Directionality of Electron-Transfer Reactions in Photosystem I of Prokaryotes: Universality of the Bidirectional Electron-Transfer Model

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The electron-transfer (ET) reactions in photosystem I (PS I) of prokaryotes have been investigated in wild-type cells of the cyanobacterium Synechocystis sp. PCC 6803, and in two site-directed mutants in which the methionine residue of the reaction center subunits Psaa and Psab, which acts as the axial ligand to the primary electron chlorophyll acceptor A0, was substituted with histidine. Analysis by pulsed electron paramagnetic resonance spectroscopy at 100 K indicates the presence of two forms of the secondary spin-correlated radical pairs, which are assigned to \([P_{700}^{+}A_{1A}^{-}]\) and \([P_{700}^{+}A_{1B}^{-}]\), where \(A_{1A}\) and \(A_{1B}\) are the phylloquinone molecules bound to the Psaa and the Psab reaction center subunits, respectively. Each of the secondary radical pair forms is selectively observed in either the Psaa-M688H or the Psab-M668H mutant, whereas both radical pairs are observed in the wild type following reduction of the iron–sulfur cluster \(FX\), the intermediate electron acceptor between \(A_1\) and the terminal acceptors \(F_A\) and \(F_B\). Analysis of the time and spectral dependence of the light-induced electron spin echo allows the resolution of structural differences between the \([P_{700}^{+}A_{1A}^{-}]\) and \([P_{700}^{+}A_{1B}^{-}]\) radical pairs. The interspin distance is 25.43 ± 0.01 Å for \([P_{700}^{+}A_{1A}^{-}]\) and 24.25 ± 0.01 Å for \([P_{700}^{+}A_{1B}^{-}]\). Moreover, the relative orientation of the interspin vector is rotated by ∼60° with respect to the \(g\)-tensor of the \(P_{700}^{+}\) radical. These estimates are in agreement with the crystallographic structural model, indicating that the cofactors bound to both reaction center subunits of prokaryotic PS I are actively involved in electron transport. This work supports the model that bidirectionality is a general property of type I reaction centers from both prokaryotes and eukaryotes, and contrasts with the situation for photosystem II and other type II reaction centers, in which ET is strongly asymmetric. A revised model that explains qualitatively the heterogeneity of ET reactions at cryogenic temperatures is discussed.

Introduction

In oxygenic photosynthesis, two macromolecular photocatalytic complexes known as photosystem (PS) I and photosystem II operate in series in order to oxidize water to molecular oxygen and, ultimately, store reducing equivalent in the form of NADPH. Each photosystem is composed of two functional moieties: the core that harbors the redox-active cofactors as well as an additional complement of chlorophyll (Chl) \(a\) and \(\beta\)-carotene cofactors that are involved in light harvesting, and the external antenna or light-harvesting complex (LHC), which serves to increase light capture.

The cores of PS I and PS II differ in a number of important features. For PS II, at least four subunits (D1, D2, CP43, and CP47) are involved in binding of chromophores, but with the electron-transfer (ET) cofactors bound primarily by a D1/D2 heterodimer, also known as the reaction center (RC).†,‡ On the other hand, the functional core of PS I consists of a heterodimer of the Psaa and Psab proteins and acts both as the inner antenna, binding about 90 chlorophyll \(a\) and about 30 \(\beta\)-carotene molecules, and as the RC, coordinating most of the ET cofactors.³,⁴

From a physicochemical point of view, the redox properties of the ET cofactors of each RC are significantly different, each being tuned to optimize the light-dependent catalytic activity of the photosystem. PS II acts as a water–plastoquinone oxidoreductase. Hence, the redox potential of the so-called donor side of PS II, where oxidizing equivalents are stored, is poised to a standard redox potential which, for the metastable cation of the photochemically active chlorophyll \(P_{680}^{+}\) to molecular oxygen, exceeds +1 V (ref 2 and references therein). A positive midpoint potential is required to oxidize water. On the other hand, the standard midpoint potential of plastoquinones \(Q_A\) and \(Q_B\), which constitute the acceptor side of PS II and are where reducing equivalents produced following primary photochemical charge separation are stored, is in the range of ±20 mV.⁵ In general, those photosynthetic RCs such as PS II that use quinones as terminal electron acceptors are

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referred to as type II RCs. Another general characteristic of type II RCs is that the terminal electron acceptor (Q_b) undergoes a two-electron reduction to the quinol form, following two consecutive charge separation events.

PS I catalyzes the oxidation of plastocyanin (or cytochrome c_6) and the reduction of ferredoxin (or flavodoxin). The standard redox potential of the metastable cation generated upon primary charge separation (P_{700+}) is titrated in the +450−500 meV range (reviewed in refs 4 and 5), whereas the redox potential of the acceptor side cofactors, the iron−sulfur clusters F_A and F_B that are bound to the PsaC subunit rather than to the PsaA−PsaB heterodimer, is poised to values in the order of −520−590 mV.4,5 Such reaction centers that have iron−sulfur clusters at the acceptor side belong to the type I RC family. Alongside PS I, this class includes the RC of heliobacteria and green sulfur bacteria.6,7 It is apparent that ET cofactors bound to PS I RCs are poised to more negative potentials than those of PS II. This is true in general when comparing the acceptor side redox potentials of type I and type II RCs. However, the standard redox potential of the donor of the purple bacteria RC8−10 is not dissimilar from that of PS I. Perhaps a more defining difference is that, unlike type II RCs, all redox reactions in type I RCs involve only one-electron chemistry.4,5

Moreover, recent investigations point toward substantial differences in the functionality of ET pathways associated with the two types of RCs. The solution of the first crystallographic model for a photosynthetic RC, that of purple bacteria RC,11,12 revealed that the redox-active cofactors were organized into two highly symmetric chains with respect to the axis perpendicular to the membrane plane. The presence of a similar C_2-fold symmetry in the arrangement of redox cofactors of oxygenic RCs, which was predicted based on sequence homology,13,14 has since been confirmed from crystallographic models of PS II15,16 and PS I.17,18 Yet, for the case of the purple bacteria RC, it is well established that only one of the two putative redox chains is involved in primary photochemical events (ref 10 and articles therein) and only the terminal acceptor Q_b, which is part of the (inactive) symmetric chain, is involved in ET. The same situation is thought to hold true in the case of PS II, because of the similarities in the acceptor side properties and structure. This process is commonly referred to as asymmetric or monodirectional electron transfer. On the other hand, an increasing body of literature based on a combination of molecular genetics and time-resolved spectroscopic analysis indicates that the two putative redox chains bound predominantly to either the PsaA (ETCA) or the PsaB (ETCB) subunits of PS I are functional in ET reactions,19−30 according to a mechanism which is often referred to as symmetric or bidirectional ET.

The organization of the two putative electron-transfer chains as revealed by the crystallographic models of PS I is shown in Figure 1. It can be noted that the ETCA and ETCB chains are not completely independent; rather, they share some of the ET cofactors. The Chl a−Chl a′ heterodimer, assigned to P_{700+}, is coordinated at the interface of the PsaA and PsaB subunits.17,18 Similarly, the electron acceptor F_B is coordinated at the interface and it is a common intermediate shared by the two electron-transfer chains.17,18 Moreover, the terminal acceptors, F_A and F_B, which are bound to the PsaC subunits are also common to both redox-active chains. The sole cofactors which are part of either ETCA or ETCB are two Chl a molecules (the so-called accessory chlorophyll, Chl_{ACC_A/B} or Chl-c2); the electron acceptor A_0 (Chl-eC3), and the following electron acceptor (A_{1(A/B)}) which is a tightly bound, nondiffusible, phylloquinone molecule (Figure 1). Recent investigations have revealed novel features of electron transfer in PS I including clear evidence of bidirectionality, particularly from studies of the eukaryotic PS I, e.g., refs 31 and 32. Photochemistry is initiated by the population of the lowest singlet excited state of the reaction center primary donor, which consists of a six Chl multimer, comprising P_{700+}, Chl_{ACC_A/B} and A_0. However, the exact details of primary charge separation are still a matter of debate.31,33−35 It was commonly accepted that P_{700+} acts as the primary electron donor while A_0 serves as an electron acceptor in a single-step charge separation reaction.4,5,33−35 This view has been questioned in recent time-resolved optical spectroscopy investigations, which suggested a more complex scenario.31,36,37

It was proposed, initially by the study of wild-type reaction centers,36,37 that stable charge separation occurs by a sequential two-step mechanism in which the accessory Chls play an active role, possibly as the primary electron donor. This suggestion received further support through analysis of mutants of the A_0 binding site on both the PsaA and the PsaB subunit of PS I.31 Muller et al.31 were able to observe the dynamics of charge primary separation on the PsaA- and PsaB-bound ET chains, providing further confirmation of the bidirectional model. Interestingly, although the population of the [P_{700+}A_0−] radical pair occurs in the 20−40 ps time range (a value similar to that obtained when considering a single-step charge separation mechanism33−35) on both electron-transfer chains,31 subtle differences in the kinetics on each redox chains were observed. These findings were interpreted in terms of different tuning of cofactors properties, mediated by the interaction with reaction centers subunits. Similarly, it has been proposed that protein−cofactor interactions lead to differences in the physical−chemical properties of the successive electron acceptors, phylloquinones...
A1A and A1B, which are manifest in the complex multieponential oxidation kinetics of the phyllosemiquinone anion (A1−) by Fx.4,5 The oxidation of A1− is described by a minimum of two kinetic phases characterized by lifetimes of 5−30 and 250−300 ns, respectively.19−21,38,39 There is substantial evidence that the faster lifetime describes, principally, the oxidation of A1B− whereas the slower lifetime relates principally to A1A− oxidation (see refs 5, 30, and 40 for recent reviews). It has been suggested that the principal reason for the differences in the observed kinetics resides in the oxidation of A1B− by Fx being thermodynamically favorable (ΔG<0), whereas the oxidation of A1A− by Fx being thermodynamically unfavorable (ΔG>0).5,30,40 Moreover, it has been proposed that such an asymmetry in the energetic properties of the phylloquinones bound to PsaA and PsaB, promotes a transient, Fx-mediated, interquinone electron transfer (i.e., A1B− → Fx → A1A−).32 Interquinone ET is a widely accepted process in type II reaction centers, but has only recently been considered for photosystems belonging to the type I family.

It is worth noting that the majority of the PS I investigations described above were carried out using wild-type and mutants forms of the eukaryotic RC, principally from the green alga Chlamydomonas reinhardtii, and strongly support a bidirectional model.21−30 However, other studies on the directionality of ET in PS I using cyanobacteria as a prokaryotic model system, e.g., refs 41−44, initially gave rise to contradictory interpretations of the experimental results, pointing toward a very asymmetric (almost monodirectional) ET in PS I of prokaryotes. This contradiction has largely been resolved as new investigations of ET in PS I of cyanobacteria29,45−49 converged to a view that bidirectional ET is a general property of PS I. Nevertheless, whereas in eukaryotic systems the fraction of electrons that are transferred via each of the two ET chains appears, on average, to be similar with values ranging from ∼1:12,23,29 to ∼1:21,25,26 in favor of ETCa, a more marked asymmetry is found in prokaryotes with reported values ranging from 1:4 to 1:94,41,42,44,49 Such a large difference in the statistical utilization of ETCa and ETCb between the eukaryotic and prokaryotic reaction centers is intriguing because the probability of utilization of each ET branch appears to be determined at the level of primary charge separation and successive stabilization of the [P700+1/2A0(A/B)]− radical pair.25,26,30,50 Yet, a comparison of structural models obtained from eukaryotic and prokaryotic organisms does not indicate any major differences in the arrangement of the cofactors engaged in charge separation reactions.

Moreover, whereas the spectroscopic characteristics of the [P700+1/2A1A−] radical pair have been extensively investigated,51−57 substantially less information is available on the (transient) [P700+1/2A1B−] radical pair23,29,45,47−49 and the (stable) A1B− radical.2,58 In particular, the spin-polarized EPR spectrum of the latter radical pair has been clearly distinguished from that of [P700+1/2A1A−] only under conditions in which A1A− was fully reduced prior to the measurements.45,47

In order to gain insight into the statistical utilization of the ETCa and ETCb branches of the prokaryotic PS I RC, and, in order to understand further the characteristics of the [P700+1/2A1B−] radical pair, we have studied two site-directed mutants of Synechocystis sp. PCC 6803 in which the axial ligand of A0 was changed from a methionine to a histidine on either the PsA (PsA-M688H) or the PsB (PsB-M666H) subunit. These are the same amino acid substitutions that were previously engineered in the PS I of C. reinhardtii22,23 and therefore allow a direct comparison of the eukaryotic and prokaryotic model systems. In this study, we show that the spin-polarized EPR spectrum associated with the [P700+1/2A1B−] radical pair can be observed under appropriate experimental conditions that lead to the reduction of the intermediate electron acceptor Fx prior to the measurements. The differences in the spin-polarized EPR spectra of the [P700+1/2A1A−] and the [P700+1/2A1B−] radical pair are interpreted as arising from both the spatial arrangement of the radical pair partners and a difference in the proton hyperfine coupling in the phylloquinone anion radical. By comparing data acquired under conditions in which the terminal acceptors Fx/A0/A1 are initially either oxidized or reduced but phylloquinone A1A− is always substantially oxidized (>95%), we propose a revised ET model which provides insight into the energetic inequivalence of the phylloquinone molecules bound to the PS I reaction center and accounts for the heterogeneity of ET reactions at low temperature.4,39 This model applies to both prokaryotic and eukaryotic PS I and therefore constitutes a generalization of the bidirectional electron-transfer mechanism in this type of reaction center.

### Material and Methods

#### Cell Growth and Thylakoid Membranes Purification.

Site-directed mutants of Synechocystis sp. PCC6803 were obtained following the procedure described in detail by Xu et al.41,42 and with appropriate codon substitutions already reported by Cohen et al.41 All strains were grown in BG11 medium59 supplemented with 5 mM glucose at 32 °C at an irradiation of 15 μE m−2 s−1 of white light. Thylakoid membranes were prepared as described by Gombos et al.60 with minor modifications. The cell suspension was disrupted by a single passage in a French press (Aminco, Urbana, IL), operated at 10 000 psi. Broken cells were centrifuged at 5500g for 5 min to remove unbroken material. The thylakoid membrane was purified by centrifuging the supernatant at 50 000g for 60 min and suspended in a buffer containing 100 mM tricine (pH 7.8), 200 mM sorbitol, and 30 mM NaCl at a Chl a concentration equivalent to 1 mg mL−1.

The samples were incubated in the presence of ascorbate (30 mM) or Na dithionite (11 mM) for 30 min under an argon atmosphere as previously described.29 The photoaccumulation procedure has been previously described in detail.29

#### Fluorescence Spectroscopy.

Fluorescence emission spectra were recorded using a Fluoromax-3 (Jobin-Yvonne, France) spectrometer equipped with a home-built liquid nitrogen bath cryostat. Fluorescence was excited at 420 nm (fwhm 5 nm), and additionally filtered through a CS 4-96 (Corning) bandpass filter to remove higher-order harmonics. The fluorescence emission was detected with a resolution of 1 nm. An OG-550 filter (Schott) was placed before the monochromator entrance slit to reduce the detection of stray and scattered light. The spectra were corrected for the sensitivity of the detector.

The cell suspensions were diluted in BG11 medium supplemented with 66% w/v glycerol to an optical density of 0.05 cm−1 at 680 nm, immediately before freezing to obtain a transparent glass. The spectra were fitted with a sum of Gaussian functions, as previously described.51

#### Time-Resolved Electron Paramagnetic Resonance.

EPR spectra, electron spin echo envelope modulation (ESEEM) time-domain spectra, and the decay of the electron spin echo (ESE) signals as a function of the delay after laser excitation time were measured using a Bruker ESP580 X-band (~9.7 GHz) spectrometer equipped with a variable Q dielectric resonator (Bruker EN4118 X-MD-4W) and fitted with an Oxford Instruments CF935 cryostat cooled with liquid nitrogen. The temperature was controlled by an Oxford Instruments ITC-5 controller.29

Actinic illumination was supplied by a frequency-doubled Nd:
YAG laser (Spectra Physics DCR-11). The pulse duration was \( \sim 10 \text{ ns (fwhm)} \) at 532 nm. The overall temporal resolution of the spectrometer is 50 ns.

ESEEM time dependences were monitored by a nonselective (“hard”) excitation two-pulse echo sequence consisting of \( \pi/2 \) pulse of 8 ns and a refocusing \( \pi \) pulse of 16 ns. ESEEM time dependences were recorded with an initial echo delay \( (\tau) \) of 124 ns, incremented in 8 ns steps. The decay of the ESE was recorded using the same pulse echo sequence \( (\pi/2-\tau-\pi, \tau = 124 \text{ ns}) \), incrementing the delay between the pulse sequence and the actinic illumination by 48 ns steps.

EPR spectra were recorded using a selective excitation (“soft”) two-pulse sequence, consisting of two equal pulses of 124 ns and the interpulse separation time, \( \tau \), was set at 304 ns. The delay after the laser excitation time was 300 ns. The initial spectra were obtained using an integration window of 40 ns centered at the maximum of the echo, scanning the \( B_0 \) field with a resolution of 0.02 mT.

In all cases, the acquisition was triggered by the laser Q-switch and the signals were corrected for imperfect phasing of the amplifier and artifacts due to possible contributions from stable radicals generated during the experiments as previously described.23,29

**Data Analysis. Decay of the Electron Spin Echo.** The time decay of the ESE signal was fitted with a sum of exponential functions using the Levenberg–Marquardt algorithm, which minimizes the sum of squared residues weighted by the number of sampled points and the degrees of freedom associated with the fit function’s adjustable parameters.23,29

\[ M(t) = \alpha_1 M_1(t) + \alpha_2 M_2(t) + \beta(t) \]  

where \( \beta(t) \) is a baseline correction term, and \( \alpha \) are weighting factors. The dipolar and exchange interactions were considered as global variables, while the relative amplitudes \( \alpha_1, \alpha_2 \), the relaxation time \( T \), and the baseline \( \beta(t) \) were independent variables in the global fitting routine.29

**Spin-Polarized Radical Pair Theory and Algorithm for the Analysis of EPR Spectra and Out-of-Phase ESEEM.**

The spin-correlated radical pair (SCRP) spectra were simulated using a linear combination of two functions, \( M(t) \), with the form given in eq 3

\[ M(t) = \alpha_1 M_1(t) + \alpha_2 M_2(t) + \beta(t) \] 

where \( \beta(t) \) is a baseline correction term, and \( \alpha \) are weighting factors. The dipolar and exchange interactions were considered as global variables, while the relative amplitudes \( \alpha_1, \alpha_2 \), the relaxation time \( T \), and the baseline \( \beta(t) \) were independent variables in the global fitting routine.29

**Field-Swept Electron Spin Echo Spectra.** In the high-field approximation the spin Hamiltonian (in angular frequency units) can be expressed as

\[ H_0 = 2\pi \mu_B h^{-1}(S_p g_p + S_q g_q) B_0 - J(S^2 - 1) + \frac{1}{2}d(3S_z^2 - S^2) \] 

where \( h \) is Planck’s constant, \( \mu_B \) is the Bohr magneton, \( g_p \) and \( g_q \) are the g-values of the primary donor cation (P) and the phyllo(semi)quinone acceptor (Q), \( B_0 \) is the applied magnetic field strength, and \( J \) is the isotropic exchange interaction energy, \( d = D(\cos^2 \Theta - \frac{1}{3}) \); \( S_p \) and \( S_q \) are the z-components of the spin operators for the P and Q electrons, and \( S \) is the total spin operator.

The spin-correlated radical pair (SCRP) spectra were simulated using Matlab software and the approach described in refs 67 and 68.

**Results and Discussion**

**Effect of the PsA-M688H and the PsB-M668H Mutations on the Stability of the PS I Reaction Center in Synechocystis sp. PCC 6803.** Figure 2 shows the fluorescence emission spectra of whole cells of wild-type Synechocystis, and of the mutants PsA-M688H and PsB-M668H recorded at 77 K upon preferential excitation of Chl \( a \) at 420 nm. In the wild type, two clear structures are observed, in agreement with several previous reports in cyanobacteria, e.g., refs 69 and 70, a group of relatively sharp bands, peaking at 674 and 682 nm, which are attributed to the fluorescence of the terminal emitter of
phycobilisomes and PS II core antenna, respectively, and a broad emission band peaking at 720 nm, which is due to PS I. Another minor emission band peaking at 695 nm is highlighted by the Gaussian deconvolution of the emission spectra. In order to compare the PS II:PS I ratios in the wild-type and the two site-directed mutants, the emission spectra were normalized at the 682 nm emission peak. The spectra presented in Figure 2 show that, although in both PsaA-M688H and PsaB-M668H the intensity of the fluorescence emission attributed to PS I is reduced, the PsaA-M688H mutant is significantly more affected.

To a first approximation, the ratio of the fluorescence emission intensities at 735 and 684 nm can be taken as an indicator of the relative stoichiometry of the photosystems. A more accurate estimate can be achieved by integrating the Gaussian bands describing the emission spectra. However, it should be noted that this description is mainly phenomenological and it is not possible to ascribe a precise physical meaning to each of the Gaussian components. Assuming the PS II accumulation is unaffected by the site-directed mutations of PS I, the level of stably accumulated PS I is decreased to about 45% and 70% of that of the wild type in the PsaB-M668H and PsaA-M688H mutants, respectively. It is interesting to note that in the PsaA-M684H and PsaB-M664H mutants of the eukaryotic alga C. reinhardtii in which the homologous residue serving as the axial ligand to A0 has been targeted for identical substitutions, 22 it was the PsaB-M664H mutant that showed a more pronounced effect on PS I accumulation, even though not as pronounced as that observed in the PsaA-M688H mutant of Synechocystis.

**Decay of the Electron Spin Echo (ESE) Arising from the \(\{P_{700}^0 A_{1}^-\} \) Radical Pair at 100 K.** Figure 3 shows the decay of the ESE signal as a function of the delay of the microwave pulse sequence with respect to the actinic flash (i.e., optical “pump”—ESE “probe” experiment), in thylakoid membranes of the wild-type and the PsaB-M668H mutants incubated in the presence of either 30 mM Na ascorbate (Figure 3A,C) or 11 mM Na dithionite (Figure 3B,D) and recorded at 100 K. It is well established that this out-of-phase ESE arises from the secondary radical pair \(\{P_{700}^0 A_{1}^-\} \) of PS I. 52-66 The ET intermediates bound to PS I are initially fully oxidized when the samples are incubated in the presence of ascorbate, while the terminal electron acceptors F8 and F6 are initially reduced in the presence of dithionite. 23,29 The decay of the ESE in the thylakoids incubated with ascorbate is described by a dominant (>98%) kinetic component characterized by lifetimes of 26.4 ± 0.2 µs and 28.2 ± 0.3 µs in the wild-type and the PsaB-M668H mutant, respectively (Table 1). Similar results were obtained in dark-adapted cells (Table 1).

The best fit of the ESE decay kinetics in thylakoid membranes isolated from the wild type and incubated with dithionite is obtained with a biexponential decay, characterized by lifetimes of 3.3 ± 0.3 and 23.3 ± 0.2 µs and associated fractional amplitudes of 0.20 ± 0.02 and 0.80 ± 0.04 (Figure 2C). The decay of the ESE in thylakoid membranes of the PsaB-M668H mutant incubated with dithionite can be satisfactorily described by a single-exponential decay, characterized by a lifetime of 26.1 ± 0.6 µs. Attempts to fit these data with a biexponential function resulted in an improvement in the statistical evaluation parameters, and yielded a second lifetime of 3.2 ± 0.7 µs associated with a fractional amplitude of 0.05–0.09 (see Figure 2D, Table 1).

We were unable to record any spin-polarized EPR signals in dark-adapted cells or in thylakoid membranes isolated from the
TABLE 1: Analysis of the Decay of the [P700⁺A1⁻] Electron Spin Echo

<table>
<thead>
<tr>
<th></th>
<th>α₁</th>
<th>t₁ (µs)</th>
<th>α₂</th>
<th>t₂ (µs)</th>
<th>tₑ,av (µs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark Adapted Cells of Synechocystis sp. PCC 6803</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>wild type</td>
<td>0.18 ± 0.06</td>
<td>1.8 ± 0.5</td>
<td>0.82 ± 0.2</td>
<td>28.1 ± 0.2</td>
<td>17.60 ± 0.2</td>
</tr>
<tr>
<td>Psb-M668H</td>
<td>0.10 ± 0.05</td>
<td>2.1 ± 0.7</td>
<td>0.90 ± 0.1</td>
<td>29.2 ± 0.1</td>
<td>28.45 ± 0.1</td>
</tr>
</tbody>
</table>

| Dark Adapted Thylakoids. Oxidized Iron–Sulfur Centers FA, FB, and FX |           |          |           |          |            |
| wild type      | 0.12 ± 0.2  | 1.8 ± 0.2 | 0.88 ± 0.2 | 26.4 ± 0.2 | 21.51 ± 0.2 |
| Psb-M668H      | 0.05 ± 0.05 | 1.6 ± 0.3 | 0.95 ± 0.05 | 28.2 ± 0.3 | 26.8 ± 0.4 |

| Dark Adapted Thylakoids Incubated with Dithionite. Reduced Iron–Sulfur Centers FA, FB |           |          |           |          |            |
| wild type      | 0.20 ± 0.2  | 3.3 ± 0.3 | 0.80 ± 0.2 | 23.3 ± 0.2 | 19.3 ± 0.4 |
| Psb-M668H      | 0.07 ± 0.02 | 3.2 ± 0.7 | 0.93 ± 0.04 | 26.1 ± 0.6 | 24.5 ± 0.6 |

| Thylakoids Preilluminated at 220 K Incubated with Dithionite. Reduced Iron–Sulfur FX |           |          |           |          |            |
| wild type      | 0.38 ± 0.2  | 2.7 ± 0.5 | 0.62 ± 0.2 | 19.8 ± 0.4 | 13.4 ± 0.4 |
| Psb-M668H      | 0.09 ± 0.02 | 2.8 ± 0.8 | 0.91 ± 0.3 | 21.8 ± 0.2 | 19.7 ± 0.4 |
| Psb-A-M688H    | 0.24 ± 0.12 | 1.4 ± 0.1 | 0.76 ± 0.1 | 4.24 ± 0.3 | 3.5 ± 0.1 |

* Results of fitting the decay of the electron spin echo associated with the spin-polarized radical pair [P700⁺A1⁻] in thylakoid membranes from the wild-type, Psb-M668H, and Psb-M688H. The samples were either dark-adapted for 30 min in the presence of the reductant dithionite, using intense white light (3000 µE m⁻² s⁻¹ at the sample) for 10 min at 220 K. It was previously shown this treatment leads to the reduction of the iron–sulfur cluster FX, as well as the terminal electron acceptors FA and FB. The data have been fitted by linear sum of exponential functions: \( y(t) = \sum_{i} \alpha_i \exp(-t/t_{e,i}) \). The average decay lifetimes is defined as \( t_{e,av} = \sum_{i} \alpha_i t_{e,i} / \sum_{i} \alpha_i \).

Figure 4. Decay of the ESE arising from the [P700⁺A1⁻] radical pair, in thylakoid membranes isolated from the wild type (A), the Psb-M668H (B), and the Psb-A-M688H mutants (C), incubated with 11 mM dithionite and preilluminated at 220 K for 10 min in order to reduce FX. Experimental conditions were as in the legend to Figure 3. Solid lines: experimental results. Dashed-dotted lines: fits to exponential decays. The fit residuals are shown in the small panels.

PsaA-M688H mutant, when incubated with either ascorbate or dithionite. However, a spin-polarized ESE signal was observed after preillumination of the thylakoids incubated with sodium dithionite, using intense white light (3000 µE m⁻² s⁻¹ at the sample) for 10 min at 220 K. It was previously shown this treatment leads to the reduction of the iron–sulfur cluster FX, as well as the terminal electron acceptors FA and FB. The comparison of the decay of the ESE signal obtained after preillumination of FX in the wild type and the mutants is presented in Figure 4. In wild-type thylakoids the decay of the ESE is described by two components characterized by lifetimes of 2.7 ± 0.5 and 19.8 ± 0.4 µs. The decay of the ESE in both site-directed mutants can be described satisfactorily by a single exponential characterized by best fit lifetimes of 3.4 ± 0.6 µs for the PsaA-M688H mutant and 19.2 ± 0.8 µs for the Psb-M668H mutant. Attempts to fit the ESE decay in the mutants with two exponentials yielded substantially the same result in the case of the Psb-M668H mutant, as a second rapidly decaying component (2.8 µs) associated with a fractional amplitude of 0.07–0.11 only marginally increased the quality of the fit (Table 1). In the case of the PsaA-M688H mutant, the data could be fitted by two exponentials yielding components of 1.4 ± 0.1 and 4.2 ± 0.2 µs, associated with weighting factors of 0.24 and 0.76, respectively (Figure 3C, Table 1). Due to the relatively low signal-to-noise ratio in these measurements, which is probably the result of the low level of PS I accumulation in this strain (Figure 2), the statistics for the monoexponential and the biexponential descriptions do not differ greatly. However, careful inspection of the residual plots suggests that the biexponential description gives a more homogeneous residual distribution. Nevertheless, the ~3.5 µs lifetime is either the only or the dominant decay component observed in the PsaA-M688H mutant. Even accounting for biexponential decay kinetics, it was not possible to detect lifetimes in the tens of microseconds time window for the PsaA-M688H mutant as observed in both the wild type and the Psb-M668H mutant (Table 1).

Electron Spin Echo Envelope Modulation (ESEEM) Arising from the [P700⁺A1⁻] Radical Pair at 100 K. Figure 5 shows the time dependences of the ESEEM signals recorded in thylakoid membranes of the wild type and the Psb-M668H mutant. The data have been fitted by linear sum of exponential functions: \( y(t) = \sum_{i} \alpha_i \exp(-t/t_{e,i}) \). The average decay lifetimes is defined as \( t_{e,av} = \sum_{i} \alpha_i t_{e,i} / \sum_{i} \alpha_i \).
Sine Fourier transformations (SFT) of both the experimental results and the fits, obtained after reconstruction of the spectrometer dead time and subtraction of a quadratic baseline, are also presented in Figure 5 (panels B and D). The best fit parameters are reported in Table 2. The ESEEM recorded in both samples is described by interaction energies \( \frac{\alpha}{D} \) and the exchange interaction energy \( \frac{\beta}{T} \), as well as the decay lifetime \( T \) evaluated for these samples, are also reported in Table 2. The dipolar and exchange interaction energies needed to describe the time dependence of the ESEEM originating from \([P700^-A_{1\alpha}^-]_{A1B}\) radical pair in the PsaB-M668H mutant it was determined that the signal associated with \([P700^-A_{1\alpha}^-]_{A1A}\) radical pair contributed only 30\% of the SFT intensity, the remaining 70\% arising from \([P700^-A_{1\alpha}^-]_{A1B}\). Moreover, despite a strong quenching of the ESE signal intensity, the ESEEM arising from both \([P700^-A_{1\alpha}^-]_{A1A}\) and \([P700^-A_{1\alpha}^-]_{A1B}\) radical pairs was detected even in the absence of prereduction of FX, in contrast to that observed in the PsaA-M668H mutant. As the intensity of the spin-polarization depends strongly on the lifetime of the precursor radical pair \([P700^-A_{1\alpha}^-]\), the most probable cause of the differences between these two mutants can be attributed to the effect of the A0 axial ligand substitution on the lifetime of the precursor. Despite these discrepancies in the detail of the results acquired for the different axial ligand substitutions, the interpretation of the results gathered both for the Met \( \rightarrow \) Arg and the Met \( \rightarrow \) His substitutions in prokaryotic RCs appears to be in general agreement with the previous suggestions derived from the analysis of ESEEM in C. reinhardtii mutants; namely, that two radical pairs, \([P700^-A_{1\alpha}^-]_{A1A}\) and \([P700^-A_{1\alpha}^-]_{A1B}\) are populated, with significant yield, in the reaction center of PS I.

Electron Paramagnetic Resonance (EPR) Spectra of the Secondary Radical Pair \([P700^-A_{1\alpha}^-]_{A1B}\). In the previous investigation of mutants of eukaryotic PS I, the \([P700^-A_{1\alpha}^-]_{A1A}\) and \([P700^-A_{1\alpha}^-]_{A1B}\) radical pairs were characterized solely on the basis of the decay rates and the modulation frequencies of the ESE \(^{12,29}\) whereas the EPR spectra associated with these radical pairs were not reported. Here we present the field-swept ESE spectra recorded in the thylakoid membranes of the wild type and the mutants of \( \textit{Synechocystis} \) (Figure 7), under the same experimental conditions in which ESEEM is detectable in all mutant strains (Figure 6), i.e., prereduction of FX.

In order to record EPR spectra that display the polarization typical of spin-correlated radical pairs, it is necessary to use microwave pulses that lead to selective hole burning in the
TABLE 2: Fit Parameters for the “Out-of-Phase” ESEEM of the Radical Pairs [P700−A1−]

<table>
<thead>
<tr>
<th></th>
<th>D (µT)</th>
<th>J (µT)</th>
<th>T (µs)</th>
<th>distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dark Adapted Thylakoids. Oxidized Iron—Sulfur Center F_X</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>−167.88 ± 0.11</td>
<td>0.01 ± 0.09</td>
<td>0.598 ± 0.02</td>
<td>25.51 ± 0.01</td>
</tr>
<tr>
<td>PsaB:M664H</td>
<td>−168.56 ± 0.08</td>
<td>0.45 ± 0.07</td>
<td>0.587 ± 0.02</td>
<td>25.48 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>220 K Preilluminated Thylakoids. Reduced Iron—Sulfur Center F_X</td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>−176.52 ± 0.25</td>
<td>1.18 ± 0.20</td>
<td>0.376 ± 0.001</td>
<td>25.08 ± 0.02</td>
</tr>
<tr>
<td>PsaB:M668H</td>
<td>−169.48 ± 0.13</td>
<td>0.38 ± 0.28</td>
<td>0.440 ± 0.001</td>
<td>25.43 ± 0.01</td>
</tr>
<tr>
<td>PsaA:M688H</td>
<td>−195.36 ± 0.11</td>
<td>3.47 ± 0.10</td>
<td>0.397 ± 0.002</td>
<td>24.25 ± 0.01</td>
</tr>
</tbody>
</table>

* Fit parameters describing the ESEEM time-dependence associated with the spin-polarized radical pair [P700−A1−] in thylakoid membranes from the wild type, Psa-A-M688H, and PsaB-M668H. The samples were either dark-adapted for 30 min in the presence of the reductant sodium dithionite (pH 8), yielding the reduction of the terminal electron acceptors FA and FB only, or preilluminated for 10 min at 220 K, yielding the reduction of FA and F_X.

Figure 6. ESEEM arising from the secondary radical pairs [P700−A1−] in thylakoid membranes isolated from the wild type, the PsaB-M668H, and the PsaA-M688H mutants, as labeled in the figure, incubated with 11 mM dithionite and preilluminated for 15 min at 220 K. Solid black lines: experimental data. Also presented are smoothed transforms. Solid circles: experimental results. Open circles: reconstruction of the instrument dead time. Solid lines: fits as described by Eq 3. Dash-dotted line: quadratic baselines. Experimental conditions were as in the legend of Figure 4.

Figure 7. Echo-detected EPR spectra arising from the secondary radical [P700−A1−] in thylakoid membranes isolated from the wild type, the PsaB-M668H, and the PsaA-M688H mutants, as labeled in the figure, incubated with 11 mM dithionite and preilluminated for 15 min at 220 K. Solid black lines: experimental data. Also presented are smoothed spectra (as a guide for the eye, WT, red; PsaB-M668H, blue; PsaA-M688H, magenta). Experimental conditions: temperature, 100 K; boxcar integration, 30 ns. The value of the static magnetic field B_0 has been corrected for the experimental frequency, to an arbitrary frequency of 9.75 GHz to allow direct comparison of the spectra, which are normalized to the maximum of the absorption.

Principal absorption peak is split, due to the large proton hyperfine coupling associated with the methyl group of the semiphylloquinone radical.54–57 This splitting of the EPR transitions appears as a clear shoulder in the absorptive transition of the spin-polarized spectrum (Figure 7).

We were not able to record an EPR signal in the thylakoids of the PsaA-M688H mutants when F_X was initially oxidized. The axial ligand of the A_0 chlorophyll has previously been mutated from a methionine to either a leucine43,51 or an arginine48,49 in *Synechocystis* 6803, on both the PsaA and PsaB subunits. It was shown that, under nonreducing conditions, the spin-polarized EPR signal is strong quenched and the spin-polarization pattern of the EPR spectrum is strongly altered in mutants of the PsaA subunit. The effect of the PsaA-M668L mutation was quantitatively described assuming a lengthening of the lifetime of the precursor radical pair [P700−A_0−] from tens of picoseconds in the wild-type, to 2–5 ns in the mutant.48,51 The estimates of Salikhov et al.51 are in agreement with ultrafast

inhomogeneously broadened spectrum. At 9–10 GHz (X-band) these conditions are obtained by the use of so-called “soft” pulses of long duration and reduced power. The EPR spectra recorded in the wild-type and PsaB-M668H mutant are very similar to those reported by Fursman et al.77 using the same technique and similar selective pulse sequence, for the PS I RC isolated from *Synechococcus elongatus*. The EPR spectra are also similar to those recorded by the direct-detection technique, which are not distorted by the use of insufficiently selective microwave pulses, in a variety of systems including *Synechocystis* 6803, e.g., refs 42, 44, 47, 51, and 54–57. The spectra exhibit a characteristic emission—absorption—emission (E/A/E) polarization pattern. The principal absorption peak is split, due to the large proton hyperfine coupling associated with the methyl group of the semiphylloquinone radical.54–57 This splitting of the EPR transitions appears as a clear shoulder in the absorptive transition of the spin-polarized spectrum (Figure 7).
time-resolved optical investigations of site-directed mutants of *C. reinhardtii* and *Synechocystis*, in which the lifetime of \( [P_{700}^{+}A_{1A}^-] \) was reported to be in the order of 1.0–1.5 ns compared to ∼30 ps in the wild-type (reviewed in refs 4 and 5). It is also worth noting that a relatively intense signal arising from \( [P_{700}^{+}A_{1A}^-] \) charge recombination (lifetime ∼30 ns) was observed at room temperature in the PsA-M684H mutants of *Chlamydomonas*. Such a long lifetime for the precursor would be sufficient to fully suppress spin polarization, at least in a fraction of PS I reaction centers. Hence, the inability to detect an EPR signal in the PsA-M684H mutant is most likely due to a combination of factors, including the low level of PS I accumulation (Figure 2), the low initial amplitude of the spin-polarized signal, and the more rapid decay of the ESE signal, as inferred from measurements obtained under reducing conditions (Figure 6).

Nevertheless, a spin-polarized EPR spectrum could be recorded after preduction of all the iron-sulfur cluster acceptors. The spectrum shows the same E/A/E spin-polarization pattern as observed in the wild type and the PsAB-M668H mutants (Figure 7). The EPR spectrum recorded in the PsAB-M668H mutant after reduction of FX is, within the signal-to-noise ratio of the measurements, identical to that recorded in the wild-type membranes when FX is initially oxidized (data not shown). Qualitative differences between the spectra recorded in the PsA-M684H and the PsAB-M668H mutants are evident; the shoulder caused by the methyl proton hyperfine interaction displays lower intensity in the \( [P_{700}^{+}A_{1A}^-] \) radical pair spectra of the PsA-M684H mutant. Second, the emissive minimum in the high-field region of the spectrum is decreased in intensity when compared to both the PsAB-M668H mutant and the wild-type spectra. These observations suggest that the relative orientation between the \( P_{700}^{+} \) and \( A_{1A}^- \) g-tensors and their orientation with respect to the interspin vector in the radical pairs monitored in the PsA-M684H and the PsAB-M668H mutants are not the same. Qualitatively similar changes in the X-band spin-polarized EPR spectra have been also observed in the PsA-M688N mutant of *Synechocystis*.

*Simulations of Field-Swept ESE Spectra.* The relative orientations of the tensors can, in principle, be determined from the fitting of the EPR spectra. A drawback associated with the field-swept ESE detection (at low microwave frequencies) is that the EPR spectrum is dependent on the pulse sequence, because distortion can arise from insufficiently selective hole-burning. Moreover, ESE detected field-swept spectra can, in part, be distorted by ESEEM contributions; however, this effect is minimized by both detection of the initial amplitude of the echo and the use of soft pulses. Hence, we have opted to simulate the spectra, based on data already available from independent measurements, rather than rely solely on the spectra determined in the present study. The results of the simulations are therefore qualitatively, rather than quantitatively, compared with those obtained in the experiments.

A host of information is available relating to the values of the g-tensor anisotropy of \( P_{700}^{+} \) and \( A_{1A}^- \) stable radicals determined from multifrequency (including very high frequency) EPR investigations. The mutual orientations of the g-tensors of \( P_{700}^{+} \) and \( A_{1A}^- \) with respect to the dipolar axis are determined with accuracy, e.g., refs 54–57. Poluektov et al. reported values for the \( A_{1B}^- \) g-tensor and its relative orientation with respect to \( P_{700}^{+} \) and the interspin vector obtained from the fitting of the spin-polarized high-frequency (130 GHz) EPR spectrum in cells of *Synechococcus lividus* after photoaccumulation of \( A_{1A}^- \). On the other hand, the strength of the dipolar (D) and exchange (J) interaction energies is determined with higher precision from the analysis of the ESEEM rather than fitting of the EPR spectra. Therefore, in the spectral simulations, we have used the values of D and J obtained in this study (Table 2).

The magnitude and relative orientation (with respect to the g-tensor) of the hyperfine tensor for the strongest coupling due to methyl protons in \( A_{1A}^- \) are also determined accurately, e.g., refs 51 and 54–57. However, significantly less information is available for the \( A_{1B}^- \) radical and this is limited to X-band. Moreover, there is, at present, no independent determination of the relative orientation of the A-tensor(s) with respect to either the g-tensor or the molecular frame for the \( A_{1B}^- \) radical. Hence, we have opted not to include hyperfine interactions in the calculations. All the quantities used to simulate the spectra of the \( [P_{700}^{+}A_{1A}^-] \) and \( [P_{700}^{+}A_{1B}^-] \) radical pairs are listed in Table 3.

Simulations were performed for microwave frequencies of 9.75 GHz (X-band) and 130 GHz (D-band); the former relates to spectra presented in this study, the latter allowed comparison with the results of Poluektov and co-workers from which the impact of the relative differences in the orientations of the radical’s g-tensors, with respect to the symmetry axis of the D-tensor is more obvious (Figure 8).

Figure 8 shows a good qualitative agreement between the experimental data and the simulations, with the exception of (i) the split feature in the main absorptive transition, which is not reproduced in the simulations; (ii) the presence in the simulation of an absorptive transition in the high-field region of the spectrum, which is more intense than in the experimental data. The inability of the simulation to reproduce the splitting of the peak absorptive transition is expected as this feature is known to derive from \(^1\)H-hyperfine couplings that are neglected in the calculations, e.g., refs 51 and 54–57.

### Table 3: Parameters Used for the Simulations of the EPR Spectra

<table>
<thead>
<tr>
<th>G tensor parameters</th>
<th></th>
<th>G tensor parameters</th>
<th>orientation parameters</th>
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<tbody>
<tr>
<td>( s_{xx} )</td>
<td>( s_{yy} )</td>
<td>( s_{zz} )</td>
<td>( R )</td>
</tr>
<tr>
<td>( P_{700}^{+} )</td>
<td>2.00322</td>
<td>2.00277</td>
<td>2.00246</td>
</tr>
<tr>
<td>( A_{1A}^- )</td>
<td>2.00636</td>
<td>2.00520</td>
<td>2.00231</td>
</tr>
<tr>
<td>( A_{1B}^- )</td>
<td>2.00629</td>
<td>2.00520</td>
<td>2.00231</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( D_B )</td>
</tr>
</tbody>
</table>

| Electron-electron couplings | Residual line width (mT) |
|---|---|---|
| \( D_X \) (\( \mu T \)) | −175.4 | 0.50 | 0.50 |
| \( J_X \) (\( \mu T \)) | 0.5 | 0.30 | 0.30 |

* Parameters used to simulate field-swept EPR spectra. Simulations are shown in Figure 7. A narrower line width is used for the sake of consistency with the data reported in ref 45 that were acquired using deuterated RCs.
The correct calculation of the high-field portion of the spectrum, at X-band, is also notoriously difficult. For a spin-correlated radical pair, the theory predicts the observation of an E/A/E/A transition pattern (reviewed in refs 65 and 68). However, due to the stochastic distribution of RC orientation with respect to the applied magnetic field in frozen solutions, and because of the small g-tensor anisotropies of both radicals, in particular that of P\textsubscript{700}\textsuperscript{+}, the higher-field transition tends to very low intensity after orientational averaging and integration. This transition is observed more clearly at higher bands or by the use of H/D and \textsuperscript{14}N/\textsuperscript{15}N isotope exchange, as both lead to associated radical pair spectra, the discrepancy in the intensity of the higher-field absorptive transition between radical pairs omitted from the simulations of both the [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1A}\textsuperscript{-}] and [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1B}\textsuperscript{-}] radical pairs at (A) 9.75 GHz (X-band) and (B) 130 GHz (D-band). All the parameters used in the simulations are reported in Table 3.

Figure 8. Simulations of the EPR spectra (eq 5) arising from the [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1A}\textsuperscript{-}] and [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1B}\textsuperscript{-}] radical pairs at (A) 9.75 GHz (X-band) and (B) 130 GHz (D-band). The effect of this change with respect to the dipolar axis, as expected from the asymmetry of P\textsubscript{700}\textsuperscript{+}, the higher-field transition tends to very low intensity after orientational averaging and integration. This transition is observed more clearly at higher bands or by the use of H/D and \textsuperscript{14}N/\textsuperscript{15}N isotope exchange, as both lead to associated radical pair spectra, the discrepancy in the intensity of the higher-field absorptive transition between radical pairs omitted from the simulations of both the [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1A}\textsuperscript{-}] and [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1B}\textsuperscript{-}] radical pairs at (A) 9.75 GHz (X-band) and (B) 130 GHz (D-band). All the parameters used in the simulations are reported in Table 3.

The relationship between the Value of Exchange Interaction Energy (J) and the Rate of Charge Recombination between P\textsubscript{700}\textsuperscript{+} and A\textsubscript{i}\textsuperscript{-}. In this study, it is observed that the value of the exchange interaction energy is significantly larger for the [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1B}\textsuperscript{-}] radical pair (J = 3.5–4.5 \textmu T) compared to the [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1A}\textsuperscript{-}] (J = 0.01–1 \textmu T) radical pair (Table 2). This is in agreement with previous results obtained by either investigating mutants of the axial ligand to A\textsubscript{i} in the PS I of C. reinhardtii or the effect of reducing the terminal electron acceptors F\textsubscript{A} in wild-type PS I reaction centers of several organisms.29 The absolute value of J is 2 orders of magnitude smaller than that of the dipolar interaction, D (Table 2).

Therefore, in relative terms, the confidence intervals associated with the estimate of this parameter are relatively large. Nevertheless, the substantial increase of J observed in [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1B}\textsuperscript{-}] in comparison to [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1A}\textsuperscript{-}] is statistically significant. It is interesting to note that the value of the exchange interaction energy scales linearly with that of the electronic donor–acceptor coupling interaction \( \langle |H_{DA}|^2 \rangle \)\textsuperscript{79–81} that determines the maximal rate of electron transfer, as both can be considered the result of electron densities overlaps. It is generally considered that the value of \( |H_{DA}|^2 \) depends exponentially on the donor–acceptor distance, so that \( |H_{DA}|^2 \approx |H_{DA}|^2 \cdot e^{-\beta r_{DA}} \), where |H\textsubscript{DA}|\textsuperscript{2} is the maximal value at “contact”; \( \beta \) is the so-called tunnelling barrier, and \( r_{DA} \) is the edge-to-edge donor–acceptor distance.\textsuperscript{81,82} Thus qualitatively the large value of J in [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1B}\textsuperscript{-}] compared to [P\textsubscript{700}\textsuperscript{+}A\textsubscript{1A}\textsuperscript{-}] is consistent with the shorter interspin distance in the former radical pair couple compared to the latter (Table 2).

The rate of (tunnelling mediated) electron transfer (\( k_{et} \)) can, in a simplified form, be described as

\[
k_{et} = \frac{2\pi}{\hbar} |H_{DA}|^2 \cdot f_{FC,DA}(T)
\]

where \( f_{FC,DA}(T) \) is a function describing the (temperature dependent) Franck–Condon factors and the other symbols have their usual physical meaning.

The kinetics of charge recombination in PS I reaction centers are often described by a biexponential decay that, when monitored by time-resolved optical spectroscopy, are characterized by lifetimes of 10–20 and 150–300 \textmu s.\textsuperscript{83} Similar differences in rates are observed by monitoring the decay of the ESE as a function of the delay from laser-flash excitation\textsuperscript{23,29} (Table 1). However, the lifetimes retrieved from analysis of pulsed-EPR data are less reliable, since both magnetic relaxation and electron-transfer processes contribute to the decay of the ESE signal.

Schloendorf and co-workers\textsuperscript{39} showed that the slower phase of charge recombination, which is characterized by a lifetime of \( \sim 150–200 \) \textmu s, is essentially temperature independent below \( \sim 150 \) K, whereas there is no similar characterization for the 10–20 \textmu s phase. Temperature independence is attained, according to Marcus treatment of tunnelling-mediated electron transfer,\textsuperscript{81} when the condition \( \Delta G^0 \approx -\lambda \) (where \( \lambda \) is the (total) reorganization energy) is verified. This is not an unrealistic proposition since the standard redox potential of P\textsubscript{700}\textsuperscript{+} is titrated at \(+450–500 \) mV while that of A\textsubscript{1A}\textsuperscript{-} has been indirectly estimated to be \( -600–780 \) mV, giving a value of \( \lambda \) of about 1–2 eV. This is within the range of reorganization energy commonly discussed for electron transfer in redox active proteins.\textsuperscript{84} Several approaches point toward a difference in standard redox potential between A\textsubscript{1A}\textsuperscript{-}/A\textsubscript{1B}\textsuperscript{-} redox couples in the order of 30–100 mV, e.g., refs 5, 30, 32, and 40. Hence the condition \( \Delta G^0 \approx -\lambda \) would also be verified for charge recombination between P\textsubscript{700}\textsuperscript{+} and A\textsubscript{1B}\textsuperscript{-}.

Alternatively, temperature independence for an electron-transfer reaction can result from coupling of the electron transfer with high-energy phonon modes.\textsuperscript{81} Considering a mean phonon frequency (\( \omega \)) that exceeds 500–700 cm\textsuperscript{-1}, the function describing the Franck–Condon factors approaches a constant, temperature-independent value. Still, it is reasonable to assume that the value of \( \omega \) is the same, or not significantly different, for the charge recombination reactions involving either A\textsubscript{1A}\textsuperscript{-} or A\textsubscript{1B}\textsuperscript{-}. Hence, whether temperature independence is due to \( \Delta G^0 \approx -\lambda \) or to coupling with high-energy phonons, \( f_{FC,P700A1A}(T) \)
oxidized, only the $[\text{P}700^-]_\text{red}$ are also observed in the wild type, with fractional amplitudes exceeding the confidence errors associated with the fit in a given condition. Then, considering $\beta$ to assume the value of 1.4 Å, commonly discussed for ET reactions involving protein-bound cofactors, taking $r$ as the edge-to-edge distance from the crystallographic data (i.e., $r_A = 19.3$ Å and $r_B = 17.3$ Å), and comparing the values of $J$ retrieved from the fitting of the ESEEM time dependence from different organisms (as this variability exceeds the confidence errors associated with the fit in a given sample), we obtain a value for $J_A/J_B$ that stands between 6 and 40. We consider this to be in reasonable agreement with both the ratio of lifetimes attributed to charge recombination (7.7 < $k_{\text{ET,B}}/k_{\text{ET,A}}$ < 30) and the value determined from edge-to-edge distance ($R \approx 16.5$ Å), taking into account that a number of (sensible) approximations and assumptions were used to derive the eq 8.

### Fractional Utilization of the PsaA-Bound and PsaB-Bound Electron-Transfer Chains

In thylakoid membranes of the wild-type RCs, under conditions in which $F_X$ is initially oxidized, only the $[\text{P}700^-/\text{A}1^-/\text{A}1^-]_\text{red}$ radical pair is observed. Under conditions which lead to $F_X$ reduction prior to the measurements, the values of the dipolar and exchange interaction energies are intermediate between those observed in the PsaA-M688H and PsaB-M668H mutants (Table 2), suggesting that a superposition of the $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ and the $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ radical pairs is observed in the wild-type RCs. In order to acquire information relating to the relative contribution of $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ and $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ in the wild type, the ESEEM time dependence was fitted by a linear combination of fit functions (eq 4) describing the signal arising from each radical pair (Figure 9A). The ESEEM is better described by a combination of two functions, each of which can be associated with the ESEEM observed in one of the mutants, weighted by amplitude factors of 0.66 and 0.34 for the $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ and the $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ radical pairs, respectively. These values are in fair agreement with the fractional amplitudes of the 2.7 and 19.8 μs phases (0.38:0.62) of the ESE decay determined under conditions of prereduced $F_X$ (Table 1).

Figure 9B also shows the reconstruction of the EPR spectrum recorded for the wild type under conditions in which $F_X$ is initially reduced, using a linear combination of the spectra recorded in the PsaA-M688H and PsaB-M668H mutants under identical experimental conditions. This very simple approach leads to a surprisingly satisfactory description of the spectra. The best fit is obtained for fractional amplitudes of the $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ and the $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ radical pair spectra of 0.78 and 0.22.

Hence, after initial prereduction of $F_X$, the two radical pairs are also observed in the wild type, with fractional amplitudes varying from 0.2:0.8 to 0.4:0.6 for the $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ and the $[\text{P}700^+/\text{A}1^{-}]_\text{red}$ radical pairs, respectively, depending on the way
would lead to either the stable reduction of $F_{AB}$ or reduction of $F_X$, followed by charge recombination in $\sim 1-3$ ms. Neither of these events contributes to the observed spin-polarized ESE signal, as the spin coherence is lost rapidly when the electrons are transferred to the first [4Fe–4S] cluster because of the very fast spin relaxation rate. It is clear that this framework explains relatively well the heterogeneity of recombination reactions observed at low temperature. In agreement with this suggestion, investigation of PsAA-M688N and PsAB-M668N strains of *Synechocystis* showed that, even though both mutants displayed a lower yield of $F_{AB}$ and $F_X$ photoreduction at low temperature, the suppression of the terminal Fe–S cluster reduction was significantly more pronounced in the case of the PsAB subunit mutant. Moreover, complete suppression of the reduction of $F_{AB}$ was observed in the PsAB-W699G mutants of *C. reinhardtii*; it was proposed that the mutation led to endergonic oxidation of $F_X$ by $A_{1B}^-$. However, in order to explain these results in this simple frame of mind, one has to put forward the hypothesis that, at a certain temperature, the RCs are frozen in a “state” (for instance, a conformer) that would utilize exclusively either ETC$_A$ or ETC$_B$. That is because, if the exergonic ETC$_B$ is used even with a low statistical probability, this would lead to the complete irreversible reduction of $F_X$ by $A_{1B}^-$. If charge separation takes place on ETC$_A$, this would result in charge recombination from *both* radical pairs, the detection of which is insensitive to the reduction state of $F_X$. The observed signal would be proportional to the simple statistical use of the ETC$_A$ and ETC$_B$ chains, which is probably determined at the level of primary charge separation. However, as spin-polarized signals arising from [$P_{700}^+A_{1B}^-$] are not observed in membranes in which $F_{AB}$ are initially oxidized (Figures 3 and 5), it can be argued that this energetic scenario is statistically very infrequent. A third limiting case is that in which the free energy for the reduction of $F_X$ by $A_{1A}^-$ is positive but that from $A_{1B}^-$ is negative, which is an “asymmetric” energetic scheme (Figure 10C). If charge separation takes place on ETC$_A$, this would result in charge recombination from the spin-correlated [$P_{700}^+A_{1A}^-$] radical pair. Charge separation events on ETC$_B$ lead to the stable reduction of $F_{AB}$ at the same time, one thing that needs to be considered is the possibility of ET between the two energetically
nonequivalent phylloquinone molecules through F_X. The occurrence of such an interquinone process has been recently discussed in order to explain the kinetics and apparent redistribution in the fractional utilization of ETC_A and ETC_B in site-directed mutants of residues involved in H-bonding to A1_A (and A1_B) in the PS I of C. reinhardtii. Since, in this case, A1_A acts as a local thermodynamic “trap”, charge recombination can then take place from the indirectly populated, and non-spin-correlated, P700^+/A1_A^- radical pair. Then, the fate of the electrons that undergo charge separation of the PsaB-bound primary radical pair is determined by the kinetic competition between F_X oxidation by A1_A and F_X. While the population of F_X leads to suppression of the transient EPR signal, the recombination between P700^- and A1_A^- (populated via A1_B^- and F_X), albeit EPR-silent, allows a repetitive flash technique experiment to be performed. This latter pathway becomes increasingly favored as the A1_A^-/A1_A redox pair becomes more oxidizing and it is therefore a function of the redox potential distribution(s). For instance, using numerical simulations, we estimated that for \( \Delta G_A^{0} \sim +60 \text{ meV} \), over 98% of the centers in such energetic configurations would undergo charge recombination reactions rather than reduction of the terminal electron acceptor at 100 K. This probability is reduced to 80% for \( \Delta G_A^{0} = +50 \text{ meV} \). In any case, it is only the direct population of [P700^-/A1_A^-] via ETC_A that is monitored. Thus, the intensity of the signal in this reaction center population is not proportional to the statistical charge separation reactions.

(iv) Finally, we consider the mirror “asymmetric” case, in which reduction of F_X by A1_A^- is exergonic and that by A1_B^- is endergonic (Figure 10D). This is obviously identical to the case discussed above but would lead to the observation of the \([P700^-/A1_B^-]\) radical pair only, independently of which ET chain is utilized.

The inability to observe the \([P700^-/A1_B^-]\) radical pair, prior to the reduction of F_X, even in the mutant in which the spin polarization of \([P700^-/A1_A^-]\) is substantially quenched, indicates that the A1_B^- oxidation is thermodynamically favorable in the vast majority of reaction centers, even in the presence of a statistical distribution of redox midpoint potentials. At the same time, it is possible to estimate that A1_A^- oxidation is energetically uphill in \( \sim 80-90% \) of the reaction centers. Still, we would like to emphasize that we are only discussing first-order approximations rather than exact estimates. In any case, even from this simplified proposition, it can be concluded that the fractional utilization of the ET chains cannot be assessed reliably by EPR methods unless the only ET reactions possible are the direct recombination from A1_A^- and A1_B^-, i.e., unless F_X is reduced prior to measurement. Under such experimental conditions, we determine (average) fractional utilization of the two electron branches in the order 0.4:0.6 in PS I of Synechocystis, which only slightly favors ETC_A. This is a substantially more symmetric distribution of electrons between ETC_A and ETC_B compared to the asymmetric figures previously reported under nonreducing conditions in the same organism, e.g., ref 41, 42, 48, and 49. The figure found here for prokaryotic RCs is in good agreement with previous estimates obtained in eukaryotic PS I either by monitoring ESE at low temperatures, \(^23,25\) i.e., monitoring electrons reaching the phylloquinones, or by ultrafast optical spectroscopy at room temperature, i.e., monitoring directly the primary charge separation reactions. \(^31\)

It is foreseeable that a more accurate description would be achieved by a careful reinvestigation of the temperature dependence of A1^- oxidation by F_X, as well as A1^- recombination with P700^+, using transient optical spectroscopy, which is not influenced by signal suppression due to quenching of spin polarization.

**Conclusion**

In the present study, we show that in the PS I reaction center of *Synechocystis 6803* it is possible to observe two spin-polarized radical pairs \([P700^-/A1_A^-]\) and \([P700^-/A1_B^-]\), which can be distinguished based on the values of spin–spin interaction (dipolar and exchange) energies, the internuclear distance, and the g-tensor orientation with respect to the internuclear axis. The two radical pairs are populated as a result of ET reactions occurring through two parallel ET chains, one principally coordinated by the PsaA subunit (ETC_A), the other by the PsaB subunit (ETC_B); i.e., electron transfer in PS I of *Synechocystis* is bidirectional. Moreover, we conclude that ETC_A and ETC_B have similar statistical utilization (branching). These results, which are in excellent agreement with previous investigations of wild-type and site-directed mutants of the eukaryotic *C. reinhardtii*, demonstrate that bidirectional electron transfer is a general property of PS I.

Moreover, we propose a revised model, which is capable of describing, from a qualitative point of view, the well-known heterogeneity of ET reactions in PS I reaction centers at low temperatures. In order to accommodate results previously reported in the literature, it is necessary to consider (i) an asymmetric driving force for A1_A^- (endothermic) and A1_B^- (exothermic) oxidation by F_X, and (ii) a distribution of driving forces associated with each of these reactions.

**List of Abbreviations.** PS I, photosystem I; RC, reaction center; P700, photosystem I primary donor (cation); A0, primary electron donor; A1^- phylloquinone acceptor (anion; semiphylo-quinone); A1_A/A1_B^- phylloquinone bound to the PsaA or PsaB reaction center subunits of PS I; F_X(A/B) iron–sulfur cluster(s) (X/A/B); EPR, electron paramagnetic resonance; ESE, electron spin echo; ESEEEM, electron spin echo envelope modulation; ETC_A/ETC_B, electron-transfer chain bound to the PsaA or PsaB subunit.

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**References and Notes**


