oxygen. However, further investigations of A_0 mutants are necessary to support this preliminary model. In particular, a direct detection of A_1^- formation with 1–2 ns kinetics, would verify the proposed model.

5. Concluding remarks

The results presented here provide further evidence for ET in both PsaA and PsaB branches of PSI in *C. reinhardtii* regardless of choices of axial ligand substitution as we reported earlier [11]. These results contrast to the earlier report [16,19] of asymmetric ET in cyanobacterial PSI in which 80% of the electron transfer proceeds along the PsaA branch. In contrast, recent work of Poluektov and coworkers [39] shows the evidence for bi-directional electron transfer in cyanobacterium *Synechococcus lividus* by observing the two distinct transient spectra of $P_{700}{}^+A_1^-$ spin-correlated radical pairs.

Further, Santabarbara et al. [40] very recently analyzed the spin-polarized echo of the [$P_{700^+}A_1^-$] radical pair of photosystem I from cyanobacteria, green algae and higher plants and interpreted their observation in terms of different proportions of the signal associated with the [$P_{700^+}A_1^-{}_A$] and [$P_{700^+}A_1^-{}_B$] radical pairs suggesting that both reaction center subunits are competent in electron transfer, supporting our conclusion that ET is occurring in both the branches of PSI. However, we cannot exclude the possibility that the effect of mutation at the A_0 level is to modify the reaction center so as to favor electron transfer through one or the other electron transfer branch as reported in [41].

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