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Effect of the P700 pre-oxidation and point mutations near A_0 on the reversibility of the primary charge separation in Photosystem I from *Chlamydomonas reinhardtii*

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ABSTRACT

Time-resolved fluorescence studies with a 3-ps temporal resolution were performed in order to: (1) test the recent model of the reversible primary charge separation in Photosystem I (Müller et al., 2003; Holwzwarth et al., 2005, 2006), and (2) to reconcile this model with a mechanism of excitation energy quenching by closed Photosystem I (with P700 pre-oxidized to $P700^+$). For these purposes, we performed experiments using Photosystem I core samples isolated from *Chlamydomonas reinhardtii* wild type, and two mutants in which the methionine axial ligand to primary electron acceptor, A_0, has been change to either histidine or serine. The temporal evolution of fluorescence spectra was recorded for each preparation under conditions where the "primary electron donor," P700, was either neutral or chemically pre-oxidized to $P700^+$. For all the preparations under study, and under neutral and oxidizing conditions, we observed multiexponential fluorescence decay with the major phases of ~7 ps and ~25 ps. The relative amplitudes and, to a minor extent the lifetimes, of these two phases were modulated by the redox state of P700 and by the mutations near A_0: both pre-oxidation of P700 and mutations caused slight deceleration of the excited state decay. These results are consistent with a model in which P700 is not the primary electron donor, but rather a secondary electron donor, with the primary charge separation event occurring between the accessory chlorophyll, A, and A_0. We assign the faster phase to the equilibration process between the excited state of the antenna/reaction center ensemble and the primary radical pair, and the slower phase to the secondary electron transfer reaction. The pre-oxidation of P700 shifts the equilibrium between the excited state and the primary radical pair towards the excited state. This shift is proposed to be induced by the presence of the positive charge on $P700^+$. The same charge is proposed to be responsible for the fast $A^+A_0^- \rightarrow AA_0$ charge recombination to the ground state and, in consequence, excitation quenching in closed reaction centers. Mutations of the A_0 axial ligand shift the equilibrium in the same direction as pre-oxidation of P700 due to the up-shift of the free energy level of the state $A^+A_0^-$.

1. Introduction

Photosynthetic reaction centers (RC), in which the energy of electronically excited molecules is converted into the energy of charge separated states, are equipped with two branches (A and B) of electron transfer cofactors embedded in a protein matrix. In a classical view, the primary electron donor is a dimer of more or less strongly interacting molecules of (bacterio)chlorophylls, depending on the organism, positioned at one end of the two branches. Whereas in purple bacterial RCs this view is commonly accepted [1], in Photosystem I (PSI) it was recently proposed that the true primary donor is in fact the accessory chlorophyll (A) positioned in between the chlorophyll dimer (P700) and the chlorophyll serving as the primary electron acceptor (A_0) [2]. According to this model, P700 is a secondary electron donor and gives the electron to A^+ only in the secondary electron transfer step, forming the state $P700^+A_0^-$. A similar sequence of primary electron transfer events was also proposed for Photosystem II [3–5].

Resolving the primary electron transfer steps in Photosystem I is difficult because it binds as many as 90 antenna Chls, in addition to 6 electron transfer Chls [6] and excitation dynamics occurs on the same time scale as that of the primary electron transfer events [7,8]. Decay of the excited states coupled to electron transfer occurs on a 20- to 30-ps time scale [9–11], whereas intrinsic primary charge separation

Abbreviations: A_0, primary acceptor; A_1, secondary acceptor; Chl, chlorophyll; LHCI, light-harvesting complex I; P, primary donor; PSI, Photosystem I; RC, reaction center; WT, wild type

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from the excited primary donor is much faster and is estimated to occur on the subpicosecond to single picoseconds time scale [12–15]. Formation of the state $P700^+A_0^-$ is followed by 10- to 30-ps electron transfer from A_0^- to A_1, the secondary phylloquinone electron acceptor [12,14,16–18]. Only the next electron transfer step, from A_1^- to first iron-sulfur cluster, F_x, occurs on a slower, nanosecond time scale [19–24], and can be easily separated from the excitated state dynamics. Electron transfer in PSI occurs along both A and B branches of cofactors [22–30].

The different kinetic schemes for excited-state dynamics in PSI discussed in the literature usually assume an irreversible character of the primary charge separation and can be divided, in general, into two models called trap-limited and transfer-to-trap-limited, respectively [31]. In the trap-limited model, the energy equilibration between the antenna and the RC is assumed to be very fast and completed before the trapping event (caused by charge separation in the RC) occurs. In the transfer-to-trap-limited model, the excitation energy transfer to the RC is assumed to be slower than the charge separation step, and in consequence, energy equilibration between the antenna and the RC is not established before trapping occurs. In recent studies of PSI from *Chlamydomonas reinhardtii* and higher plants, the trap-limited model was proposed to describe the observed excited-states dynamics [11,32–35]. The overall kinetics in cyanobacterial PSI complexes was suggested by different groups to be either trap-limited [36,37] or transfer-to-trap-limited [9,10,14,15,38]. Recently, models assuming reversibility of the primary charge separation in PSI, and demonstrating the impact of this reversibility on the excitation dynamics, have been proposed [32,33,35,37].

In order to best resolve the kinetics of pure electron transfer reactions in PSI, not contaminated by the excitation energy dynamics, a few groups have the approach of subtracting the time-resolved transient absorption spectra recorded for the closed state of PSI (P700 pre-oxidized to $P700^+$) from those recorded for the open state (P700 neutral) [39–42]. This approach assumes that the excitation dynamics is identical for the two states, an assumption that is supported by experiments showing roughly similar monoexpoenential excited state decay in these two cases [39,43–47]. To rationalize this similarity, it is assumed that the quenching efficiency of P700 and $P700^+$ are identical, although the mechanism underlying the quenching mechanism by $P700^+$ is unknown. The reversible model for the primary charge separation in PSI, implying a more complex multiexponential decay of excited states [2,32,33], is at odds with the idea that P700 and $P700^+$ have identical quenching properties, and challenges the correctness of the subtraction procedure described above.

In order to critically test the recent models of energy transfer and reversible charge separation in *C. reinhardtii* PSI, and to gain a deeper insight into the mechanism of the excitation energy quenching in closed PSI, we have performed time-resolved fluorescence measurements on PSI-core preparations from wild type (WT) and two mutants (MHB and MSB, or PsaB: M664H and M664S) with open or closed RCs. In the mutants, the methionine axial ligand to the primary acceptor A_0 in the B-branch of the electron transfer cofactors was replaced by histidine (MHB) or serine (MSB). By using optical methods, these mutations have been previously shown to block the electron transfer from A_0^- to the secondary electron acceptor A_1 by modifying the properties of A_0 [28–30] (similar mutations were also characterized by using EPR [25–27]). Both pre-oxidation of P700, and mutations of the A_0 axial ligands, were expected to influence the primary electron transfer events.

The dynamics of the excited states strongly depends on the presence of so-called red chlorophylls in the investigated PSI particles that makes the interpretation of the experimental data more difficult. However, in contrast to what is observed in PSI from cyanobacteria and higher plants [9,10,35,37,48], no indications of red Chls absorbing above 700 nm were found in the PSI core particles isolated from CC 2696 strain of *C. reinhardtii* [11,49], which were investigated in this contribution.

2. Materials and methods

The experiments were performed on wild type (WT) and on MHB and MSB mutants in which the methionine axial ligand to the primary electron acceptor A_0 in branch B of electron transfer cofactors (Met B664) was replaced with histidine (MHB) or serine (MSB) using methods described previously [50,51]. The *C. reinhardtii* strain for transformation was CC2696 in WT and mutants, and was obtained from the *Chlamydomonas* Culture Collection at Duke University. The CC2696 strain carries a deletion in the chloroplast *psbA* gene that causes a complete loss of Photosystem II, and also contains the DS-521 nuclear mutation leading to a 90% reduction in LHC II content. The cells were grown in CC liquid medium [51] and then the thylakoid membranes were isolated according to the method presented in [52]. The PSI complexes were extracted from thylakoid membranes and purified using protocols described previously [28], and finally suspended in a buffer containing 50 mM HEPES (pH = 7.2), 5 mM $MgCl_2$, 12 mM $CaCl_2$, 20% glycerol (v/v), 1 mM benzamidine, 1 mM aminocapric acid, 1 mM EDTA, and 0.03% dodecyl maltoside. During experiments the primary donor was kept neutral (open RC) by addition of 20 mM sodium ascorbate and 20 μM phenazine methosulfate or oxidized (closed RC) by addition 3 mM $K_3(FeCN)_6$.

The time-resolved fluorescence measurements were carried out with a Streak camera setup. Excitation pulses of 400 nm with a time duration of ~100 fs and were generated in a system composed of a titanium:sapphire laser (Coherent, Vitesse), a regenerative amplifier (Coherent, RegA) and a double pass optical parametric amplifier (Coherent, OPA). The sample was excited with vertically polarized 1.2-nJ pulses (which corresponds to ~0.1 excitations per PS1) and a repetition rate of 125 kHz. The fluorescence was detected without any polarizer at a right angle with respect to the excitation beam using a spectrograph (Chromex 250IS) and streak camera (Hamamatsu C5680), and recorded by a CCD camera (Hamamatasu C4880). The temporal width of the detection system response function was ~3 ps (FWHM). The sample was placed in a rotating cuvette to ensure that each laser pulse illuminated a fully relaxed sample. The exposure times per image were 8–12 min and 4–6 min for the time windows of 200 ps and 500 ps, respectively. The detected streak images from both time windows were analyzed globally together from 635 to 780 nm with 5 nm resolution and decay-associated spectra (DAS) were obtained. The data were also modeled using the target analysis method [53,54].

3. Results

Fig. 1 shows decay associated spectra (DAS) of three preparations: WT and two A_0 mutants of PSI from *C. reinhardtii* in either the open or closed state. DAS are the wavelength-dependent pre-exponential factors, $A_i(\lambda)$, of the multiexponential fluorescence (Fl) decay components, $Fl = \Sigma\ A_i(\lambda)\exp(-t/\tau_i)$, associated with particular exponential lifetimes, τ_i. They were determined from the global fitting performed simultaneously for all wavelengths from the recorded spectral range of fluorescence emission (see [53,54] for further details on global fitting). The subpicosecond spectra are assigned to internal conversion of Chls from their Soret to Q_y state. The negative amplitudes of these spectra are due to the appearance of fluorescence in Q_y region. The 4.5- to 5-ns spectra are assigned to Chls uncoupled from the electron transfer reactions, whereas the ~100- to 200-ps spectra with very small amplitudes are assigned to a minor fraction of PSI particles showing either slow excitation energy transfer to RCs or nonphotochemical quenching. On the basis of the spectra presented in Fig. 1, the contribution of PSI particles showing these two types of slow decay is estimated to be below 15%. Both these phases were often observed in PSI core preparations from *C. reinhardtii* [11,29,32] and will not be discussed further here.