THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

pubs.acs.org/JPCB

Primary Electron Donor(s) in Isolated Reaction Center of Photosystem II from *Chlamydomonas reinhardtii*

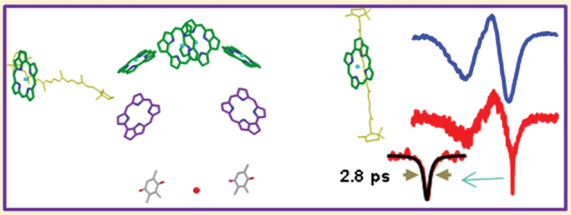
Khem Acharya,[†] Valter Zazubovich,[§] Mike Reppert,[∥] and Ryszard Jankowiak*[,†,‡]

[†]Department of Chemistry and [‡]Department of Physics, Kansas State University, Manhattan, Kansas 66506, United States

[§]Department of Physics, Concordia University, Montreal, Quebec, Canada

[∥]Department of Chemistry, MIT, Cambridge, Massachusetts 02139, United States

ABSTRACT: Isolated reaction centers (RCs) from wild-type *Chlamydomonas (C.) reinhardtii* of Photosystem II (PSII), at different levels of intactness, were studied to provide more insight into the nature of the charge-separation (CS) pathway(s). We argue that previously studied D1/D2/Cyt_{b559} complexes (referred to as RC680), with Chl_{D1} serving as the primary electron donor, contain destabilized D1 and D2 polypeptides and, as a result, do not provide a representative model system for the intact RC within the PSII core.



The shapes of nonresonant transient hole-burned (HB) spectra obtained for more intact RCs (referred to as RC684) are very similar to $P^+Q_A^- - PQ_A$ absorbance difference and triplet minus singlet spectra measured in PSII core complexes from *Synechocystis* PCC 6803 [Schlodder et al. *Philos. Trans. R. Soc. London, Ser. B* **2008**, *363*, 1197]. We show that in the RC684 complexes, both P_{D1} and Chl_{D1} may serve as primary electron donors, leading to two different charge separation pathways. Resonant HB spectra cannot distinguish the CS times corresponding to different paths, but it is likely that the zero-phonon holes (ZPHs) observed in the 680−685 nm region (corresponding to CS times of ∼1.4−4.4 ps) reveal the Chl_{D1} pathway; conversely, the observation of charge-transfer (CT) state(s) in RC684 (in the 686−695 nm range) and the absence of ZPHs at $\lambda_B > 685$ nm likely stem from the P_{D1} pathway, for which CS could be faster than 1 ps. This is consistent with the finding of Krausz et al. [*Photochem. Photobiol. Sci.* **2005**, *4*, 744] that CS in intact PSII core complexes can be initiated at low temperatures with fairly long-wavelength excitation. The lack of a clear shift of HB spectra as a function of excitation wavelength within the red-tail of the absorption (i.e., 686−695 nm) and the absence of ZPHs suggest that the lowest-energy CT state is largely homogeneously broadened. On the other hand, in usually studied destabilized RCs, that is, RC680, for which CT states have never been experimentally observed, Chl_{D1} is the most likely electron donor.

1. INTRODUCTION

Photosystem II (PSII), the only protein complex capable of evolving oxygen, performs the primary charge separation (CS) in the D1/D2/Cyt_{b559} reaction center (RC)[1] in oxygenic photosynthesis (plants, cyanobacteria, and algae). According to the recent X-ray crystal structure[2] (from *T. vulcanus*), the PSII RC complex contains six chlorophyll (Chl) and two pheophytin (Pheo) molecules, two plastoquinones (Q_A and Q_B), two β-carotene molecules (Car_{D1} and Car_{D2}), a Cytochrome *b*-559 (Cyt_{b559}), and a nonheme iron. Figure 1, based on the recent crystal structure of PSII (PDB ID 3ARC),[2] shows a schematic arrangement of the six Chls, two Pheos, Q_A, Q_B, Car_{D1}, and Car_{D2} molecules, and a nonheme iron (Fe). This structure further confirmed that the amino acid sequences of the membrane bound polypeptides D1 and D2 subunits of PSII RC are homologous to their counterparts L and M subunits, respectively, of purple bacterial RC (BRC), whose crystal structure[3] has been solved at atomic resolution earlier. The P_{D1} and P_{D2} Chls are structurally analogous to the P_L and P_M BChls of the bacterial special pair, respectively, while the $Chl_{D1,D2}$ and $Pheo_{D1,D2}$ molecules correspond to the monomeric $BChl_{L,M}$ and $BPheo_{L,M}$ molecules (where the subscripts represent the respective polypeptide chains to which the chlorines are bound).[4] Unlike in BRC, PSII RC contains two additional peripheral Chls, bound by the histidines of D1 and D2 polypeptides, and sometimes referred to as $Chlz_{D1}$ and $Chlz_{D2}$.[5] In both BRC and PSII RCs, the pigments are organized and function in a similar way, with a structural pseudo-C_2 structural symmetry but functional asymmetry of their two branches D1/L and D2/M.[6] By analogy with the BRC, it is believed that the D1 protein chain of PSII RC is photochemically active, and thus the P_{D1}/P_{D2}, Chl_{D1}, and $Pheo_{D1}$ molecules participate in primary charge separation.[1] The two Chl monomers, P_{D1} and P_{D2}, form a dimer with a partial structural overlap, stabilized by van der Waals interaction of about −17 kcal mol^{-1}.[7] Differences in the immediate environment of the two pigments of the dimer cause localization of the major portion of dimer's HOMO at the monomer P_{D1}.[7] It has also been recently shown that redox potential (E_m) of P_{D1} for one electron oxidation ($E_m(P_{D1})$) is lower than that of P_{D2}, favoring the localization of the cationic charge of the primary charge transfer (CT) state on P_{D1}.[8]

Received: March 25, 2012
Revised: March 29, 2012
Published: March 30, 2012

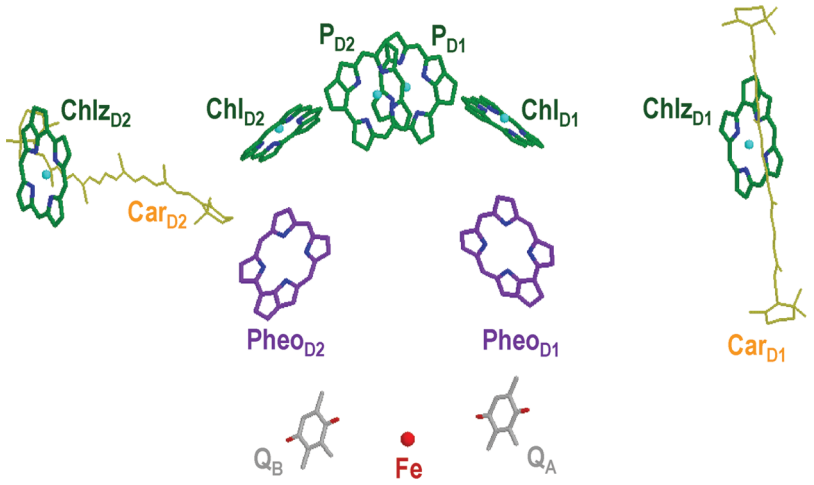


Figure 1. Cofactor arrangement in the active (D1) and inactive (D2) branches of Photosystem II reaction center from the crystal structure of *T. vulcanus* at 1.9 Å resolution, PDB ID 3ARC.[2] The cofactors are color coded as chls, green; carotenes, yellow; pheophytins, purple; plastoquinones, gray; nonheme iron, red; and nitrogen, blue. The substituents of the cofactors are truncated for clarity.

Since the first isolation of the PSII RC in 1987 by Nanba and Satoh,[9] its biophysical processes, including excitation energy transfer (EET) and primary charge separation (CS), have been the subject of many studies: spectral hole-burning (SHB),[10−14] stark spectroscopy,[15] photon echo,[16] time-resolved fluorescence,[17] visible pump−probe,[18] 2D spectroscopy,[19] mutagenesis studies,[20−23] and theoretical calculations.[24−26] Although in recent years consistent progress has been made in comprehending the electronic structure and charge transfer dynamics of PSII RC,[25,27,28] an adequate global understanding of this complex system has yet to be achieved.

One significant obstacle to our understanding of the PSII RC is that, in contrast to the BRC (which can be isolated and crystallized while maintaining full functional), the PSII RC is considerably more fragile during isolation and interrogation procedures[29] and retains only limited PSII functionality in its isolated form.[28] Although isolation and purification protocols have been consistently improved over the years, these complexes are highly fragile and the right choice of buffer and detergent is critical; for example, use of Triton X-100 has been found to significantly affect the low temperature absorption and persistent hole spectra and to disrupt the charge transfer from accessory Chl *a* to the active pheophytin ($Pheo_{D1}$).[11] Moreover, it has been reported that some integral parts of the native PSII RC, that is, Q_A and Q_B secondary electron acceptors, as well as 4-Mn clusters, are lost during the isolation.[9,21] It has also been suggested that the isolated RC samples possess two subsets of RCs, destabilized RC680 and more intact RC684, the latter being vulnerable and changing to RC680 upon biochemical manipulation.[13,30] In this regard, it is likely that after isolation, the coupling between P_{D1} and P_{D2} is weakened, resulting in the blue-shifted absorption spectrum. Our current data also suggest (see section 3.1) that all previously studied RCs from spinach were destabilized with the electronic structure significantly different with respect to that observed in intact PSII core[27,31] complexes, calling into question the validity of the use of the isolated RCs as model systems of intact RC in PSII core.

In addition to these sample preparation considerations, the PSII RC is considerably more difficult to study than its purple bacterial counterpart due to the inherent spectral congestion of the Q_y absorption region of the PSII RC.[32] In contrast to the three well-resolved absorption bands per 6 pigments of the BRC from *Rps. Viridis*,[33] the low temperature Q_y-region absorption spectrum of the PSII RC displays only two obvious Q_y bands corresponding to 8 pigments (6 Chl and 2 Pheo). This severe spectral congestion both makes it very difficult experimentally to selectively excite any particular state (or pigment) and introduces significant uncertainty in theoretical interpretation of the transient absorption data in both time- and frequency-domain experiments because ultrafast energy-equilibration, radical pair formation, and excitation energy transfer all happen in the same narrow spectral region.[34]

Thanks in part to these technical difficulties, there are still numerous issues to be addressed regarding primary CS processes in the PSII RC. For example, the question of whether the primary electron donor is P_{D1} or Chl_{D1}[27,28,34,35] is still controversial. Also, the site energies of RC pigments reported in various papers are not consistent.[17,24,26,35,36] A recent modeling study[24,36] of the electronic structure of PSII RC has suggested that the mean site energy of $Pheo_{D1}$ is near 672 nm, whereas that of $Pheo_{D2}$ is at ∼677.5 nm. We show that the Q_x-/Q_y-region site energies of $Pheo_{D1}$ and $Pheo_{D2}$ in PSII RC are ∼545/680 nm and ∼541.5/670 nm, respectively.[37] Furthermore, although spectral features interpreted as belonging to the primary CT state were experimentally observed in the intact PSII core,[31] such features have never been identified in the isolated PSII RC; moreover, the nature of the suggested CT state still remains to be unequivocally resolved.

To provide more insight into the nature of the PSII RC, and resolve the aforementioned issues, we focus our SHB studies on the isolated RCs obtained from *Chlamydomonas (C.) reinhardtii*. To our knowledge, there are only a few studies of the isolated algal PSII RC from *C. reinhardtii* because of the difficulties of their preparation.[5,20,21,38] *C. reinhardtii*, a unicellular green alga flagellate possessing a single chloroplast, is an important model for the fundamental studies of photosynthesis as well as for molecular biology studies.[39] In this Article, we present optical spectra such as low-T absorption, and transient hole-burned (HB) spectra obtained for isolated PSII RC from *C. reinhardtii*, at different levels of intactness, studied during the last 5 years. A new assignment of the Q_x- and Q_y-region site energies of $Pheo_{D1}$ and $Pheo_{D2}$ (based on mutational and modeling study, and incompatible

dx.doi.org/10.1021/jp302849d | J. Phys. Chem. B 2012, 116, 4860−4870

with refs 24,36) is published elsewhere.[37] Here, we report the comparison of our RC data with those previously obtained for the isolated spinach RC[10−14] and the PSII core,[27,31,36] and demonstrate that previously studied isolated D1/D2/Cyt$_{b559}$ complexes had destabilized D1 and D2 polypeptides and therefore do not provide a proper model system for the intact RC within the PSII core. Our results reported below indicate that the nature of the primary electron donor in isolated PSII RC depends on its intactness. Our findings should be useful for future modeling of the excitonic structure, which should provide a more complete picture of the EET pathways and charge separation (CS) in PSII core complexes.

2. EXPERIMENTAL SECTION

Photosystem II RC complexes from *C. reinhardtii* containing 6 Chls per 2 Pheos from both thylakoids and PSII-enriched membranes following the method of Nanba and Satoh,[9] with important modifications,[21] were prepared by Dr. R. Picorel in Dr. M. Seibert's laboratory at NREL (Golden, CO). Preparation of isolated RCs from both thylakoids or PSII-enriched membranes and their basic spectroscopic characterization and pigment analysis are described in ref 37.

The hole burning apparatus and measurements were described in detail elsewhere.[40] Briefly, a Bruker HR125 Fourier transform spectrometer and a Janis 8-DT Super Vari-Temp liquid helium cryostat were used to measure the absorption and HB spectra at 5 K. Nonresonant and resonant HB spectra were recorded at a resolution of 4 and 0.5 cm^{-1}, respectively. For some nonresonant HB, a 496.5 nm Coherent Innova 200 argon ion laser was employed. For resonant HB experiments, a tunable Coherent CR699-21 ring dye laser (Exciton LD688; range 650−710 nm; line width of 0.07 cm^{-1}) was used, pumped by a 532 nm Spectra-Physics Millennia diode laser. The latter laser system was also used to obtain nonresonant HB spectra with 665.0 nm excitation. The laser output was stabilized using LPC from Brockton Electro-Optics Corp. Sample temperature was read and stabilized with a Lakeshore Cryotronic model 330 temperature controller. The transient spectra reported in this work correspond to the difference of absorption spectrum with laser on and absorption spectrum with laser off. This difference is due to dynamic depopulation of the singlet ground state for the duration of the (long) lifetime of either triplet state (triplet-bottleneck hole) or charge-separated state. Burn intensities and times are given in the figure captions.

3. RESULTS

3.1. Low Temperature Absorption Spectra for Isolated RC of *C. reinhardtii*. Frames A−C of Figure 2 show the Q_y- (main frames) and Q_x-region (respective insets) absorption spectra obtained for three RC samples from *C. reinhardtii* (out of 10 samples studied). The gray dashed curves in frames A and B of Figure 2 correspond to the absorption spectrum from frame C (shown for comparison). These data are representative of variability observed in both absorption and transient HB spectra (frames E, F) of isolated RCs studied in our laboratory. Samples whose absorption spectra are shown in frames A−C of Figure 2 will be referred to as RC$_{S1}$, RC$_{S2}$, and RC$_{S3}$, respectively. Absorption spectra are normalized at the Q_x transition of the pheophytins (Pheos) and are similar (but not identical) to those typically observed in the PSII RC of spinach.[10−14,32] The integrated absorption in all three samples

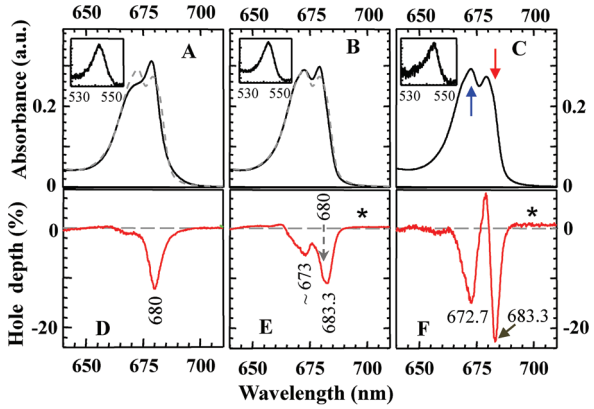


Figure 2. Normalized absorption spectra (at Q_x transition of Pheos) of three representative RC samples RC$_{S1}$, RC$_{S2}$, and RC$_{S3}$ (obtained from *C. reinhardtii*) are shown in the frames A, B, and C, respectively. Corresponding transient HB spectra are shown in frames D, E, and F. The insets in frames A, B, and C show the Q_x absorption band of pheophytins. All HB spectra were obtained with λ_B = 665.0 nm and laser intensity of ∼100 mW/cm^2. The dotted gray curve in frames A and B is the absorption spectrum from frame C and is shown for easy comparison. All spectra were measured at T = 5 K.

is similar, in agreement with pigment extraction analysis, which showed that all preparations had a similar number of Chls per two Pheos (i.e., 6.0 ± 0.5 Chl *a* per 2 Pheo *a*), as discussed in ref 37. Furthermore, there was no indication that any of these samples were contaminated with antenna complexes; that is, no bleaching and/or emission characteristic for PSII antenna complexes[41,42] was observed. Comparison of the three frames reveals that the major differences between absorption spectra of RC$_{S1}$, RC$_{S2}$, and RC$_{S3}$ samples occur near 673 and 684 nm. The comparison of the Q_x-regions, on the other hand, shows similar spectral shapes with the same integrated area, but with a slightly red-shifted Q_x-band of pheophytins in RC$_{S3}$. The maxima of the Pheo Q_x-band in RC$_{S1}$, RC$_{S2}$, and RC$_{S3}$ are at 542.5, 543.0 (most frequently observed Q_x-transition wavelength of both pheophytins[43−45]), and 544.2 nm, respectively. The position of the most red-shifted RC$_{S3}$ Q_x-band is similar to that observed in intact PSII core complexes,[31,46] suggesting that the RC$_{S3}$ sample is the most intact one. On the basis of the above-mentioned discrepancies in the Q_x- and Q_y-regions (along with varied intensities near ∼673 and at ∼684 nm), we suggest that RC$_{S1}$, RC$_{S2}$, and RC$_{S3}$ consist of varying proportions of different subpopulation of RCs, in agreement with our earlier RC680/RC684 model for isolated RCs from spinach;[13,32] see section 4.1 for discussion.

3.2. Nonresonant Transient HB Spectra. Frames D, E, and F in Figure 2 show transient holes obtained for RC$_{S1}$, RC$_{S2}$, and RC$_{S3}$, samples, respectively, with excitation wavelength (λ_B) of 665.0 nm at T = 5 K. As described in the Experimental Section, transient holes in our measurements are due to dynamic depopulation of the singlet ground state for the duration of the (long) lifetime of either triplet state (triplet-bottleneck hole) or charge-separated state. The transient HB signal thus serves as a specific indicator of the presence of these types of excited states. Note that the positions/shapes and depths of the transient holes in frames D, E, and F are significantly different. For example, the transient hole spectrum of RC$_{S1}$ (frame D) has a bleach at ∼680 nm, typically observed in isolated RC from spinach,[11,12,14] while those of RC$_{S2}$ (frame E) and RC$_{S3}$ (frame F) have pronounced bleaches at ∼673 nm

dx.doi.org/10.1021/jp302849d | J. Phys. Chem. B 2012, 116, 4860−4870

and at ~683−684 nm. Also note that the delta absorption in the longer wavelength (>690 nm) region in frame D (and in refs 11,12,14) is zero, while a weak positive delta absorption (see asterisks) is observed in frame E and, especially, in frame F (see also Figure 5). Note that while the transient hole of RC_{S1} is similar to those of previously studied RCs,[10,32,47] and to flash-induced ($^3P - {}^1P$) absorption spectra of PSII cores,[23,48] the transient hole obtained for RC_{S3} is similar (although not perfectly identical) to flash-induced ($P680^+Q_A^- - P680Q_A$) absorption difference spectra of the intact PSII core.[22,27,30,48] These differences in optical spectra of different RC preparations, in particular in transient holes, suggest a large heterogeneity of all isolated RCs studied so far, most likely due to the presence of different RC subpopulations, with the RC_{S1} transient HB features arising largely from a long-lived triplet state and the RC_{S3} spectra from transient chemical oxidation and accompanying electrochromic shifts (i.e., $P^+Q_A^- - PQ_A$). In this light, we will argue below that some of our more intact RCs contain plastoquinone, Q_A, which is lost in isolated RCs from spinach.[9,21] We will also argue that the spectra shown for RC_{S1} in frames A and D of Figure 2 correspond to the destabilized RCs (referred to as RC680), which are similar to the often studied isolated RCs from spinach,[11,12,14] whereas the data shown for RC_{S3} in frames C and F represent the most intact isolated RCs studied so far. The latter closely resembles the RCs within PSII cores.[27,31,36] Intact RCs will be referred to as RC684. The data in frames B and E can be understood assuming that RC_{S2} sample represents a mixture of RC_{S1} and RC_{S3} samples (see Figure 4A for details).

Figure 3 shows the comparison of two transient holes (λ_B = 665.0 nm) obtained for our most intact sample (RC_{S3}; curve a)

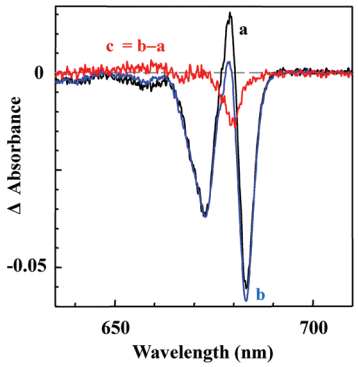


Figure 3. Transient HB spectra. Curves a and b represent the transient HB spectra of intact and partly damaged RC_{S3d} obtained with λ_B = 665.0 nm and laser intensities I = 100 mW/cm^2, respectively. The difference between curves b and a is shown as curve c.

and partly damaged RC_{S3d} (obtained by allowing the RC_{S3} sample to interact with a buffer/glycerol mixture at room temperature in the presence of light for about 5 min; curve b). Curves a and b are normalized at ~673 nm. Interestingly, the difference between the spectra of damaged and intact samples (red curve c = b − a) reveals contribution resembling a transient hole typically observed in RC680 (i.e., to transient spectra of many previously studied RCs isolated from spinach[10,32,47]), clearly indicating that RC684 are not stable after isolation and are very sensitive to sample handling procedures. Note that the hole in curve c (peaking at ~680 nm) is also very similar to the transient hole reported in frame D of Figure 2 (RC_{S1} sample).

Frames A and B of Figure 4 show that the shapes of transient HB spectra in RC_{S2} and RC_{S3} depend on excitation wavelength

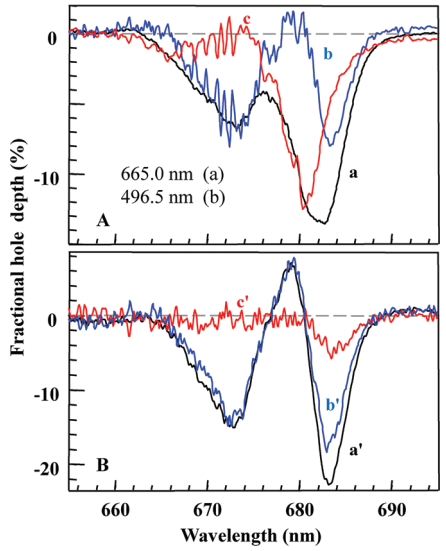


Figure 4. Transient HB spectra. Frames A and B represent the transient HB spectra of RC_{S2} and RC_{S3}, respectively, obtained with λ_B of 665.0 nm (curves, a/a′) and 496.5 nm (curves, b/b′). The red curves c/c′ are obtained as the difference of a/b and a′/b′, which correspond to triplet bleach in RC680 and RC684, respectively.

(although for RC_{S3} the effect is significantly smaller than for RC_{S2}). The curves a (a′) and b (b′) were obtained with λ_B of 665.0 and 496.5 nm, respectively. Note that curves a and b in frame A for RC_{S2}, when normalized at ~673 nm bleach, have significantly different behavior near 680 nm, in contrast to curves a′ and b′ in frame B (RC_{S3}). Although we will not make any assumptions as to why exactly excitation at different wavelengths results in preferential transient bleaching of different pigments in the RC or of different RC subpopulations in the heterogeneous samples, the very existence of qualitative differences between transient holes obtained with λ_B of 665.0 and 496.5 nm indicates the presence of such different subpopulations. Thus, the differences between transient holes reflect selected/amplified contributions from one or another subpopulation. The difference between spectra a and b in frame A (RC_{S2}; curve c) exhibits a major bleach at 680 nm. This bleach is very similar to that observed in RC_{S1} (Figure 2D) and isolated spinach RCs.[10,32,47] Note that this hole is also nearly indistinguishable from curve c in Figure 3 corresponding to deliberately destabilized sample RC_{S3d}. On the other hand, the difference between spectra a′ and b′, in frame B, exhibits major contribution at ~684 nm. The fact that near 684 nm one transient hole (curve a′) is deeper than another (curve b′) may have interesting implications that are discussed in section 4.3. Here, we only mention that these differences are likely connected to the presence of two different subpopulations of intact isolated RCs, with Q_A present and absent. Note that a similar band near 684 nm (manifested as a weak shoulder) was often observed in isolated spinach RC in transient HB experiments,[13,32,49] and by a selective 685 nm excitation in evolution-associated difference spectra (EADS) in time-resolved experiments,[34] although the origin of such contribution was interpreted differently (see section 4.3). Spectrum b′ (frame B) has more positive absorbance near 680 nm than does spectrum b (in frame A). This is consistent with RC_{S2} having

dx.doi.org/10.1021/jp302849d | J. Phys. Chem. B 2012, 116, 4860−4870

more contribution from the destabilized RC680 complexes. This also agrees with the major bleach in curve a′ (RC_{S3} sample; Figure 4B) being red-shifted (to ~683.3 nm) in comparison with curve a obtained for RC_{S2} and shown in Figure 4A (~682 nm).

A weak but distinct positive absorption feature is observed in the transient HB spectra of the RC_{S3} and RC_{S2} samples in the long wavelength region (690−860 nm). The mainframe of Figure 5 shows a close-up view of this feature observed in the

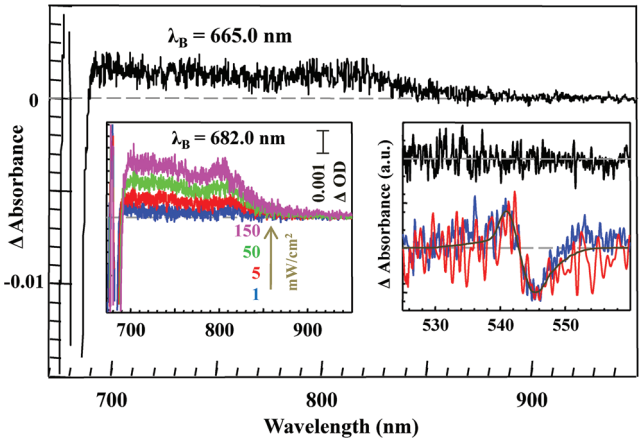


Figure 5. Transient hole-burned spectra of various RC samples. In the mainframe, the black curve represents the absorbance due to the oxidation of P_{D1} of RC_{S3}, obtained with λ_B of 665 nm and intensity of 100 mW/cm^2. Left inset corresponds to the absorption of P_{D1}^+ in RC_{S2} obtained with λ_B of 682 nm and laser burn intensities (I) of 1, 5, 50, and 150 mW/cm^2. Right inset represents the electrochromic shift in Pheo Q_x-band of spinach RC sample (black), RC_{S2} (blue), and RC_{S3} (red) obtained with λ_B of 665.0 nm.

nonresonant transient HB spectrum (λ_B = 665 nm, I = 100 mW/cm^2). The positive transient response is the strongest in RC_{S3} sample, although similar behavior was also observed in RC_{S2}, in particular for the resonant excitation at λ_B = 682.0 nm as shown in the left inset of Figure 5 for four different laser burn intensities (1, 5, 50, and 150 mW/cm^2). Although a full discussion of these features will be provided in section 4.2, we should note here that similar absorbance increases have been observed in chemically oxidized RCs[50] and flash-induced ($P684^+Q_A^-$ − $P684Q_A$) absorbance difference spectra of PSII core complexes from WT *Synechocystis* sp. PCC6803,[22,27,48] which should possess intact RCs. We stress that such behavior has never been observed in (isolated) destabilized RC680 complexes previously studied under identical conditions. In agreement with our earlier observations on transient response in the 670−690 nm region, the similarity of these spectra to flash-induced ($P684^+Q_A^-$ − $P684Q_A$) difference spectra suggests that at least some fraction of the RC684 samples studied here retain the attached plastoquinone Q_A and are capable of forming the transient $P684^+Q_A^-$ state. By the same token, the absence of this response in RC680 preparations suggests that isolated RC680 and RC684 samples may possess different charge separation pathways as will be discussed in more detail in sections 4.1−4.3. Note, however, that our attempts to directly identify the presence of Q_A in RC_{S3} via extraction of cofactors and subsequent mass spectrometry and NMR experiments were inconclusive, most likely due to very small amounts of these highest-intactness RCs available for these measurements.

3.3. Resonant Hole-Burned (HB) Spectra. Figure 6 shows nonresonant transient HB spectrum (4 cm^{-1} resolution)

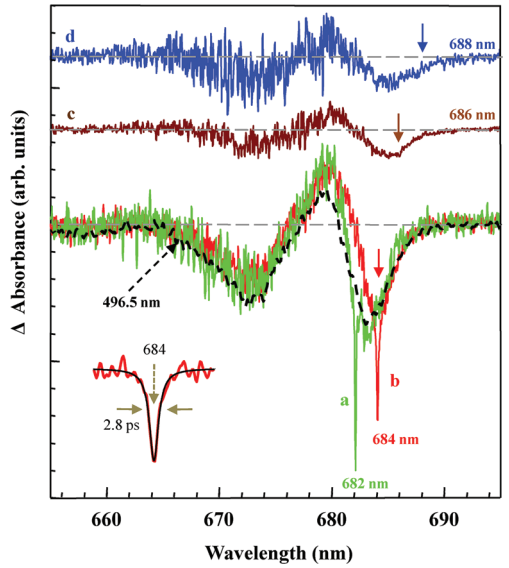


Figure 6. Resonant transient HB spectra obtained for RC_{S3} sample. Spectra a, b, c, and d (resolution 0.5 cm^{-1}) were obtained with λ_B of 682.0, 684.0, 686.0, and 688.0 nm, respectively. Black dashed curve was obtained at λ_B = 496.5 nm. The inset corresponds to the Lorentzian fit (black curve) of ZPH of curve b.

obtained with λ_B = 496.5 nm (dashed black line), and resonant transient HB spectra (0.5 cm^{-1} resolution) for RC_{S3} sample obtained with λ_B = 682.0 (green curve a), 684.0 (red curve b), 686.0 (brown curve c), and 688.0 nm (blue spectrum d). Curve c reveals an extremely weak ZPH, as indicated by the brown arrow at 686.0 nm. Similar spectra were obtained for RC_{S2} at $\lambda_B \geq$ 682 nm. Note that all spectra exhibit bleaching near 673 nm (revealed for the first time in the resonant transient HB spectra of isolated PSII RCs) and in the 684−686 nm region. However, a ZPH coincident with the burn wavelength is clearly observed only for λ_B in the 680−685 nm range. Both depth and width of the ZPH increase with illumination intensity. We note that ZPH widths (2.4−7.6 cm^{-1} at 682 nm, depending on depth/intensity, data not shown for brevity) are related to the primary CS time (τ_{CS}). These ZPH widths (with an example shown in the inset of Figure 6) correspond to τ_{CS} in *C. reinhardtii* (at 682−684 nm) in the range of 1.4−4.4 ps, in agreement with previous data obtained for primary CS in spinach RCs.[13,14,18,19,35] The values of τ_{CS} were obtained by using[41,51]

$$\Gamma_{hom}(cm^{-1}) = (1/2\pi c\tau_{fl} + 1/2\pi c\tau_{CS}) + 1/\pi cT_2^* \approx 1/2\pi c\tau_{CS}$$

where τ_{fl} is the fluorescence lifetime and T_2^* is the "pure" dephasing time. At T = 5 K, $\tau_{fl} \gg T_2^* \gg \tau_{CS}$, and charge separation time has the major effect on the ZPH width.

ZPHs were nearly absent at 686.0 nm and entirely absent at 688.0 nm and longer wavelengths; the spectra obtained at λ_B of 690−695 nm region (not shown here) were very noisy due to extremely low absorption in that spectral region, but their shape was similar to that represented by curve d. Given the optical density changes in the hole profiles shown in Figure 6, it is apparent that the spectra obtained at longer λ_B are a manifestation of stronger electron−(protein) phonon coupling.

dx.doi.org/10.1021/jp302849d | J. Phys. Chem. B 2012, 116, 4860−4870

This is a signature of the state with significant charge transfer (CT) character (possibly either between two of the P chlorophylls or involving P_{D1} and Chl_{D1}). We also stress that illumination at 690−695 nm results in formation of a bleach near 673 nm and positive absorption (in the 690−860 nm region), which is a signature of a cation residing on P_{D1} (i.e., P_{D1}^{+}).[23,48]

4. DISCUSSION

Taken as a whole, the HB results presented above establish that isolated RCs from *C. reinhardtii* contain a mixture of destabilized (RC680) and intact RCs (RC684), with RC_{S3} samples containing the largest fraction of RC684. Moreover, on the basis of long-wavelength transient absorption features typically ascribed to the $P684^{+}Q_A^{-}$ state, it would seem that the more intact RC684 fraction most likely contains RCs both with and without Q_A; the presence of Q_A allows electron transfer beyond $Pheo_{D1}$ leading to a long-lived $P^{+}Q_A^{-}$ state. This state is responsible for significant bleaching near 673 nm and electrochromic shifts (both observed in the isolated PSII RC for the first time). The absence of a quinone leads to charge recombination resulting in triplet formation, with the associated triplet bleach located near 680 nm (in RC680) or near 684 nm (in RC684). In this context, it is useful to note that recent mutational studies by Schlodder et al.[23] have suggested that the ~673 nm bleach in flash-induced ($P680^{+}Q_A^{-}$ − $P680Q_A$) difference spectra is due to an excitonic state with the strongest contribution from the P_{D1} pigment of the special pair. On the other hand, the triplet-minus-singlet ($^3P - {}^1P$) response near 684 nm is likely due to a triplet state localized on Chl_{D1}. Because of the observed P^{+} transient absorption, stronger P_{D1}−P_{D2} interaction expected in RC684, a bleach near 673 nm, and the electrochromic shift of $Pheo_{D1}$ due to Q_A^{-} formation, we suggest that the primary electron donor in RC684, by analogy to BRC,[52,53] might be one of the Chls in the so-called P_{D1}/P_{D2} special pair. Because the major portion of the HOMO is localized on P_{D1} chlorophyll,[7] the electron release most likely occurs along the P_{D1} path. In what follows, we further discuss the possibility that isolated RCs from *C. reinhardtii* possess two alternative CS pathways within the RC684 (P_{D1} and Chl_{D1} primary donors). We also consider the localization of the cation radical and provide more insight into the triplet state formed in RC680 (near 680 nm) and intact RC684 (near 684 nm) by charge recombination of the primary radical pair(s), in a fraction of RC684 without Q_A. A possibility of triplet−triplet transfer in RC684 (i.e., $^3P_{D1} \rightarrow {}^3Chl_{D1}$) is also briefly discussed.

4.1. Absorption Spectra of RC684 and RC680 RCs Isolated from *C. reinhardtii*. The results presented above indicate that RC_{S3} sample contains largest subpopulation of intact RC684 (with or without Q_A). However, comparison of many different optical spectra obtained for 10 RC preparations revealed that RC_{S3} also likely contains a subpopulation (~40%; see discussion below related to Figure 7) of RC680 complexes. Its presence (manifested via the 680 nm transient hole) is not directly revealed in the transient spectra of RC_{S3} (see Figure 4B), due to the significantly deeper transient holes formed in the intact RC684 fraction of the sample; for example, the maximum fractional transient hole depth at 683.3 nm (I = 100 mW/cm^2) is ~22% in RC_{S3} (see frame F) as compared to ~10% in RC_{S2} (frame E). The latter, with the relatively higher content of RC684 in RC_{S3} sample, minimizes the relative contribution from the transient holes near 680 nm originating from destabilized RC680. The contribution from RC680 near 680 nm in RC_{S3} must be small, as 496.5 and 665.0 nm excitation (Figure 4B), in contrast to RC_{S2} preparation (see data in Figure 4A), did not result in any difference in transient holes near 680 nm.

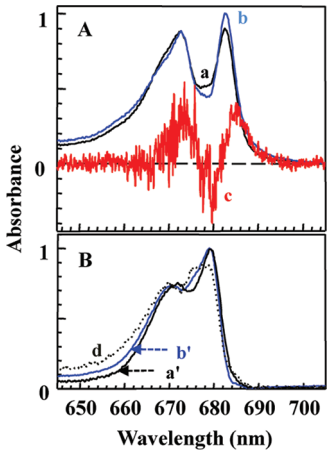


Figure 7. Extracted absorption spectra for RC684 (frame A) and RC680 (frame B) complexes. The curves a/b and a′/b′ correspond to the extracted absorption spectra of two subsets of RCs, RC684 (intact) and RC680 (destablished) complexes, respectively (see text for details). Transient spectrum c (inverted curve d from Figure 6) shows a good agreement with the low-energy absorption tail of extracted absorption spectrum of RC684. Curve d (in frame B) was obtained as the difference of absorption spectra of intact and partly damaged RC_{S3} preparation, which corresponds to the destabilized RC680.

Guided by the data shown in Figures 2−4, and unpublished results, as well as earlier studies,[13,31,32] we attempted to extract individual absorption spectra of the RC684 and RC680 subpopulations. The resulting putative absorption spectra are shown in Figure 7 (frames A and B, respectively). Curve a in Figure 7A (assigned to the absorption of intact RC684 complexes) was obtained by subtracting scaled contributions of the absorption spectra of RC_{S2} (a mixture of RC680 and RC684) and RC_{S1} (mostly RC680) from the absorption spectrum of RC_{S3}. Curve b was obtained likewise by subtracting a typical absorption spectrum of spinach RC[13,14] (instead of the RC_{S1}) from RC_{S3}. Note that the low-energy part of spectrum c in Figure 7A, corresponding to the inverted HB spectrum (curve d in Figure 6) obtained at λ_B = 688.0 nm, fits very well the low-energy absorption tail of the intact RC684 complexes.

On the basis of this putative absorption spectrum for the intact RC684 complex, one can extract the representative absorption spectra of RC680 (curves a′ and b′ in Figure 7B) by subtracting the RC684 absorption spectrum from the absorption spectra measured for various less-intact RC preparations. Such difference spectra (curves a′ and b′) are indeed very similar to the absorption spectra of typically studied RC680 complexes, which exhibited similar absorption maximum and triplet-bottleneck hole (bleach) near 680 nm, as well as no response near 673 nm. To demonstrate that this assignment is realistic, one should compare curves a′ and b′ with spectrum d (in frame B of Figure 7), which was obtained as the difference between the absorption spectra of intact RC_{S3} sample (Figure 2C) and partly damaged RC_{S3} (i.e., RC_{S3d}; not shown for brevity), respectively. Recall that the difference between transient holes shown in Figure 3 (obtained for the above two samples) revealed more transient hole with bleaching near 680 nm in the damaged sample (see curve c in Figure 3) proving that RC684 indeed converts to RC680. This is why spectrum d

dx.doi.org/10.1021/jp302849d | J. Phys. Chem. B 2012, 116, 4860−4870

in Figure 7B is similar to a typical absorption of destabilized RC680. Thus, we conclude that our analysis is consistent with our RC680/RC684 model for isolated spinach RCs,[13,32,49] proving that RC680 are destabilized products of more intact RC684 complexes. None of the spinach RCs discussed in refs 13,32,49, however, contained Q_A as it was lost during the preparation procedure. This is most likely why in spinach RCs (mostly RC680 with a small contribution from RC684, both fractions without Q_A) only the triplet-bottleneck holes near 680 nm (major) and 684 nm (minor) have been observed, but not the 673 nm hole.

These findings pose many relevant questions, for example: (1) Which optical spectra should be fitted using theoretical structure-based models to describe electronic structure and/or electron transfer dynamics in intact RCs? (2) Are both channels of charge separation (i.e., P_{D1} and Chl_{D1} paths) operational in intact RCs (i.e., RC684) and destabilized RC680? (3) Why does the triplet-bottleneck bleach in RC680 (no Q_A) shift to the blue by ~4 nm in comparison with that in RC684 (also with no Q_A)? (4) Does the Chl_{D1} act as the primary electron donor at physiological temperatures? Some of these questions will be briefly addressed in section 4.3. First, we focus on the origin of electrochromic shift and the possibility that a fraction of intact RC684 from *C. reinhardtii* may possess the secondary plastoquinone, Q_A.

4.2. On Triplet Formation and the Nature of the Electrochromic Shift in RC684. For a moment, we focus only on the electrochromic shift and the general shapes of transient HB spectra discussed above. The blue and red curves in the right inset of Figure 5 show the effect of $P^+Q_A^-$ formation on the Q_x-region of pheophytins in the RC_{S2} and RC_{S3} samples, respectively. These curves correspond to transient spectra after the persistent hole was saturated. A clear blue-shift of the Q_x-transition of pheophytins is observed, in agreement with data obtained for intact PSII core samples,[31] and suggesting that a significant subpopulation of RCs in RC_{S3} (and RC_{S2}) contains plastoquinone Q_A, whose reduction to Q_A^- leads to the electrochromic shift of the active $Pheo_{D1}$ residing in the vicinity of Q_A. The gray (smooth) curve is shown to help the reader to perceive the electrochromic shift. (Modeling study of the optical spectra reported here is beyond the scope of this Article and will be published elsewhere.) Note that no electrochromic shifts have ever been observed in the spinach RC samples, as shown by the black top curve in the right inset of Figure 5. The same is true for positive absorption increase due to P^+ formation. This is consistent with the absence of Q_A in the isolated RC680 complexes from spinach and RC_{S1} sample from *C. reinhardtti*. As shown in section 3.2, nonresonant transient holes of RC_{S2} and RC_{S3} samples (Figures 2 and 4) are very different from the nonresonant transient holes typically observed in spinach, as well as from those observed for RC_{S1} (Figure 2D), for which the major bleach is near 680 nm, with no accompanying bleach near 673 nm or positive absorption increase in the long-wavelength region (>690 nm). The most interesting finding, however, is that RC_{S3} sample (extracted directly from thylakoids)[37] has a Q_y transient hole spectrum similar to that of the flash-induced ($P^+Q_A^-$ $-PQ_A$) absorbance difference spectrum obtained for PSII core.[22,27,30,48]

Curve a in Figure 8 corresponds to spectrum b′ of Figure 4B. We believe that this spectrum may still contain a small contribution from the triplet-bottleneck hole due to $^3Chl_{D1}$ with a main bleach near 684 nm, observed in intact RC684

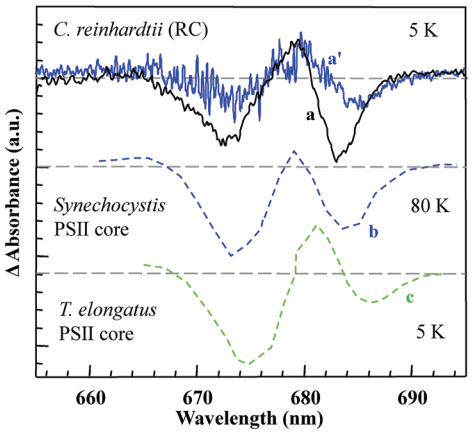


Figure 8. Comparison of various ($P^+Q_A^-$ − PQ_A) spectra: curve a (black) represents the ($P^+Q_A^-$ − PQ_A) obtained for the isolated RC_{S3} from *C. reinhardtii*, adopted from curve b′ of Figure 4B. Curve a′ is the resonant transient HB spectrum of RC_{S3} sample obtained at 688.0 nm. Spectra b and c are the flash induced absorption difference ($P^+Q_A^-$ − PQ_A) spectra of PSII core complexes from *Synechocystis* sp. PCC 6803 at 80 K[23] and *T. elongatus* at 5 K,[30] respectively.

without Q_A (see curve c′ in Figure 4B). That is, spectrum a might be a mixture of triplet and ($P^+Q_A^-$ − PQ_A) contributions. Spectrum a′ was obtained with λ_B of 688.0 nm and is shown for comparison. Spectra b and c in Figure 8 correspond to transient ($P^+Q_A^-$ − PQ_A) spectra obtained for *Synechocystis* sp. PCC6803[22,27,48] and *T. elongatus*[27,30] PSII core complexes, at 80 and 5 K, respectively. Comparison of spectra a/a′, b, and c clearly suggests that transient spectra measured for our sample RC_{S3} are similar to transient ($P^+Q_A^-$ − PQ_A) spectra observed in more intact PSII core complexes,[22,27,30,48] where RCs are not affected by the removal of the CP43 and CP47 antenna complexes. We note that the entire shapes of both resonant and nonresonant transient holes in our isolated RCs and in the PSII cores[22,27,30,50] are not only contributed to by the CS per se, but also by the electrochromic shifts of Chl_{D1} and $Pheo_{D1}$ due to charges residing on P_{D1} and Q_A, respectively, during the transient hole measurement.

Recombination from the $P^+Q_A^-$ state occurs with a characteristic time of about 2−5 ms.[54] In our experimental approach, the 684 nm part of the contribution of this state to transient holes cannot be directly distinguished from the holes of triplet-bottleneck nature (see next paragraph). Thus, it is possible that bleaching near 684 nm in RC_{S3} (curve a of Figure 8) still carries a small contribution from the Chl_{D1} triplet. In fact, the differences between the depths of 684 nm transient holes a′ and b′ (Figure 4) and differences in 673/684 nm hole depth ratios for RC_{S3} and PSII cores (Figure 8) speak in favor of such a possibility. Taking into account that in PSII core the RC triplet is only observed if Q_A is removed or reduced,[48,55] it is natural to suggest that in our transient HB experiments in either RC680 or RC684 the triplet is also observed only in the absence of Q_A. The triplet in RC684 (with Q_A lost) is most likely localized on Chl_{D1} ($^3Chl_{D1}$), as $^3P_{D1}$ triplet would yield a hole that is significantly blue-shifted (to about 675 nm),[24] in agreement with our calculations (data not shown). The lifetime of such triplet state should be similar to that of the Chl *a* at 5 K (τ = 1.4 ms)[56] and thus should be shorter than the lifetime expected for $P684^+Q_A^-$, that is, shorter than 2−5 ms.[54,57−59] Because of the longer lifetime of $P_{D1}{}^+Q_A^-$ (in RC684 with Q_A present) in comparison with the lifetime of the triplet state in

dx.doi.org/10.1021/jp302849d | J. Phys. Chem. B 2012, 116, 4860−4870

RC684 or RC680 (no Q_A), the contribution to a triplet-bottleneck hole near 680 nm (from a fraction of RC680 still present in RC_{S3}) can be expected to be minor and buried under other features in spectra shown in Figure 4B. The triplet-bottleneck hole in RC684 (no Q_A) is observed at 684 nm (see curve c′ in Figure 4B). However, it is not clear if $^3Chl_{D1}$ in intact RC684 without Q_A is formed directly by charge recombination ($Chl_{D1}^+Pheo_{D1}^- \rightarrow {}^3Chl_{D1}$) or if $^3P_{D1}$ triplet is formed originally, followed by a fast triplet energy transfer to Chl_{D1} (i.e., $P_{D1}^+Chl_{D1}^-Pheo_{D1} \rightarrow P_{D1}^+Chl_{D1}Pheo_{D1}^- \rightarrow {}^3P_{D1} \rightarrow {}^3Chl_{D1}$).[56] Assuming that the presence or absence of Q_A does not affect the very first step of the charge separation, and that P_{D1} is the primary electron donor in RC684, one should favor the latter mechanism of triplet formation on Chl_{D1}. No response near 673 nm is expected if $^3P_{D1} \rightarrow {}^3Chl_{D1}$ transfer is fast enough (~50 ns)[56] as compared to $^3Chl_{D1}$ lifetime. The shape and the minimum of the nonresonant hole c′ in Figure 4B are consistent with measured T − S spectra for PSII core from *Synechocystis* with Q_A reduced (blocking electron transfer beyond $Pheo_{D1}$) where it was shown that (at low temperatures) the main bleach is located near 683 nm, in agreement with the RC triplet being localized on accessory Chl_{D1}.[48]

The presence of well-defined ZPH for λ_B of 682−684 nm, combined with the absence of ZPH for $\lambda_B \geq 686$ nm (see Figure 6), suggests that mixing between the CT state(s) and lowest-energy excited state increases at longer wavelengths. Holes burned at $\lambda_B \geq 688.0$ nm (the noisy spectra for λ_B of 690−695 nm are not shown) were very similar to those obtained at λ_B = 686.0 and 688.0 nm, suggesting that there is(are) a low-lying CT state(s) that might be largely homogeneously broadened (although see discussion below of homogeneous vs inhomogeneous broadening). It is also tempting to suggest, although results presented in this Article do not provide enough evidence, that narrow ZPHs, which are not due to the CT state, belong to the Chl_{D1} primary CS path. Thus, the CS may occur from both the special pair (most likely P_{D1}) and Chl_{D1}, depending on a particular realization of the static energetic disorder.[26] We anticipate that theoretical modeling of our transient HB spectra (research in progress) will shed more light on excitonic structure of intact RC684 and on the mixing of the special pair Chls with a low-energy CT state(s) whose maximum(a) is(are) likely near 688−695 nm.

4.3. Charge Separation Pathway(s) in RC680 and RC684. Our transient HB spectra provide a new insight into the Chls that contribute to the long-lived (>1 ms) excited and radical pair state(s) in *C. reinhardtii*. In RC684 with Q_A present, the shape of the transient HB spectrum is due to formation of a long-lived (2−5 ms) $P_{D1}^+Q_A^-$ state, accompanied by electrochromic shifts. That is, the negative and positive changes in the transient spectra near the 682−690 and 677−681 nm regions, respectively, are contributed to by the electrochromic shifts of pigments residing close to P_{D1} and Q_A, that is, Chl_{D1} and $Pheo_{D1}$, respectively. The bleach near 673 nm and the increase of absorption at >690 nm are signatures of the $P_{D1}^+Q_A^-$ formation (specifically P_{D1}^+); see data in Figure 8. However, transient HB spectra in RC684 complexes (with Q_A present) cannot distinguish between the following sequences of events: $P_{D1}\mathbf{Chl_{D1}^+Pheo_{D1}^-}Q_A \rightarrow \mathbf{P_{D1}^+}Chl_{D1}\mathbf{Pheo_{D1}^-}Q_A \rightarrow \mathbf{P_{D1}^+Q_A^-}$ and/or $\mathbf{P_{D1}^+Chl_{D1}^-}Pheo_{D1}Q_A \rightarrow \mathbf{P_{D1}^+}Chl_{D1}\mathbf{Pheo_{D1}^-}Q_A \rightarrow \mathbf{P_{D1}^+Q_A^-}$ as the lifetime of the final state is much longer than the characteristic times of the intermediate steps. That is, observation of $P_{D1}^+Q_A^-$ does not exclude the possibility that Chl_{D1} can serve as a primary electron donor. In addition, the shape of nonresonant transient HB spectra characterized by a single bleach near 680 nm (in RC680) or 684 nm (in RC684) in a subpopulation of RCs without Q_A (and no evidence of oxidation of P_{D1}) does not prove that electron transfer starts at Chl_{D1} ($P_{D1}\mathbf{Chl_{D1}^+Pheo_{D1}^-} \rightarrow {}^3Chl_{D1}$), as $^3Chl_{D1}$ in RC684 can be also obtained via the sequence: $\mathbf{P_{D1}^+Chl_{D1}^-}Pheo_{D1} \rightarrow \mathbf{P_{D1}^+}Chl_{D1}\mathbf{Pheo_{D1}^-} \rightarrow {}^3P_{D1} \rightarrow {}^3Chl_{D1}$, especially if the triplet−triplet transfer is fast.[56] Thus, it is likely that Chl_{D1} can also serve as primary electron donor in PSII RC if it is strongly enough contributing to the lowest exciton state. Therefore, our data are consistent with the recent conclusion reached by Romero et al.,[34] based on time-resolved spectroscopic experiments, that both P_{D1} and Chl_{D1} paths are possible. In particular, it has been suggested in ref 34 that the very weak shoulder near 672−673 nm observed in recent 77 K transient absorption experiments on spinach RCs (i.e., in the species-associated difference spectra (SADS) obtained with laser pump pulse duration of ~100 fs and 10 nm fwhm) reflects a contribution from the P_{D1} path for CS, although no clear evidence was presented. Therefore, this pathway, if present in the spinach RC,[34,35] most likely operates only in a very small subpopulation of intact spinach RCs (i.e., RC684), but not in destabilized RC680, which (with weakened P_{D1}−P_{D2} coupling) are responsible for the major bleach near 680 nm in all spinach RC transient absorption spectra published so far.

The fact that RC680-dominated samples reveal a major response (bleaching) near 680 nm[11,12,14] (and not at 684 nm as in RC684 complexes) shows that these RCs have lost Q_A and the interaction between D1 and D2 proteins in RC680 is altered. Weaker coupling (i.e., smaller wave function overlap) between the Chls constituting the special pair (P_{D1}/P_{D2}) in RC680 could lead to the well-documented (preferential) charge separation pathway in RC680 starting from the accessory Chl_{D1} (Chl_{D1} path), with the major bleach near 680 nm[1,24] as a signature. In these destabilized RC680 (D1/D2/Cyt_{b559} proteins), one would not expect the negative signal (bleach) near 673 nm due to oxidation of P_{D1}. This is consistent with the major ~680 nm bleach observed in isolated RCs from spinach; SADS indicated that the $(Chl_{D1}Pheo_{D1})^*$ state decays in 3 ps forming the $Chl_{D1}^+Pheo_{D1}^-$ radical pair,[34] while the $P_{D1}^+Pheo_{D1}^-$ formation was assigned to be faster. Regarding the latter pathway, the authors claimed that contributions from high exciton states, P_{D1} and/or P_{D2}, and Chl_{D1}, were observed at 660, 667, and 680 nm, respectively,[34] in agreement with the $(P_{D1}P_{D2}Chl_{D1})^* \rightarrow P_{D2}^+P_{D1}^- \rightarrow P_{D1}^+Chl_{D1}^-$ process. However, clear bleaches near 672/673 and 684 nm (as in our RC684) have not been observed, suggesting that this P_{D1} pathway in spinach RCs was present only in a minor subpopulation of more intact RC684. On the other hand, very recent modeling of the same transient absorption kinetics data (by the same group) suggested[35] that it is the $Chl_{D1}^+Pheo_{D1}^-$ formation that corresponds to the fastest component (0.7 ps).

The fact that there is a distribution of CS times and variable λ_B-dependent (due to disorder) mixing with CT states complicates the so-called model-dependent "target analysis" of composite transient absorption spectra. The absence of ZPHs at $\lambda_B \geq 686$ nm (see Figure 6) suggests the presence in RC684 of low-lying CT state(s) characterized by very strong electron−phonon coupling, in agreement with Krausz et al. who showed that excitation wavelengths as long as 695.0 nm (T = 1.7 K) can induce CS in PSII core and thus Q_A^- formation.[31,60] On the other hand, a very small shift of the $P^+Q_A^-$ holes near 673 and 684 nm for λ_B of 686, 688, 690, and

dx.doi.org/10.1021/jp302849d | J. Phys. Chem. B 2012, 116, 4860−4870

695 nm is consistent with the presence of a weakly absorbing (and predominantly homogeneously broadened) CT state(s) lying near 688−695 nm. This assignment is in agreement with ref 31 where a weak (homogeneously broadened) CT state was hypothesized to be present in the PSII core (hidden beneath the CP47 lowest-energy state centered at 690 nm). The homogeneously broadened character of the CT state is analogous to that observed in bacterial RCs, with well-established strong electron phonon coupling.[61,62] However, Novoderezhkin et al.[26] suggested that the absence of ZPHs does not automatically imply very large S-values (leading to large homogeneous broadening) because the relative intensities of zero-phonon and vibrationally excited transitions will depend on the degree of mixing of each CT mode with higher-energy exciton states, which in turn depends on the relative displacement of the CT and exciton state potential energy surfaces along each vibrational mode. In short, they argued that preferential mixing of vibrationally excited modes of the (dark) CT state with the (bright) exciton states may lead to an effective suppression of the CT state ZPL even in the absence of strong local electron−phonon coupling. Furthermore, it should be noted that Krausz et al.[31] argued that the CT state in PSII core extends far beyond 700 nm (700−730 nm), a notion that requires further confirmation, as the long absorption tail in PSII core is strongly contributed to by antenna complexes, and no such absorption was revealed in our intact isolated RC684 complexes. Note that RC684 has a major bleach ∼4 nm to the red (at ∼684 nm; see curve c′ in Figure 4B) in comparison with the ∼680 nm bleach observed in destabilized RC680. It remains to be determined whether the 4 nm blue-shift of the triplet-bottleneck hole in RC680 is due to a shift in the site energy of $Pheo_{D1}$, or, as arbitrarily assumed in ref 24, due to a blue-shift of the site energy of Chl_{D1}. Both states of affairs are possible because, based on our preliminary modeling studies, both $Pheo_{D1}$ and Chl_{D1} contribute to the lowest-energy excitonic state.[37] It cannot be excluded that the blue-shift of the site energy of $Pheo_{D1}$ in RC680 is due to the H-bond to $Pheo_{D1}$[1,54] being broken.[37,63] This would be consistent with the observed blue-shift of the Q_x- transition of $Pheo_{D1}$ and a blue-shift of the broad nonresonant triplet-bottleneck hole (λ_B = 665.0 nm) in RC680 when compared to RC684.

In summary, our data suggest the following:

(1) Spectra shown in Figures 6 and 8 can be explained only assuming that the major cation in intact RCs is localized on P_{D1}.[27]

(2) A fraction of intact RC684 must contain Q_A (with a positive charge on P_{D1} and negative charge on Q_A during our transient HB measurements, $P_{D1}^{+}Q_A^{-} - P_{D1}Q_A$). An electrochromic shift with a zero crossing near 681 nm in the Q_y and near 543 nm in the Q_x regions is consistent with a blue-shift of the site energies of active $Pheo_{D1}$ and in part Chl_{D1} (see curve a in Figure 8 and the right inset (lower curves) in Figure 5). These data are in agreement with transient profiles reported for the PSII core in refs 22,27,30,48.

(3) Charge separation in intact RCs in *C. reinhardtii* (RC684) most likely starts from P_{D1} pigment, although Chl_{D1} being a primary donor for a fraction of RC684 cannot be excluded.

Although our findings are consistent with the original suggestion that isolated RCs (in spinach) may possess two parallel electron-transfer pathways, one starting at Chl_{D1} and one at P_{D1},[34] with the relative contribution of each pathway determined by the particular realization of disorder,[34,35] we suggest that the major transient contribution peaked at 680 nm observed in these papers originated from the Chl_{D1} path in destabilized RC680, while the minor P_{D1} path contribution was due to a small subpopulation of more intact RC684 complexes. (Recall that triplet localization on Chl_{D1} in RC680 and RC684 leads to transient holes near 680 nm (see curves c in Figures 3 and 4) and 684 nm (curve c′ in Figure 4), respectively). Bear in mind that these contributions can be only observed in RCs without Q_A. Our assignment is also supported by the fact that transient absorption spectra reported in ref 34 exhibited a small red-shift to ∼682 nm of the major bleach for 685 nm excitation with fs laser with a bandwidth of ∼10 nm. The explanation provided in refs 34,35 was that the red-shift is caused by photoselection of a subpopulation from the inhomogeneous distribution. Although under such experimental conditions a weak photoselection from the inhomogeneous distribution can be present, it is much more likely that, in agreement with transient HB spectra, these spectra were contributed to by two distinct subpopulations, as discussed above. In other words, transient spectra with bleaching near ∼673/685 nm and ∼680 nm correspond to different subsets of the ensemble of RCs, that is, intact RC684 (with P_{D1} and Chl_{D1} as the primary electron donor) and destabilized RC680 (with only Chl_{D1} as the primary electron donor) complexes, respectively. We think both P_{D1} and Chl_{D1} pathways may be highly operational for particular realizations of disorder being responsible for the very efficient CS in PSII RC at physiological temperatures, as room temperature protein motions can lead to large conformational protein disorder.[17] Thus, it appears the conformational protein dynamics modulating pigment−pigment and pigment−protein interactions leads not only to efficient excitation energy transfer but also to efficient and fast electron transfer times, utilizing two different CS pathways.

5. CONCLUDING REMARKS

Comprehensive HB studies of a large number of isolated PSII RC samples with different levels of intactness indicate that isolated RCs from *C. reinhardtii* are highly fragile and sensitive to the isolation/purification protocols and sample handling/preparation procedures. This is in agreement with previous reports that harsh biochemical treatment (necessary to isolate RCs) can modify both optical spectra and redox properties of protein's cofactors.[13,32,64] As a result, the nature of the primary electron donor in isolated PSII RCs from *C. reinhardtii* depends on the intactness of the protein. Our data clearly suggest that isolated RCs from *C. reinhardtii* possess three RC fractions referred to as destabilized RC680 (no Q_A), more native RC684 (no Q_A), and a small fraction of native RC684 with Q_A present, in different proportions. We argue that all isolated RCs studied so far contained mostly RC680 (i.e., destabilized RCs; no Q_A), with a major single-band bleaching near 680 nm in the transient hole spectra as well as Chl_{D1} path for CS. On the other hand, two different CS pathways (P_{D1} and Chl_{D1}) are possible in RC684, with the former likely being dominant. We show, for the first time, that transient HB spectra obtained for our most intact RCs (RC_{S3} sample; with a significant fraction of RC684 possessing Q_A) are similar to transient delta absorption spectra of ($P^{+}Q_A^{-} - PQ_A$) observed in the PSII core.[27,30] In particular, transient HB spectra in RC684 revealed contributions from both a longer lived $P^{+}Q_A^{-}$ state (most likely formed by both P_{D1} and Chl_{D1} pathways for CS) and triplet-bottleneck holes

near 684 nm (localized on Chl_{D1}) formed either directly via the Chl_{D1} CS path or via the P_{D1} CS path plus triplet−triplet transfer ($^3P_{D1} \rightarrow {}^3Chl_{D1}$). Resonant HB cannot distinguish different paths by the CS times, but it is clear that primary CS (most likely via the Chl_{D1} pathway) occurs on the picosecond time scale of about 1.4−4.4 ps. In contrast, we propose that CS in RC684 via the P_{D1} pathway could be even faster; because HB spectra burned at $\lambda_B > 686$ nm do not show ZPHs, the CS time via the P_{D1} pathway cannot be obtained from HB spectra. We demonstrate that the destabilization of isolated D1/D2/Cyt_{b559} protein complexes (i.e., RC680) not only eliminates (or significantly decreases) contribution from the RC684 (and, as a result, the P_{D1} CS pathway), as observed in spinach RCs,[34] but also modifies the site energies of the pigments participating in the Chl_{D1} path for CS along the D1 protein (section 4.3), shifting its triplet-bottleneck hole by ∼90 cm^{-1} (∼4 nm) toward high energy. The lack of P_{D1} path in RC680 is most likely caused by a modified (i.e., decreased) overlap of the π-electron wave functions between P_{D1} and P_{D2} and/or P_{D1}/P_{D2} and Chl_{D1} cofactors, as well as possible conformational changes in the D1/D2 protein.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ryszard@ksu.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy, grant EC9987 to R.J. Partial support at the early stage of this project was provided by the DOE EPSCoR Grant (DE-FG02-08ER46504) and DOE BES to R.J. V.Z. acknowledges support by NSERC. We thank Chen Lin for mass spectrometry and NMR measurements using extracts of RC_{S3} sample and acknowledge our collaborators Dr. Michael Seibert (from NREL, Golden, CO) and Dr. Rafael Picorel (CSIC, Zaragoza, Spain) for kindly providing us with the isolated RC complexes and useful discussions.

REFERENCES

(1) Diner, B. A.; Rappaport, F. *Annu. Rev. Plant Biol.* **2002**, *53*, 551−580.

(2) Umena, Y.; Kawakami, K.; Shen, J.-R.; Kamiya, N. *Nature* **2011**, *473*, 55−60.

(3) Deisenhofer, J.; Epp, O.; Miki, K.; Huber, R.; Michel, H. *Nature* **1985**, *318*, 618−624.

(4) Deisenhofer, J.; Epp, O.; Miki, K.; Huber, R.; Michel, H. *J. Mol. Biol.* **1984**, *180*, 385−398.

(5) Xiong, J.; Subramanian, S.; Govindjee. *Photosynth. Res.* **1998**, *56*, 229−254.

(6) Trebst, A. *Z. Naturforsch., C: J. Biosci.* **1986**, *41*, 240−245.

(7) Lill, N. S. O. *Phys. Chem. Chem. Phys.* **2011**, *13*, 16022−16027.

(8) Saito, K.; Ishida, T.; Sugiura, M.; Kawakami, K.; Umena, Y.; Kamiya, N.; Shen, J.-R.; Ishikita, H. *J. Am. Chem. Soc.* **2011**, *133*, 14379−14388.

(9) Nanba, O.; Satoh, N. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 109−122.

(10) Tang, D.; Jankowiak, R.; Seibert, M.; Yocum, C. F.; Small, G. J. *J. Phys. Chem.* **1990**, *17*, 6519−6522.

(11) Tang, D.; Jankowiak, R.; Seibert, M.; Small, J. G. *Photosynth. Res.* **1991**, *27*, 19−29.

(12) Zazubovich, V.; Jankowiak, R.; Riley, K.; Picorel, R.; Seibert, M.; Small, G. J. *J. Phys. Chem. B* **2003**, *107*, 2862−2866.

(13) Riley, K. J.; Jankowiak, R.; Rätsep, M.; Small, G. J.; Zazubovich, V. *J. Phys. Chem. B* **2004**, *108*, 10346−10356.

(14) Jankowiak, R.; Rätsep, M.; Hayes, J.; Zazubovich, V.; Picorel, R.; Seibert, M.; Small, G. J. *J. Phys. Chem. B* **2003**, *107*, 2068−2074.

(15) Frese, R. N.; Germano, M.; de Weerd, F. L.; Van Stokkum, I. H. M.; Shkuropatov, A. Y.; Shuvalov, V. A.; van Grokon, H. J.; Van Grondelle, R.; Dekker, J. P. *Biochemistry* **2003**, *42*, 9205−9213.

(16) Prokhorenko, V. I.; Holzwarth, A. R. *J. Phys. Chem. B* **2000**, *104*, 11563−11578.

(17) Novoderezhkin, V. I.; Andrizhiyevskaya, E. G.; Dekker, J. P.; van Grondelle, R. *Biophys. J.* **2005**, *89*, 1464−1481.

(18) Groot, M.-L.; van Mourik, F.; Eijckelhoff, C.; van Stokkum, I. H. M.; Dekker, J. P.; van Grondelle, R. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4389−4394.

(19) Myers, J. A.; Lewis, K. L. M.; Fuller, F. D.; Tekavec, P. F.; Yocum, C. F.; Ogilvie, J. P. *J. Phys. Chem. Lett.* **2010**, *1*, 2774−2780.

(20) Alizadeh, S.; Nixon, P. J.; Telfer, A.; Barber, J. *Photosynth. Res.* **1995**, *43*, 165−171.

(21) Wang, J.; Gosztola, D.; Ruffle, S. V.; Hemann, C.; Seibert, M.; Wasielewski, M. R.; Hille, R.; Gustafson, T. L.; Sayre, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 4091−4096.

(22) Diner, B. A.; Schlodder, E.; Nixon, P. J.; Coleman, W. J.; Rappaport, F.; Levergne, J.; Vermaas, W. F. J.; Chisholm, D. A. *Biochemistry* **2001**, *40*, 9265−9281.

(23) Schlodder, E.; Coleman, W. J.; Nixon, P. J.; Cohen, R. O.; Renger, T.; Diner, B. A. *Philos. Trans. R. Soc. London, Ser. B* **2008**, *363*, 1197−1202.

(24) Raszewski, G.; Saenger, W.; Renger, T. *Biophys. J.* **2005**, *88*, 986−998.

(25) Raszewski, G.; Diner, B. A.; Schlodder, E.; Renger, T. *Biophys. J.* **2008**, *95*, 105−119.

(26) Novoderezhkin, V. I.; Dekker, J. P.; van Grondelle, R. *Biophys. J.* **2007**, *93*, 1293−1311.

(27) Renger, T.; Schlodder, E. *ChemPhysChem* **2010**, *11*, 1141−1153.

(28) Cox, N.; Hughes, J.; Rutherford, A. W.; Krausz, E. *Phys. Procedia* **2010**, *3*, 1601−1605.

(29) Seibert, M. In *The Photosynthetic Reaction Center*; Deisenhofer, J., Norris, J., Eds.; Academic Press: New York, 1993; Vol. *I*, pp 319−356.

(30) Hillmann, B.; Brettel, J. K.; van Mieghem, F.; Kamlowski, A.; Rutherford, A. W.; Schloder, E. *Biochemistry* **1995**, *34*, 4814−4827.

(31) Krausz, E.; Hughes, J. L.; Smith, P.; Pace, R.; Årsköld, S. P. *Photochem. Photobiol. Sci.* **2005**, *4*, 744−753.

(32) Chang, H.-C.; Jankowiak, R.; Reddy, N. R. S.; Yocum, C. F.; Picorel, R.; Seibert, M.; Small, G. J. *J. Phys. Chem.* **1994**, *98*, 7725−7735.

(33) Reddy, N. R. S.; Kolaczkowski, S. V.; Small, G. J. *Science* **1993**, *260*, 68−71.

(34) Romero, E.; van Stokkum, I. H.; Novoderezhkin, V. I.; Dekker, J. P.; van Grondelle, R. *Biochemistry* **2010**, *49*, 4300−4307.

(35) Novoderezhkin, V. I.; Romero, E.; Dekker, J. P.; van Grondelle, R. *ChemPhysChem* **2011**, *12*, 681−688.

(36) Cox, N.; Hughes, J. L.; Steffen, R.; Smith, P. J.; Rutherford, W.; Pace, R. J.; Krausz, E. *J. Phys. Chem. B* **2009**, *113*, 12364−12374.

(37) Acharya, K.; Neupane, B.; Zazubovich, V.; Sayre, R. T.; Picorel, R.; Seibert, M.; Jankowiak, R. *J. Phys. Chem. B* **2012**, *116*, 3890−3899.

(38) Xiong, L.; Seibert, M.; Gusev, A. V.; Wasielewski, M. R.; Hemann, C.; Hille, C. R.; Sayre, R. T. *J. Phys. Chem. B* **2004**, *108*, 16904−16991.

(39) Rochaix, J.-D. *Annu. Rev. Genet.* **1995**, *29*, 209−230.

(40) Riley, K. J.; Zazubovich, V.; Jankowiak, R. *J. Phys. Chem. B* **2006**, *110*, 22436−22446.

(41) Jankowiak, R.; Reppert, M.; Zazubovich, V.; Pieper, J.; Reinot, T. *Chem. Rev.* **2011**, *111*, 4546−4598.

(42) Jankowiak, R.; Small, G. J. In *The Photosynthetic Reaction Centers*; Norris, J., Deisenhofer, J., Eds.; Academic Press: New York, 1993; pp 133−177.

(43) Germano, M.; Shkuropatov, A. Ya.; Permentier, H.; de Wijn, R.; Hoff, A. J.; Shuvalov, V. A.; van Gorkom, H. J. *Biochemistry* **2001**, *40*, 11472−11482.
(44) Dědic, R.; Lovčinskŷ, M.; Vácha, F.; Hala, J. *J. Lumin.* **2000**, *87*−*89*, 809−811.
(45) Germano, M.; Shkuropatov, A. Ya.; Permentier, H.; Khatypov, R. A.; Shuvalov, V. A.; Hoff, A. J.; van Gorkom, H. J. *Photosynth. Res.* **2000**, *64*, 189−198.
(46) Hughes, J. L.; Smith, P.; Pace, R.; Krausz, E. *Biochim. Biophys. Acta* **2006**, *1757*, 841−851.
(47) Jankowiak, R.; Rätsep, M.; Picorel, R.; Seibert, M.; Small, G. J. *J. Phys. Chem. B* **1999**, *103*, 9759−9769.
(48) Schlodder, E.; Renger, T.; Raszewski, G.; Coleman, W. J.; Nixon, P. J.; Cohen, R. O.; Diner, B. A. *Biochemistry* **2008**, *47*, 3143−3154.
(49) Herascu, N.; Ahmouda, S.; Picorel, R.; Seibert, M.; Jankowiak, R.; Zazubovich, V. *J. Phys. Chem. B* **2011**, *115*, 15098−15109.
(50) Allakhverdiev, S. I.; Ahmed, A.; Tajmir-Riahi, H.-A.; Klimov, V. V.; Carpentier, R. *FEBS Lett.* **1994**, *339*, 151−154.
(51) Jankowiak, R.; Hayes, J. M.; Small, G. J. *Chem. Rev.* **1993**, *93*, 1471−1502.
(52) Madjet, M. E.; Müh, F.; Renger, T. *J. Phys. Chem. B* **2009**, *113*, 12603−12614.
(53) Kálmán, L.; Williams, J. C.; Allen, J. P. *Photosynth. Res.* **2008**, *98*, 643−655.
(54) Moënne-Loccoz, P.; Robert, B.; Latz, M. *Biochemistry* **1989**, *28*, 3641−3645.
(55) Vass, I.; Styring, S.; Hundal, T.; Koivuniemi, A.; Aro, E.-M.; Anderson, B. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 1408−1412.
(56) Rutherford, A. W.; Paterson, D. R.; Mullet, J. E. *Biochim. Biophys. Acta* **1981**, *635*, 205−214.
(57) Noguchi, T.; Tomo, T.; Kato, C. *Biochemistry* **2001**, *40*, 2176−2185.
(58) Mathis, P.; Vermeglio, A. *Biochim. Biophys. Acta* **1975**, *396*, 371−381.
(59) Hillmann, B.; Schlodder, E. *Biochim. Biophys. Acta* **1995**, *1231*, 76−88.
(60) Hughes, J. L.; Prince, B. J.; Krausz, E.; Smith, P. J; Pace, R. J.; Riesen, H. *J. Phys. Chem. B* **2004**, *108*, 10428−10439.
(61) Renger, T. *Phys. Rev. Lett.* **2004**, *93*, 188101−1−188101−4.
(62) Neupane, B.; Jaschke, P.; Saer, R.; Zazubovich, V.; Beatty, J. T.; Jankowiak, R. *J. Phys. Chem. B* **2012**, *116*, 3457−3466.
(63) Hughes, J. L.; Cox, N.; Rutherford, A. W.; Krausz, E.; Lai, T.-L.; Boussac, A.; Sugiura, M. *Biochim. Biophys. Acta* **2010**, *1797*, 11−19.
(64) Krausz, E.; Cox, N.; Årsköld, S. P. *Photosynth. Res.* **2008**, *98*, 207−217.