Site-Energies of Active and Inactive Pheophytins in the Reaction Center of Photosystem II from *Chlamydomonas reinhardtii*

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Abstract

It is widely accepted that the primary electron acceptor in various Photosystem II (PSII) reaction center (RC) preparations is pheophytin *a* (Pheo *a*) within the D1 protein ($Pheo_{D1}$), while $Pheo_{D2}$ (within the D2 protein) is photochemically inactive. The Pheo site energies, however, have remained elusive, due to inherent spectral congestion. While most researchers over the last two decades placed the Q_y-states of $Pheo_{D1}$ and $Pheo_{D2}$ bands near 678–684 nm and 668–672 nm, respectively, recent modeling [Raszewski et al. *Biophys. J.* **2005**, *88*, 986–998; Cox et al. *J. Phys. Chem.* B **2009**, *113*, 12364–12374] of the electronic structure of the PSII RC reversed the assignment of the active and inactive Pheos, suggesting that the mean site energy of $Pheo_{D1}$ is near 672 nm, whereas $Pheo_{D2}$ (~677.5 nm) and Chl_{D1} (~680 nm) have the lowest energies (*i.e.*, the $Pheo_{D2}$-dominated exciton is the *lowest* excited state). In contrast, chemical pigment exchange experiments on isolated RCs suggested that both pheophytins have their Q_y absorption maxima at 676–680 nm [Germano et al. *Biochem.* **2001**, *40*, 11472–11482; Germano et al. *Biophys. J.* **2004**, *86*, 1664–1672]. To provide more insight into the site energies of both $Pheo_{D1}$ and $Pheo_{D2}$ (including the corresponding Q_x transitions, which are often claimed to be degenerate at 543 nm) and to attest that the above two assignments are most likely incorrect, we studied a large number of isolated RC preparations from spinach and wild-type *Chlamydomonas reinhardtii* (at different levels of intactness) as well as the *Chlamydomonas reinhardtii* mutant (D2-L209H), in which the active branch $Pheo_{D1}$ is genetically replaced with chlorophyll *a* (Chl *a*). We show that the Q_x-/Q_y-region site-energies of $Pheo_{D1}$ and $Pheo_{D2}$ are ~545/680 nm and ~541.5/670 nm, respectively, in good agreement with our previous assignment [Jankowiak et al. *J. Phys. Chem. B* **2002**, *106*, 8803–8814]. The latter values should be used to model excitonic structure and excitation energy transfer dynamics of the PSII RCs.

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Abbreviations: Bacterial reaction center (BRC); charge separation (CS); charge separation time (τ_{CS}); chlorophyll (Chl); continuous wave (CW); Cytochrome b_{559} (Cytb_{559}); D1/D2-side pheophytins ($Pheo_{D1}$/$Pheo_{D2}$); D1/D2-side special pair chlorophylls (P_{D1}/P_{D2}); D1-side accessory chlorophyll (Chl_{D1}); excitation energy transfer (EET); fluorescence lifetime (τ_{fl}); Glutamine (Gln); Huang-Rhys factor (S); inhomogeneous broadening (Γ_{inh}); L/M-side bacteriochlorophylls ($BChl_{L,M}$); L/M-side bacteriopheophytins ($BPheo_{L,M}$); laser intensity (I); non-photochemical hole burning (NPHB); photochemically active/inactive polypeptide chains in PSII RC (D1/D2) and in BRC (L/M); Photosystem II (PSII); reaction center (RC); single site absorption (SSA); site distribution function (SDF); triplet-bottleneck hole burning (TBHB); wild-type (WT); zero-phonon action (ZPA); zero-phonon hole (ZPH).

1. Introduction

The recent X-ray structure of the Photosystem II (PSII) reaction center (RC) at 1.9 Å resolution[1] has confirmed that some of the general features of the PSII RC are homologous to those of the bacterial RC (BRC).[2] The $S_1(Q_y)$-electronic structure, excitation energy transfer (EET), and primary charge separation (CS) processes in the D_1-D_2-Cytb_{559} RC complex of PSII (PSII RC) of plants,[3-9] cyanobacteria,[10-14] and algae[15-18] have been the subjects of many time-domain[18-22] and frequency-domain studies[23-28]. Although in recent years consistent progress has been made in our understanding of the electronic structure and CS dynamics of the isolated PSII RC,[10,29-31] an adequate global understanding has yet to be achieved. The key uncertainty in modeling of its optical spectra (independent of the level of the excitonic theory involved) is that different sets of data are interpreted in the context of different pigment site-energies[10,22,29,31-33] that cannot be reliably calculated due to the complex protein environment. Instead, the search for realistic site energies is always guided by experimental constraints and aided by fitting algorithms.[31,32,34-37] In particular, the focus of the current study is on the site energies of the active and inactive pheophytins ($Pheo_{D1}$ and $Pheo_{D2}$, respectively) in the isolated PSII RC from wild-type (WT) *Chlamydomonas (C.) reinhardtii* and its D2-L209H mutant. While most researchers over the last two decades surmised that the Q_y-states of $Pheo_{D1}$ and $Pheo_{D2}$ bands

were at ~678–684 nm and ~668–672 nm, respectively, recent modeling[11,14,38] of the electronic structure of the PSII RC reversed the location of the active and inactive Pheos, suggesting that the mean site energy of $Pheo_{D1}$ is near 672 nm, whereas $Pheo_{D2}$ (~677.5 nm) and Chl_{D1} (~680 nm)[11,14,38] have the lowest energies (i.e., the $Pheo_{D2}$-dominated exciton is the *lowest* excited state of the RC). The latter would allow one to explain the presence of narrow (persistent) holes burned into the lowest energy state spectrum, as excitation of $Pheo_{D2}$ would not lead to charge separation.[38] This assumption, however, is not necessary, as a broad distribution of charge separation rates[4,6,21] can explain the possibility of burning very narrow holes. For example, it has been shown that there are fractions of both P680- and P684-type RCs, where charge separation is too slow to compete with fluorescence at 5K.[4,39,40] Besides, a typical fractional depth of saturated ZPHs at 680 nm are 25%[4] and not 15%, as reported in[38]. Such holes are not achievable within the Cox *et al.* model[38], as $Pheo_{D2}$ contributes only 17% to the lowest state in their model. Thus we favor an interpretation that is also consistent with recent transient absorption experiments at 77K on the isolated PSII RCs from spinach, where the existence of a very slow channel for charge separation was observed.[5] The combination of femtosecond pump-probe transient absorption experiments with exchange of selected Pheos (*i.e.*, chemical exchange of $Pheo_{D1}$ and $Pheo_{D2}$ with 13′-deoxy-13′-hydroxy-pheophytin *a*) suggested that both pheophytins in the PSII RC have their Q_y-transitions at 676–681 nm.[7,41,42] To provide more insight into the site energies of both $Pheo_{D1}$ and $Pheo_{D2}$, and to prove that the above[7,41,42] assignment is unlikely correct, we compare data obtained for the RC from spinach with those generated for WT *C. reinhardtii* and its D2-L209H mutant. In this mutant the active branch $Pheo_{D1}$ is replaced with chlorophyll *a* (Chl *a*) by site-directed mutagenesis[43] on the *psbD* gene.

Figure 1 (left frame) shows a schematic arrangement of the six Chl and two Pheo molecules for the PSII RC from *Thermosynechococcus (T.) vulcanus* based on Ref.[1]; the P_{D1} and

P_{D2} Chls are analogous to the P_L and P_M BChls of the bacterial special pair, respectively, while the $Chl_{D1,D2}$ an $Pheo_{D1,D2}$ molecules correspond to the monomeric $BChl_{L,M}$ and the $BPheo_{L,M}$ molecules of the BRC.[2] The D1 and D2 polypeptides are homologous with the L and M polypeptides of the BRC, respectively.[17,44] By analogy with the BRC, it is also believed that the P_{D1}/P_{D2}, Chl_{D1}, and $Pheo_{D1}$ molecules participate in primary charge separation in the PSII RC.[29-32] An obvious difference between the PSII RCs and BRCs is that the former contains two additional (peripheral) Chls, which are also shown in the left frame of Figure 1. In addition, a sample heterogeneity and spectral congestion of pigments (with comparable couplings) in the PSII RC and the lack of crystals of isolated D1-D2-Cyt b_{559} complexes, make it difficult to describe the excitonic structure of the isolated PSII RC. The right frame of Figure 1 shows the pigments along with their ligands of the D2-L209H mutant mentioned above, in which the active $Pheo_{D1}$ has been replaced with chlorophyll a (Chl *a*). The cofactor arrangement in the active (D1) and inactive (D2) branches of the PSII RC was taken from the very recent crystal structure of *T. vulcanus* at 1.9 Å resolution, PDB ID 3ARC.[1]

To our knowledge, there are only a few studies of the isolated algal PSII RC from *C. reinhardtii* because such complexes are difficult to prepare.[10,15-18] We anticipate that the structural asymmetry introduced into the RC complex in the case of the *C. reinhardtii* D2-L209H mutant will shed more light on the site-energy of the active pheophytin. This strategy of replacing selected bacteriopheophytins (BPheos) with bacteriochlorophylls (BChls) was originally developed in BRCs.[45-47] Similar pigment replacements have been accomplished in PSII RCs using *chemically modified* Pheos along with detergent extraction and pigment reconstitution approaches.[7,41,48] However, it is not clear to what extent the latter approach modifies and/or destabilizes the excitonic structure of the RCs. Site-directed mutagenesis approaches should, in principle, give more precise replacement and a less distorted system than chemical modification,

and have the additional advantage that intact PSII complexes (thylakoids) as well as smaller PSII sub-fractions (including RCs) can be characterized biophysically. It has recently been demonstrated that the introduction of a potential Mg ligand (D1-L210H) over the center of the $Pheo_{D2}$ macrocycle ring resulted in the replacement of $Pheo_{D2}$ with a Chl *a*.[10] In this case, the kinetics of primary charge separation were not substantially altered in the D1-L210H mutant, indicating that the Chl substitution for the $Pheo_{D2}$ in the inactive branch did not significantly perturb the energetics of the primary electron donor/acceptor pair.[10] It was also shown that $Pheo_{D2}$ Q_x band is blue shifted in comparison with the Q_x band of $Pheo_{D1}$ (active).[10]

We present below absorption and hole burned (HB) spectra of WT *C. reinhardtii* and its D2- mutant (D2-L209H), obtained under various conditions as well as some new results from our modeling studies. A discussion of the primary electron donor(s) and the possibility of P_{D1} and Chl_{D1} electron transfer pathways being present in isolated PSII RC is reported in[49]. In the current study we focus on the assignment of the Q_y- and Q_x-states of $Pheo_{D1}$ and $Pheo_{D2}$. The results reported below indicate that the $Pheo_{D1}$-Q_x and $Pheo_{D2}$-Q_x transitions lie on the low and high energy sides, respectively, of the single Pheo-Q_x absorption band, in perfect agreement with data obtained previously with the PSII RCs from spinach.[10,26,34,50] We also demonstrate that the replacement of the $Pheo_{D1}$ with a Chl *a* leads to results which are in agreement with simple modeling studies of the absorption difference spectrum. We show that in contrast to the D1-L210H mutant (with $Pheo_{D2}$ replaced by Chl *a*[10]), where the kinetics of primary charge separation were not substantially altered,[10] the D2-L209H mutant exhibits significant changes in the energetics of the primary electron donor/acceptor pair. Finally, we argue that it is unlikely that $Pheo_{D2}$ dominates the lowest-energy excited state (and contributes to the zero-phonon action (ZPA) spectra) as suggested recently.[38] A new interpretation of the origin of the nonresonant (persistent) hole bleached in the isolated PSII RC is also provided. We anticipate that the data

presented in this paper will allow us to refine the models of the electronic structure of the PSII RC by providing additional constraints (*i.e.,* proper site energies of both the active and inactive pheophytins) for future excitonic calculations of optical spectra (research in progress).

2. Materials and Methods

2.1. Preparation of PSII RC from WT C. reinhardtii and its D2-L209H mutant.

Photosystem II RC preparations from *C. reinhardtii* containing ~6 Chls per 2 Pheos in the WT, and ~7 Chls and 1 Pheo in the D2-L209H mutant (note this mutant is incapable of photosynthetic growth because of the absence of an active-Pheo and thus was grown in the dark in TAP medium) were prepared from both thylakoids and PSII-enriched membranes following the method of Nanba and Satoh[3] with important modifications.[18] To obtain the D2-L209H mutant studied in the present work, we used a transgenic (site-directed mutagenesis) approach to modify the pigment composition of the *C. reinhardtii* PSII RC. A histidine, a common Chl ligand, was introduced to the D2 protein over the macrocycle ring of the active branch $Pheo_{D1}$. Thylakoids or PSII-enriched membranes (around 100 mg Chl) were resuspended in buffer A (20 mM Bis-Tris, pH = 6.5, 1.5 mM (w/v) taurine, and 35 mM NaCl), and treated with 6% (w/v) Triton X-100 (final concentration) at a final chlorophyll (Chl) concentration of 0.5 mg Chl/mL. The detergent was added drop-wise from a 30% (w/v) solution, and the suspension was incubated for 2 h at 4^{o}C with gentle stirring in the dark. The solution was centrifuged at 40,000 x g for 30 min, and the resultant supernatant was loaded onto a Toyopearl TSK DEAE 650s column (30 x 1 cm), pre-equilibrated with buffer B (20 mM Bis-Tris, pH = 6.5, 1.5 mM taurine, 35 mM NaCl, and 0.35% [w/v] Triton X-100) at a flow rate of 3–4 mL/min. The large pellet from the centrifugation was still quite green, and it was re-suspended in the same buffer without Triton, incubated for 10 min, and centrifuged again under the same conditions. The new green supernatant was added to

the previous one on the column. Note that the final bound green material remained at about the middle of the column in contrast to the observation with similar material isolated from spinach, which remained at the very top of the column. Spinach material seems to bind more strongly to this column than that from Chlamydomonas. A similar observation was reported by Alizadeh *et al.*[16] using the *Chlamydomonas* F54-14 mutant (a PSI- and chloroplastic ATPase-defective double mutant). The column was then washed overnight with the same buffer B at a flow-rate of 3 mL/min until the peak at 435 nm was a little lower that that at 417 nm. At this point, the remaining green material was eluted with a 35–300 mM NaCl linear gradient in the same buffer, 3-mL fractions were collected, and the RCs eluted at around 90 mM NaCl. In the case of the D2-L209H mutant, most of the recovered material showed a room temperature absorption peak at around 675 nm, and the material was stored without further treatment under liquid N_2 until use. WT RCs exhibited an absorption peak at around 675.5 nm (the first 3–4 colored fractions that eluted at around 90 mM NaCl), but the other fractions showed maxima at ≥676 nm, probably due to some contamination with LHCII or PSI components. The fractions with maxima at around 675.5 nm were pooled for Cu^{2+}-IMAC chromatography as in Ref.[51], using a Chelating Sepharose Fast-Flow (GE Healthcare) column to eliminate some minor Chl *b*-containing pigment–protein complex contaminant. The green material was eluted with a 0–50 mM Imidazol gradient. Pigment quantification of the preparations was done after extraction with 80% (v/v) acetone using the method in Ref.[52] Our typical RC preparation from spinach contained 5.85 Chl/2 Pheo and that from WT Chlamydomonas contained 5.55 Chl/2 Pheo. If we assume 6.0 Chl/2Pheo in spinach we obtained about 5.7 Chl/2 Pheo in our WT Chlamydomonas PSII RC preparation. This somewhat lower Chl content of our preparations compared with other PSII RC material is most probably due to a reproducibly lower carotenoid content in our preparations as compared to those from other laboratories; carotenoid bands were taken into account in our pigment

calculations). Using the same quantification methodology, our non-active D2-L209H mutant RCs contained about seven Chl per one Pheo, demonstrating that in this mutant one Pheo is been replaced for one Chl.

2.2. Spectroscopic measurements. The hole burning apparatus used for this study was described in detail elsewhere.[53,54] Briefly, absorption and hole spectra were recorded with a Bruker HR120 Fourier transform spectrometer. Absorption and hole-burned spectra were obtained at a resolution of 4 cm^{-1} and 0.5 cm^{-1}, respectively. A Coherent CR699-21 ring dye laser (linewidth of 0.07 cm^{-1}), pumped with a 6 W Coherent Innova argon-ion laser, was used to burn the holes. The persistent NPHB spectra reported in the figures correspond to the post-burn absorption spectrum minus the pre-burn absorption spectrum. The triplet bottleneck hole spectra correspond to absorption spectrum with laser on minus the spectrum with laser off. Burn intensities and times are given in the figure captions. The sample temperature was maintained at 5 K using a Janis 8-DT Super Vari-Temp liquid helium cryostat. The excitation source for the fluorescence experiments was a Coherent UV argon-ion laser operating at 496.5 nm. Fluorescence was dispersed at a resolution of 0.1 nm by a Princeton Instrument Acton SP-2300 monochromator, equipped with a back-illuminated, N_2-cooled CCD camera. Care was taken to ensure that reabsorption effects were negligible.

2.3. Monte Carlo simulations of the WT minus the mutant RC absorption difference spectrum. To model absorption changes introduced by the mutation, we carried out simple excitonic calculations using MathCad 12.0. Transition dipole moments and coupling between chromophores were calculated as in Ref.[11] However, due to the significant shift in the position of the central magnesium out of the chlorin plane, which was revealed in the recent crystal structure, the transition dipole moments were taken to extend from the ring I nitrogen (N-B) to

the ring III nitrogen (N-D) rather than from the central Mg to N-D, and the center of the Chl molecule was taken to be at the averaged position of the four ring nitrogen atoms, rather than at the central magnesium. A Frenkel Hamiltonian matrix (static lattice approximation) for the PSII RC chromophores was constructed (see section 3.5); for each iteration of the Monte Carlo simulations, site energies were added randomly according to a Gaussian distribution, mimicking the site distribution function (SDF) of the chromophore. A simple fitting algorithm was used to find optimized parameters (*i.e.,* site energies and the width of the SDF) to achieve simultaneously good fits to the absorption for the RCs from WT *C. reinhardtii* and the D2-L209H mutant, as well as the difference between the latter two spectra.

3. Results

3.1. Low temperature absorption spectra of the RC from WT C. reinhardtii and its D2-L209H mutant—site energies of $Pheo_{D1}$ and $Pheo_{D2}$. Absorption spectra of the WT RC from *C. reinhardtii* and its D2-mutant are shown in Figure 2, as blue (a) and black (b) curves, respectively. According to pigment extraction with 80% (v/v) acetone and comparison with spinach RC data obtained as in Ref.,[52] we established that our WT RC and D2 mutant contained about 6 Chl *a*/2 Pheo *a* and 7 Chl *a*/1 Pheo *a*, respectively (the measured values were 5.7 and 6.5-7, respectively; however, see the comments in Material and Methods, Section 2.1). Note that our WT RC preparation (see curve a in Figure 2) has a 5K absorption maximum near 679.5 nm with a noticeable shoulder near 685 nm. Spectra a and b were normalized over a broad spectral range of 500–700 nm, assuming that the integrated absorbance of the WT PSII and D2-L209H mutant RCs should be about 7.0 (in Chl *a* units) and 7.5, respectively. The difference originates from the fact that the mutant has Chl *a* (instead of $Pheo_{D1}$) in the active D1-protein branch and the Pheo oscillator strength in the protein environment is 50% that of Chl *a*[7,55], for convenience

normalized to unity. The difference spectrum between mutant and WT RC is given by the red curve (c) in Figure 2. The overall absorption decrease near 684 nm in curve (c) corresponds to about 0.3 in Chl *a* units. The intensity gain at ~679 nm is bigger than the loss at ~684 nm because the oscillator strength of the Chl *a* is higher than that of Pheo *a*. A theoretical description of this spectrum is shown in Figure 2. Here we note only that the shape of curve (c) suggests that the site energy of Chl *a* in the $Pheo_{D1}$ binding pocket shifts to the blue. The fluorescence from WT *C. reinhardtii* RCs peaks near 685 nm (data not shown).

Curves a′ and b′ in the inset of Figure 2 correspond to the normalized Q_x band of the WT *C. reinhardtii* RC and its D2-L209H mutant. The difference between curves a′ and b′ (curve c′) corresponds to the removal of $Pheo_{D1}$ (red curve in the inset), proving that the absorbance difference in Q_y near 684 nm (see curve c) indeed originates from the genetically replaced mutation of Pheo *a* ($Pheo_{D1}$) to Chl *a*. We would like to make it clear that the 684 nm bleach does not reflect the site energy of $Pheo_{D1}$; rather, it reflects changes to the lowest exciton band in the RC mutant (*vide infra*). We hasten to add that the coupling constants between chlorins in the Q_x region are about one order of magnitude weaker than those in the Q_y-region.[56] This is why the *entire* Q_x absorption band near 544 nm in WT RC corresponds both to the active and inactive pheophytins, since the Q_x band of Chl *a* lies near 580 nm. Thus the bleach at ~545 and ~684 nm corresponds definitively to the replacement of active $Pheo_{D1}$ with Chl *a*. This in turn indicates that the Q_x-transition of $Pheo_{D2}$ must be near 541.5 nm; the site energy of Q_y $Pheo_{D2}$ will be discussed below. Here, it suffice to say that the relative positions of the Q_x-transitions of active and inactive Pheos in *C. reinhardtii* are in good agreement with the same Q_x-transitions observed in isolated RC from spinach, where the localized Q_x-transitions of $Pheo_{D1}$ and $Pheo_{D2}$ were demonstrated to lie at 544.4 and 541.2, respectively.[26,34] In addition, we found that in our most

intact WT RC from *C. reinhardtii* the entire Q_x-band lies near 544 nm (not at 543 nm, as typically observed in isolated spinach RCs[7,24,41]). The red-shifted Q_x bleach of active $Pheo_{D1}$ near 545 nm is in perfect agreement with data obtained for intact spinach PSII core,[58,59] suggesting that our isolated RCs are intact. The green curve d is the theoretical fit of curve c that was obtained using $Pheo_{D1}$ site energy in WT RC at 679.8 nm, which was blue-shifted by 125 cm^{-1} (due to $Pheo_{D1}$ replacement with Chl *a*) in the mutant (see section 3.5. for details). Here we just note that the $Pheo_{D1}$ site energy used in this fit is 679.8 nm. In view of the above data, we conclude that it is doubtful that the site energy of $Pheo_{D1}$ lies near 672 nm, as assumed recently in Refs.[11,38] (*vide infra*). Thus, it is not likely that the exciton realizations may lead to a significant $Pheo_{D2}$ contribution to the lowest energy exciton, as suggested by Cox *et al.*[38].

3.2. Comparison of absorption, persistent, and transient hole spectra obtained for the RCs from C. reinhardtii and spinach. Frames A and B in Figure 3 show Q_y absorption spectra obtained for RCs from *C. reinhardtii* and spinach, respectively. There is no indication that any of these two samples are contaminated with antenna complexes, *i.e.,* both have 6 Chl *a* /2 Pheo *a*, and exhibit no bleaching and/or emission bands typically observed in PSII antenna complexes.[40,60] The corresponding insets in frames A and B show Q_x absorption band of pheophytins located near 544 nm (frame A) and 543 nm (frame B). As expected, the more intact sample (frames A, C, and E) has red-shifted peaks of Q_y- and Q_x-absorption bands, as well as the position of the lowest-energy bands, in agreement with spectra obtained for RC from intact PSII core samples.[14,58,59] Frames C and D show (nonresonant) persistent saturated holes obtained with λ_B of 665.0 nm. Non-resonant holes appear as a result of downhill energy transfer. The hole depths of the broad (saturated) holes, obtained under identical conditions and shown in frames C (*C. reinhardtii*) and D (spinach), are 4.6% and 3.5%, respectively. Although hole shapes are similar, the broad hole in *C. reinhardtii,* located at 683.8 nm, is about 3.2 nm red-shifted in

comparison with the hole typically obtained for RCs isolated from spinach.[25,26,61] The origin of the broad 683.8 and 680.6 nm holes will be discussed in section 4.1. Here we only note that both persistent holes shown in frames C and D have weak responses in the Q_x region of $Pheo_{D1}$ near 545 ± 1 and 544 ± 1 nm, respectively. An example of Q_x bleach in spinach RC is shown in the inset of frame D.

In contrast to persistent holes, the transient, non-line narrowed spectra shown in frames E and F (λ_B = 665.0 nm) are very different; the hole in *C. reinhardtii* (frame E) has two components at 672.7 and 683.3 nm with a positive feature near 679 nm. The fractional hole depth at 683.3 nm (measured with a laser intensity (I) of 100 mW) is 22.5%. The large nonresonant transient hole, obtained under identical conditions for spinach RCs, is blue shifted to 680.0 nm (although slightly red-shifted holes near 681–682 nm have been previously observed in isolated RCs from spinach[25,26,61]) and has a smaller fractional depth of 9.8%. Note the shape of this transient hole is exactly the same as previously observed in spinach PSII RCs[25,26,61] with a bleach near 680/681 nm and a weak shoulder near 683/684 nm[26], which we assigned previously to P680 and P684 RCs, respectively[4]. That is, we proposed that in isolated spinach RCs, transient holes at ~680–681 nm and ~684 nm correspond to the triplet bottleneck holes, originating from P680- and P684-type RCs and reflecting the gross heterogeneity of PSII RCs (the P684–band is a subset of RCs that are more intact than P680-type RCs).[4] This suggestion is consistent with data presented in Figure 3. Another striking difference between the transient holes obtained for *C. reinhardtii* and spinach RCs is in the Q_x region. As previously observed there is no bleaching in this region for transient spectra obtained for spinach RCs (see the inset in frame F), whereas the Q_x Pheo band clearly shifts in *C. reinhardtii* RCs as shown in the inset of frame E.

3.3. Optical spectra obtained for the C. reinhardtii D2-L209H mutant. Spectra a and a′ in Figure 4 show absorption and emission (with a (0,0)-band maximum near 681 nm) spectra of the D2-L209H mutant. The lowest-energy state (see curve b corresponding to a nonresonant persistent hole obtained with 665 nm excitation) is near 680 nm, revealing a very small Stokes shift of ~20 cm^{-1}. This means that electron-phonon coupling is weak (with an estimated Huang-Rhys factor, S of ~0.6, assuming ω_m of 17 cm^{-1}). The fluorescence maximum of the D2-L209H mutant, in comparison to WT RCs, is blue shifted due to a different pigments contributing to the lowest-energy excitonic state. Recall that the absorption spectrum of the D2-L209H mutant, in which the active branch $Pheo_{D1}$ is replaced with Chl *a* (see inset of Figure 2), did not reveal (as expected) the Q_x-absorption band near 545 nm assigned to $Pheo_{D1}$ (*vide supra*). In light of the above observations, it is interesting to note that the persistent (nonresonant) hole spectrum obtained for the mutant with λ_B = 665.0 nm (curve b in Figure 4) has a ~93 cm^{-1} wide hole centered at ~680 nm but no response in the Q_x pheophytin region (see inset below curve b), proving again that $Pheo_{D2}$ does not contribute to the lowest-energy state near 680 nm. The hole near 680 nm can be attributed to downward energy transfer from pigments excited at 665.0 nm to the (blue shifted) lowest-energy excitonic band in the mutant, which is strongly contributed to by Chls (in particular the Chl *a* residing in the $Pheo_{D1}$ binding pocket and Chl_{D1}). This is consistent with data shown in Figure 2, as the substituted Chl *a* strongly contributes to the absorption spectrum near 680 nm. As expected, WT RC samples intentionally degraded by leaving them on the bench at room temperature for many hours do not reveal any broad-band bleach near 680 nm in agreement with data shown in Figure S1 (see curves d) in Supporting Information. The identity of the pigment(s) contributing to the low-energy states of WT and mutant RCs will be discussed in section 4.3. The weak (~6% hole depth) symmetric transient hole (Figure 4, curve c)

is most likely formed via intersystem crossing and corresponds to the lowest-energy band (~680 nm) of the mutant, although it cannot be excluded that the shallow transient hole reflects a triplet-bottleneck hole after recombination of the $Chl_{D1}^{+}Chl a^{-}$ state (if formed). (Recall that Chl *a* mentioned above resides in the $Pheo_{D1}$ binding pocket). This suggestion needs to be tested via resonant HB and time resolved spectroscopies. Curve b, however, is asymmetric in the higher frequency range due to the presence of an anti-hole typically observed in persistent HB spectra.[40,62] Nevertheless, these data clearly support our above assignment that the mean site energy of $Pheo_{D2}$ is unlikely to lie anywhere close to 680 nm and dominate the lowest-energy excited state, as suggested in Refs[29,38]. We hasten to add that our findings also question the assignment of site energies of pheophytins obtained with the *in vitro* exchange experiments, where $Pheo_{D1}$ and/or $Pheo_{D2}$ were replaced by 13^1-deoxo-13^1-hydroxy pheophytin (13^1-OH Pheo).[7,41] The observation reported in Refs[7,41] that both $Pheo_{D1}$ and $Pheo_{D2}$ contribute at the same wavelength to the absorption spectrum, both in the Q_y- and Q_x-regions, could indicate that exchange experiments destabilize RCs or perturb excitonic interactions. The observed bleach near 680 nm and the absorption increase at ~654 nm (the Q_y-transition of replaced 13^1-OH Pheo[7,41]) are not necessarily correlated, *i.e.*, some changes observed near 680 nm could be due to partial damage of RCs and the accompanying changes in the excitonic interactions. Therefore, in light of the data presented in this work, we cannot exclude the possibility that some delta absorbance changes (near 680 nm region) observed in samples going through the chemical exchange process and purification are induced by RC destabilization. In fact, difference spectra reported in Refs[7,41] if not normalized at 624 nm, would be similar to spectra c shown in Figure S1 for destabilized RCs in Supporting Information.

3.4. Monte Carlo simulations of the absorption difference spectra of C. reinhardtii and its D2-L209H mutant. The red curve (c) in Figure 2 corresponds to delta absorbance spectra

obtained for WT RC and its mutant, assuming a dipole strength ratio of Pheo a/Chl a of 0.5.[55] The green curve (spectrum d) in Figure 2 was obtained using excitonic calculations for the Q_y-states using the Frenkel Hamiltonian mentioned above. We used coupling constants calculated by the *ab initio* TrEsp method[11] and the recent X-ray structural data (3ARC PDB, file)[1]. The coupling constants (with the exception of the coupling constant (V) for the special pair $V_{PD1\text{-}PD2}$) were very similar to those reported in[11] and are listed in Table S1 in the Supporting Information. Taking into account a considerable wavefunction overlap between P_{D1} and P_{D2} Chls, resulting in Dexter-type exchange coupling, the coupling constant $V_{PD1\text{-}PD2}$ of 150 cm^{-1} was taken from Ref.[11] The site energies were adjusted using our experimental constraints obtained for the site energies of both $Pheo_{D1}$ and of $Pheo_{D2}$, as discussed above. The site energies used for $Pheo_{D1}$ and $Pheo_{D2}$ are ~ 679.8 and ~ 670 nm, respectively. The inhomogeneous width of 165 cm^{-1} was used in the Monte Carlo simulations. Absorption spectra of *C. reinhardtii* and its D2-L209H mutant were calculated with the same set of parameters with the only adjustment that $Pheo_{D1}$ in WT RC was replaced with Chl *a* in the mutant, and the site energy of Chl a (residing in the $Pheo_{D1}$ binding pocket) was shifted to the blue by 125 cm^{-1} from the site energy of $Pheo_{D1}$ at 14710 cm^{-1} (679.8 nm). This shift is consistent with the loss of H-bonding (with Glutamine 130) in Chl *a* residing in the $Pheo_{D1}$ binding pocket. The nice agreement between experimental and calculated difference spectra in the low energy spectral range provides further evidence that $Pheo_{D1}$ in WT RC significantly contributes to the lowest energy exciton state near 684 nm and has a site energy near 680 nm (see section 4.3 for more details).

4. Discussion

4.1. Nonresonant persistent holes. Historically, the original assignment of the broad (~120 cm^{-1}) nonresonant persistent hole, typically observed near 680–682 nm[25,26,61] for 665 nm

excitation in isolated RCs from spinach, was to a Q_y-state localized on $Pheo_{D1}$.[25] This was proposed prior to the introduction of the multimer model.[34,63,64] Subsequent research revealed that *only nonresonant* excitation near 660–670 nm reveals persistent bleaching at the Q_x band assigned to $Pheo_{D1}$,[25] meaning that the Q_x bleach is never observed for saturated holes burned *resonantly* in the 680–686 nm region. In Ref.[4] we provided evidence for highly dispersive primary charge separation kinetics; that is, we have shown that population of either P680* (in RC680, where * ≡ the Q_y-state) or P684* (in RC684, the subset of RCs more intact than RC680) results in both resonant *transient* (due to charge recombination of the primary radical pair) and resonant *persistent* (nonphotochemical) holes. Indeed, one set of parameters (*e.g.* electron-phonon coupling and site distribution function) could be used to simulate all transient and persistent holes burned in the 680–686 nm region. Thus the question arises what is the origin of the broad nonresonant ~680 and ~684 nm persistent holes (as shown in Figure 3, frames C and D) accompanied by a clear bleach in the Q_x-region of $Pheo_{D1}$?

We suggest that our original interpretation,[25] namely that this broad hole belongs to the quasi-localized Q_y-band of $Pheo_{D1}$ may actually be correct. We propose that these non-resonant persistent HB spectra could be formed (via CW excitation) during the relatively long lifetime (~2 ms[9,65]) of the $^3P680$ or $^3P684$ triplet states that are formed (in the absence of Q_A) after recombination of the $P680^+Pheo_{D1}^-$ and $P684^+Pheo_{D1}^-$ states, respectively. In these cases, the formation of $^3P680$ or $^3P684$ (if the triplet is localized on Chl_{D1}[13]) could partially decouple $Pheo_{D1}$ from the rest of the RC pigments. If $Pheo_{D1}$ has a site energy of ~680 nm, as suggested in this work, it would contribute to the energy trap for EET from states at ~665 nm which are excited by the laser. This indeed could lead to weak broad (~120 cm^{-1}) satellite holes with hole minima near 680 nm and/or 684 nm, depending on the quality (*i.e.*, intactness) of the RC

sample.[25,26,61] Note that the same process is highly improbable in the case of resonant burning. The RCs, which are resonantly excited at 680-684 nm, may exhibit either slow or fast primary charge separation[5], with persistent HB favoring slow charge separation RCs very strongly (i.e., RCs with fast charge separation and likely triplet formation do not contribute much). On the other hand, in RCs exhibiting fast charge separation the NPHB into the original lowest-energy state should be very inefficient based on the equation for the spectral hole burning yield[39]:

$$\phi_{SHB} = \frac{\Omega_0 e^{-2\lambda}}{\Omega_0 e^{-2\lambda} + \tau_{CS}^{-1} + \tau_{fl}^{-1}} \quad ,$$

where $\Omega_0 e^{-2\lambda}$ is the NPHB rate, and τ_{cs} and τ_{fl} are the charge separation time and fluorescence lifetime, respectively. Then during the lifetime of the ^{3}P680- or ^{3}P684-triplet states, the narrow zero-phonon lines (ZPL) of the lowest state (now localized on $Pheo_{D1}$) would not be in resonance with the laser, and, as a result, these RCs will become unavailable for further absorption of resonant photons and NPHB. This escape of the lowest-energy state from the resonance with the laser due to triplet formation is obviously irrelevant for nonresonant excitation at 665 nm, as EET would occur anyway.

The only difference between (nonresonant) saturated persistent holes (quasi-localized on $Pheo_{D1}$) burned with λ_B = 665 nm (during the ^{3}P680 and ^{3}P684 triplets, in RC680 and RC684, respectively) is that the corresponding Q_x-/Q_y-transitions depending on sample intactness are at ~544±1/~680.6 nm and ~545±1/683.8 nm, respectively, as further discussed in the Supporting Information. This is why Q_y- nonresonant persistent holes have a profile similar to that obtained due to formation (in the presence of dithionite) of stable $Pheo_{D1}^-$.[26,34] However, the situation is more complicated in intact RC684 from *C. reinhardtti*, which could contain plastoquinone Q_A, and possibly possess two electron donors, P_{D1} and/or Chl_{D1} (*i.e.*, two charge separation pathways, as recently proposed in Ref[5]). That is, the transient hole in Figure 3E may have

contributions from both the ^{3}P684 triplet and the long-lived $P^+Q_A^-$ state[66] for a subpopulation of RCs that contain Q_A. The nature of the electron donor(s) and the feasibility of multiple electron transfer pathways in *C. reinhardtii* (including measurements of electron transfer times via resonant HB) is discussed in[49]. Here we only note that the ratio of the nonresonant TBHB spectrum (I = 100 mW) to NPHB (saturated persistent hole) in spinach (see Figure 3) is about 3.5, while the same ratio in *C. reinhardtii* (obtained under identical conditions; see Figure 3) is about 5.5. Interestingly, the ratio of ~3.6 was also observed in destabilized P680-type RCs obtained for *C. reinhardtii*. Since the depth of the saturated persistent hole (nonresonant, λ_B = 665.0 nm, obtained under similar conditions) in: (1) spinach RC near ~680 nm; (2) destabilized RC from *C. reinhardtii* (also at ~680 nm; data not shown); and (3) intact RC from *C. reinhardtii* (~683.8 nm), is only ~3–4%, it is not surprising that the *persistent (nonresonant) hole* from a small subset of intact P684-type RCs near 684 nm in *spinach* RCs (revealed as a weak shoulder near 684 nm in a transient hole[61]) has been never observed.

4.2. Site energies of Pheo$_{D1}$ and Pheo$_{D2}$. We emphasize that knowledge of the site energies of the Chls and Pheos is important to understand the excitonic structure and excitation energy transfer in PSII RCs, as they determine the direction of the energy flow. Recall that conclusions made from the experiments with borohydride reduction of the inactive branch Pheo$_{D2}$[67]and exchange of Pheo$_{D2}$[7,41,68], as well as with partial exchange of Pheo$_{D1}$[7,41] with 13^1-hydroxy Pheo (*vide supra*)[7,41], which suggested that both pheophytins have their Q_y absorption maxima at 676–680 nm (at 6 K), are inconsistent with data shown above. In addition, the blue shift of both the Q_x and Q_y transitions of Pheo$_{D1}$ in destabilized RCs (see Figure S1; Supporting Information) is consistent with resonance Raman spectroscopy data[69], where it was suggested that Pheo$_{D1}$ in the PSII RC is likely H-bonded (as BPheo$_L$ in the BRC[70,71]), with the glutamine

residue D1-Gln130 being the likely homologue of the glutamic acid residue L-104 in BRC. That is, in the *Synechocystis* site-directed mutation, D1-Gln130Leu results in the shifting of the Q_x band to shorter wavelength consistent with a disappearance of the $C13^1$=O hydrogen bond. Different Q_x and Q_y positions of $Pheo_{D1}$ and $Pheo_{D2}$ also suggest that only $Pheo_{D1}$ is H-bonded[72]. Thus we suggest that the H-bond of $Pheo_{D1}$ can be easily broken in destabilized RCs, as shown by the blue shift of the Q_y-absorption band in Figure S1 (see Supporting Information). Finally, the different Q_y positions of $Pheo_{D1}$ and $Pheo_{D2}$ are consistent with an earlier suggestion in Ref.[69] that one possible distinction between the active and inactive branches of the PSII RC might be the presence of a H-bonded pheophytin on the active branch and the absence of a hydrogen bond on the inactive branch. The latter is consistent with data presented above, but contradicts the conclusions reached in[72] that the Q_x transitions of both pheophytins located at 543 nm imply that both pheophytins have a H-bond to the $C_{13}{}^1$=O of equal strength. Other groups also concluded that the $Pheo_{D1}$ contributes to the low-energy state near 681 nm.[3,37,42,73-75] Several earlier studies also indicated that the site-energy of $Pheo_{D2}$ Q_y-state must be located near 668–672 nm[26,34,37,73-75], in agreement with the results presented in this paper.

4.3. Excitonic calculations. In calculations of absorption spectra (and of the difference spectrum shown in Figure 2 (curve d)), inhomogeneous broadening (Γ_{inh} = 165 cm^{-1}) was treated via Monte Carlo methods, while homogenous broadening was included by convoluting the calculated spectra with an assumed single site (SSA) spectrum for absorption. The parameters used for the SSA curve (*i.e.*, zero phonon line width = 0.5 cm^{-1}, S = 0.8, ω_m = 17 cm^{-1}) were taken from[4]. Vibrational modes of Chl *a* were adopted from[76]. The other common property of the parameter sets resulting in good fits to the difference spectrum shown in Figure 2 is that the site energy of $Pheo_{D2}$ used in the modeling was near 670 nm. In addition, our modeling data confirm

that the site energies of P_{D1} and P_{D2} found by Renger's group[11,29] are correct and must lie in the 665–667 nm region. We also confirm that the Chl_{D1} site energy must be red shifted to about 678 nm in agreement with Ref.[29] and various calculations of triplet-bottleneck spectra.[11] Discussion of site energies for PSII RCs, along with simultaneous fits of WT and mutant RC absorption, emission, triplet-bottleneck holes, and transient $P_{D1}^{+}Q_A^{-}$ - $P_{D1}Q_A$ spectra will be reported elsewhere as fits of absorption spectra alone cannot provide the final set of site energies. Nevertheless, all pigment site energies tested so far, which can simultaneously describe the absorption spectra of WT RC and its D2-L209H mutant, as well as their difference shown in Figure 2, have in common that the site energy of $Pheo_{D1}$ must be near 679–681 nm, in agreement with our experimental data reported above, and previously published results. [3,26,34,37,42,73-75]

4.4. Transient holes. The transient spectrum obtained for isolated RC from *C. reinhardtti* (frame C, Figure 3) is very similar to the flash-induced ($P684^{+}Q_A^{-}$ - $P684Q_A$) absorbance difference spectra of PSII core complexes from WT *Synechocystis sp.* PCC 6803.[13] The latter suggests the presence of Q_A; therefore, an electrochromic shift in the Q_x-region of the pheophytins is possibly due to the formation of the $P^{+}Q_A^{-}$ state, as previously observed in bacterial RCs[60,77,78] and PSII cores.[14,58,59] As expected, there was no bleach or shift in the 530–560 nm region in the D2-L209H mutant, since there is no formation of a $P^{+}Q_A^{-}$ state, even if Q_A is present, due to the lack of active $Pheo_{D1}$. This proves that our isolated RCs from *C. reinhardtti* are to a large extent intact with a bleach at 672.7 nm being due to the oxidation of P (mostlikely P_{D1}), or, expressed more properly and as discussed by Schlodder *et al*. in the case of PSII core from *Synechocystis*[13], due to the bleaching of an exciton state dominated by contributions from P_{D1}. This is consistent with recent finding that the redox potential (E_m) of P_{D1} for a one-electron oxidation ($E_m(P_{D1})$) is lower than that of P_{D2}, favoring localization of the cationic charge state

more on P_{D1}.[79] The positive peak in the transient spectrum (see frame E in Figure 3) is related to an electrochromic shift of the pigment(s) contributing to the lowest-energy state (most likely Chl_{D1} and $Pheo_{D1}$), induced by the positive charge on P (P_{D1}[12,13,79]) and a negative charge on plastoquinone Q_A (Q_A^-). A very different shape of the transient hole observed in spinach RC (frame F) is most likely the result of disruptive protein-pigment structural perturbations introduced by the isolation procedure and absence of Q_A. We suggest that in spinach RCs (see frame F) Chl_{D1} is the preferred electron donor with the triplet also localized on Chl_{D1}.[13,80,] The lack of response near 672.7 nm in spinach RCs supports the notion that RC680 (in contrast to RC684) has destabilized D1 and D2 proteins with a significantly weakened excitonic coupling between the P_{D1} and P_{D2} Chls, and as a result, a much weaker absorption band near 673 nm.

V. Conclusions.

A large number of isolated RC preparations from spinach and wild-type *C. reinhardtii* (at different levels of intactness), as well as the D2-L209H mutant, in which the active branch $Pheo_{D1}$ has been genetically replaced with Chl *a*, have been studied by HB spectroscopy. This work provides direct evidence that the biochemical treatment used to isolate D1-D2-Cytb_{559} RC complex may change optical properties of the RC pigments. It appears, however, that RCs with similar properties to those observed in intact PSII cores can be obtained; the latter is of critical importance, as optical spectra of PSII cores are more difficult to interpret due to possible contributions from CP43 and CP47 antenna pigment complexes. The results presented here offer no support for the recent assignments[11,14,29,38] that site energies of $Pheo_{D1}$ and $Pheo_{D2}$ are at 672 nm and 677.5 nm, respectively. It is also unlikely that both pheophytins contribute to the absorption near 676–681 nm region as proposed in Refs.[7,33,41,42] In contrast, we have demonstrated that the Q_x-/Q_y-region site-energies of $Pheo_{D1}$ and $Pheo_{D2}$ are most likely at ~545/~680 nm and ~541.5/~670 nm, respectively, in good agreement with our previous

assignment.[26,34] These values should be used to model excitonic structure and excitation energy transfer dynamics of the PSII RC. We also propose that the nonphotochemical (nonresonant) persistent holes at ~680 and ~684 nm (depending on the sample intactness), obtained via higher-energy excitation (e.g., λ_B = 665.0 nm) are, at least in part, the result of burning in the decoupled $Pheo_{D1}$ Q_y-state during the lifetime of the long lived $^3Chl_{D1}$ triplet bottleneck-state in RCs without Q_A. On the other hand, the narrow (lifetime limited) resonant holes[23,81] as well as the saturated resonant holes burned in the 678–686 nm spectral region of the PSII RC (with no bleaching in the Q_x band of the pheophytins) are not bleached in the $Pheo_{D2}$ dominated low-energy exciton band, as suggested in[11,29,38]. On the contrary, the experimentally observed distribution of hole widths in both persistent and transient HB spectra[4,39,82] must be associated with highly dispersive primary charge separation kinetics in destabilized RC680 and intact RC684.

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Supporting Information Available: This material contains information on spectroscopic measurements of destabilized RCs as well as the coupling constants used for the excitonic calculation. This material is available free of charge via the Internet at http://pubs.acs.org.

Figure captions

Figure 1. Cofactor arrangement in the active (D1) and inactive (D2) branches of the PSII RC based on the crystal structure of *T. vulcanus* at 1.9 Å resolution, PDB ID 3ARC[1]. The left frame shows the arrangement in WT RCs (chls, green; carotenes, yellow; pheophytins, purple; plastoquinones, gray; non-heme iron, red; and nitrogen, blue). The right frame shows the arrangement of selected pigments and their ligands (water, orange; histidine, red; leucine, cyan) in the D2-L209H RC mutant. The substituents of the cofactors are truncated for clarity.

Figure 2. Spectra a and b correspond to the absorption spectra of intact WT and D2-L209H mutant RCs, respectively, measured at T = 5 K. The areas of curve a and b are scaled to 7.0 and 7.5, respectively, based on the normalized oscillator strength of the cofactors. Curve c is the difference between spectra b and a, and curve d is the calculated difference spectrum. The inset shows the corresponding Q_x-region (see text for detail).

Figure 3. Absorption, nonresonant persistent hole, and transient HB spectra obtained for RCs from *C. reinhardtii* and spinach RC are shown in frames A/C/E and B/D/F, respectively. The insets in frames A and B show the Q_x absorption band of both pheophytins near 544 and 543 nm, while those in frames E and F illustrate the Q_x response (in the pheophytin region) in the transient spectra. All HB spectra were obtained with a λ_B of 665 nm and were measured at 5K. The inset in frame D (~544 nm) corresponds to Q_x bleach of $Pheo_{D1}$ (see text).

Figure 4. Curves a, a', b, and c correspond to the absorption, fluorescence, persistent, and transient HB spectra obtained for the D2-L209H mutant of *C. reinhardtii*. Both HB spectra were obtained with a λ_B of 665.0 nm, and recorded at 5 K. The inset below curve b shows that there is no bleaching in the Q_x-region of $Pheo_{D2}$ (note, $Pheo_{D1}$ is absent in D2-mutant).

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