Site-directed Mutations at D1-Thr179 of Photosystem II in *Synechocystis* sp. PCC 6803 Modify the Spectroscopic Properties of the Accessory Chlorophyll in the D1-branch of the Reaction Center[†]

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ABSTRACT: D1-Thr179, which overlies the reaction center chlorophyll Chl_{D1} of Photosystem II was replaced with His and Glu through site-directed mutation in *Synechocystis* sp. PCC 6803. Spectroscopic characterization of the mutants indicates that, compared to wild type, the main bleaching in the triplet-minus-singlet absorbance difference spectrum and the electrochromic band shift in the ($P680^+Q_A^-$−$P680Q_A$) absorbance difference spectrum are displaced to the red by ∼2 nm in the D1-Thr179His mutant and to the blue by ∼1 nm in the D1-Thr179Glu mutant. These difference spectra are compared with the absorbance difference spectra, measured on the same states in the D1-His198Gln mutant in which the axial ligand D1-His198 of the special pair chlorophyll, P_{D1}, was replaced by glutamine. Together, these results give direct evidence that (a) the reaction center triplet state, produced upon charge recombination from $^3[P^+Pheo^-]$, is primarily localized on Chl_{D1}; (b) the cation of the oxidized donor P^+ is predominantly localized on chlorophyll P_{D1} of the special pair; and (c) the Q_Y band of the accessory chlorophyll Chl_{D1} is electrochromically shifted in response to charges on P^+ and Q_A^-. Light-induced absorbance difference spectra (between 650 and 710 nm), associated with the oxidation of secondary donors and the reduction of Q_A, exhibit a bleaching attributed to the oxidation of a Chl_Z and strong electrochromic band shifts. On the basis of mutation-induced spectroscopic changes and of structure-based calculations, we conclude that the experimental spectra are best explained by a blue-shift of the Q_Y band of the accessory chlorophyll Chl_{D1}, arising from charges on Car_{D2}^+ and Chl_{ZD2}^+ and on reduced Q_A.

Photosystem II (PS II)[1] of plants, algae, and cyanobacteria is a membrane-bound pigment–protein complex that mediates light-induced electron transfer from water to plastoquinone (for a review see refs *1, 2*. PS II of higher plants and algae consists of the PS II core complex and intrinsic membrane peripheral light-harvesting chlorophyll−protein complexes. In cyanobacteria, in which the latter have been replaced by phycobilisomes, membrane-bound PS II is composed of four large subunits (D1, D2, CP43, and CP47), cyt *b559*, which is constituted by subunits PsbE and PsbF, and a number of low molecular weight membrane-intrinsic subunits. Three extrinsic subunits (PsbO (33 kDa), PsbU (12 kDa), and PsbV (cyt *c550*)) are located on the lumenal side of the membrane.

The structure of the PS II core complex from *Thermosynechococcus elongatus* has been determined by X-ray crystallographic analysis to a resolution of 3.0–3.5 Å (*3–6*) The most recently published X-ray structure of dimeric PS II (*6*) identifies 36 transmembrane helices and some 77 cofactors that include 35 Chl *a*, 11 *β*-carotene, 2 pheophytin *a* (Pheo), 1 *b*-type cytochrome (cyt *b559*), 1 *c*-type cytochrome (cyt *c*550), 2 plastoquinones (Q_A and Q_B), a nonheme-iron, and the Mn_4Ca cluster responsible for water oxidation. The D1 and D2 protein subunits coordinate the cofactors involved in the light-induced electron transfer reactions.

P680, a dimer of two chlorophyll *a* molecules, P_{D1} and P_{D2}, is located on the lumenal side of the thylakoid membrane. Two transmembrane branches of cofactors related by pseudo-*C*2

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[1] Abbreviations: ΔA, absorbance change; Car, *β*-carotene; Chl_{D1} and Chl_{D2}, monomeric accessory Chl on the active D1 branch and on the inactive D2 branch of the reaction center; Chl, chlorophyll; D1 and D2, the D1 and D2 polypeptides of the PS II reaction center; *β*-DM, n-dodecyl-*β*-maltoside; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared spectroscopy; MES, 2-(*N*-morpholino)ethanesulfonic acid; P, the primary electron donor of PS II comprised of P_{D1} and P_{D2}; P_{D1}, the chlorophyll coordinated by D1-His198; P_{D2}, the chlorophyll coordinated by D2-His197; ^3P, the triplet state formed by charge recombination in PS II, Pheo, pheophytin; PS II, Photosystem II; Q_A and Q_B the primary and secondary plastoquinone electron acceptors of PS II; RC, reaction center; WT, wild type *Synechocystis* sp. PCC 6803; Tyr_Z, D1-Tyr161, the redox active tyrosine of PS II on the D1 polypeptide, T−S, triplet-minus-singlet, $t_{½}$, half-life.

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symmetry connect P680 and the plastoquinones. Each branch (D1 or A and D2 or B) is composed of the so-called accessory chlorophyll (denoted Chl_{D1} and Chl_{D2} in ref *6*), a pheophytin *a* ($Pheo_{D1}$ and $Pheo_{D2}$), and one plastoquinone (Q_A and Q_B, respectively).

After absorption of light, the excitation energy is efficiently trapped via charge separation in the reaction center (RC), leading to the generation of the radical pair $P680^+Pheo_{D1}^-$ within about 50 ps. More recently, it has been proposed that the initial charge separation starts from the excited accessory chlorophyll Chl_{D1}*, at least at low temperature (*2, 7–10*). The site energy of Chl_{D1} is so low that the lowest excited-state of the PS II RCs is largely localized on this pigment (*9, 10*). At liquid helium temperatures, the excitation energy is so highly localized on Chl_{D1} that it is likely that charge separation, which still occurs at this temperature, will be initiated by Chl_{D1}* (*2, 9, 10*), rather than from the excited P_{D1}/P_{D2} special pair. Reports from two groups (*11, 12*) have provided recent evidence that the reduction of $Pheo_{D1}$ occurs even at ambient temperature prior to the oxidation of P_{D1}/P_{D2} ($P680^+$), implying that the cation of the primary radical pair is localized elsewhere, most likely Chl_{D1}.

Subsequent stabilization of charge separation is achieved by electron transfer from $Pheo_{D1}^-$ to Q_A in 200–500 ps. The $P680^+$ located on the donor side is a very strong oxidant capable of driving the oxidation of water to O_2. The midpoint potential of the couple $P680^+$/P680 has been estimated to be about +1.26 V (*13*). Spectroscopic studies of site-directed mutants have provided evidence favoring the localization of the positive charge of $P680^+$ mainly on P_{D1} (*9*). Such localization is in agreement with ENDOR (*14, 15*) and FTIR (*16*) measurements, which indicate a very asymmetric charge distribution within $P680^+$. The oxidized P_{D1} is reduced by Tyr_Z, the redox active tyrosine 161 of the D1 polypeptide. Tyr_Z^{ox} is in turn reduced by the Mn_4Ca cluster. The manganese cluster stores the oxidizing equivalents required for the oxidation of two water molecules to molecular oxygen. In this way, the one-electron photo-oxidation of P680 is integrated with the overall four-electron process of water oxidation.

At low temperatures ($T < 100$ K), electron transfer from Q_A to Q_B and from Tyr_Z to $P680^+$ is blocked, and charge recombination from $P680^+Q_A^-$ occurs with a half-life of between 2 and 5 ms (*17, 18*). However, the initial signal amplitude attributed to the formation of $P680^+Q_A^-$ decreases progressively with successive flashes. The decrease can be explained by an electron transfer from secondary electron donors (cyt *b559*, Car, Chl_Z) to $P680^+$ that occurs with a low quantum yield in competition with charge recombination of $P680^+Q_A^-$(see ref *19* for a recent review). More recently, the oxidation of Tyr_Z has also been reported to occur with low yield at low temperature (*20, 21*). Such secondary electron transfer leads to a progressive accumulation of long-lived charge-separated states (e.g., $Car^+Q_A^-$) with increasing flash number. The electric field caused by the negative charge located on Q_A^- and the positive charge located on the secondary donor leads to shifts of the transition energies of nearby pigments (Stark effect). The absorbance changes accompanying the formation of these long-lived states can be obtained by subtracting the absorbance spectrum of PS II in the dark-adapted state from that measured after illumination (*18, 22–25*). A characteristic electrochromic blue shift of the Q_X band of $Pheo_{D1}$ is observed around 550 nm (the so-called C550 band shift) and is an indicator of the formation of Q_A^- (*26*). In the Q_Y region, the assignment of the observed electrochromic band shifts is complicated by the strong overlap of the electronic transitions of the pigments in the PS II RC and by the stronger exciton coupling in the Q_Y region. The predominant feature of the absorbance difference spectrum observed with PS II core complexes from *Synechocystis* sp. PCC 6803 looks like a blue shift of an absorbance band, giving rise to a positive peak at 681 nm and a negative peak at 684 nm (*23, 24, 27, 28*). In addition, any pigment that changes its electronic state also contributes to the difference spectra. For example, the formation of the Car and/or Chl cation can be monitored by an absorbance increase in the near-infrared (*27, 28*). The formation of a Chl cation should also be accompanied by a bleaching in the Q_Y region. However, there is a considerable debate regarding the position and the extent of such bleaching (*18, 23, 29*).

When electron transfer beyond Pheo is blocked by prereduction or by removal of the primary quinone acceptor Q_A, then the RC triplet state ^3P is formed, in high yield at low temperatures, by charge recombination of $P680^+Pheo_{D1}^-$ following singlet–triplet mixing in the radical pair state (*30*). EPR studies with oriented PS II samples have shown that the plane of the Chl, which carries the triplet state, is oriented like that of the accessory chlorophylls (*31*), that is, the triplet state is localized on one of the accessory chlorophylls, most probably on Chl_{D1} (*9, 31, 32*). It is not clear if $^3Chl_{D1}$ is directly formed by charge recombination of $P_{D1}^+Pheo_{D1}^-$ or if $^3P_{D1}$ is formed initially followed by a fast triplet energy transfer to Chl_{D1}.

Site-directed mutagenesis, to introduce perturbations affecting individual chlorophyll molecules, in combination with difference spectroscopy, to examine the consequences these have on the absorbance changes associated with the formation of functional states, provide a powerful means to identify those chlorophyll molecules contributing to each of these states. Mutations that replace the axial ligand (*9*) or which perturb a hydrogen bond to the 13^1-keto group of a particular chlorophyll (*33*) modify the absorbance and vibrational spectra and provide spectroscopic labels on individual chlorophylls. Therefore, this approach offers the possibility to assign the bands in the difference spectra and to elucidate the electronic (excitonic) states of the RC chlorins.

In this work, we analyzed PS II core complexes isolated from mutants of *Synechocystis* sp. PCC 6803 constructed at D1-Thr179 using low-temperature absorbance difference spectroscopy. D1-Thr179 is the closest residue to the accessory chlorophyll Chl_{D1} and overlies the coordinated Mg^{2+} but is not a direct ligand (see Figure 1). It has been suggested that axial coordination to Chl_{D1} is probably provided by a water molecule H-bonded to D1-Thr179Oγ (*6*). This Thr was replaced by His and Glu by site directed mutation in *psb*A3, which encodes polypeptide D1. While His and Glu are, in principle, capable of direct coordination of Mg^{2+}, they may still be too distant in the case of D1–179 to provide axial ligation to the accessory Chl (see below). Nonetheless, these mutations cause a shift of the main bleaching band of the triplet-minus-singlet (T−S) ($^3P - {}^1P$) absorbance difference spectrum and of a spectral feature of the ($P680^+Q_A^- - P680Q_A$) difference spectrum attributed to an electrochromic blue shift of the Q_Y band of Chl_{D1} (*9*).

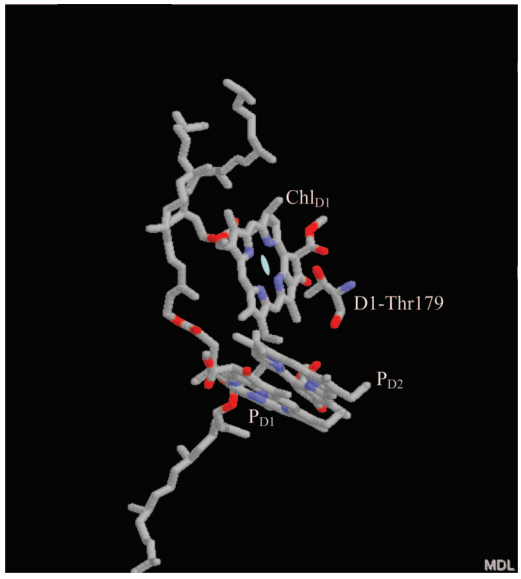

FIGURE 1: Environment of P_{D1}, P_{D2}, and Chl_{D1} showing the location of D1-Thr179. The figure was based on the coordinates of the PS II structure of *T. elongatus* (*5, 6*) using the program MDLISIS. View direction is along the membrane normal.

Our results support these assignments and give the first direct evidence that the triplet state is localized on Chl_{D1}. To better understand the electrochromic band shifts induced by illumination at low temperature, light-minus-dark absorbance difference spectra of PS II from wild type and mutants were measured. Based on the effects induced by the mutations, the predominant electrochromic feature of the absorbance difference spectrum associated with the reduction of Q_A and the oxidation of a secondary donor (mainly Car and Chl_Z) can clearly be assigned to a blue shift of Chl_{D1}.

MATERIALS AND METHODS

Mutant Construction. The glucose-tolerant strain of the cyanobacterium *Synechocystis* sp. PCC 6803 (*34*) was used for the construction of the site-directed mutants described in this paper. Mutations at codons 179 and 198 of the *psbA*3 gene encoding the D1 polypeptide were introduced into the TD41 *psbA* triple deletion strain of *Synechocystis* sp. PCC6803 as described by Nixon et al. (*35*). All strains were grown on BG-11 medium as described by Williams (*34*) and Metz et al. (*36*). Five millimolar glucose was added to the medium to allow the propagation of mutants inactive in PS II.

Isolation of PS II Complexes. PS II core complexes were isolated according to the combined procedures of Tang and Diner (*37*) and Rögner et al. (*38*), in that order, and were stored at −80 °C until use.

Absorbance Spectroscopy. Light-minus-dark absorbance difference spectra at low temperature were measured on PS II core complexes diluted to about 10 μM Chl in 20 mM MES (pH 6.5), 10 mM $MgCl_2$, 20 mM $CaCl_2$, 0.02% β-DM, and 65% glycerol. $K_3(Fe(CN)_6$ (3 mM) was added to oxidize cyt *b*559. The sample was dark adapted and frozen in an Oxford Instruments cryostat (model CF1204 or DN 1704). Light-minus-dark difference spectra at low temperature were obtained by subtracting the absorbance spectrum in the dark-adapted state from that after illumination with continuous white light for about 30 s. Absorbance spectra were measured in a Cary1E UV–vis spectrophotometer (Varian) using a home-built cryostat holder. Spectra were recorded with data intervals of 0.1 nm, a scan speed of 20 nm/min, and a spectral bandwidth of 1 nm.

Transient Absorbance Spectroscopy. Flash-induced absorbance difference spectra were recorded at low temperature as previously described (*18*) using a laboratory-built flash spectrometer. The ($^3P - {}^1P$) absorbance difference spectra were monitored in the presence of doubly reduced Q_A. Q_A was doubly reduced by preillumination at room temperature under anaerobic conditions in the presence of 10 mM $Na_2S_2O_4$. Under these conditions, the lifetime of ^3P is similar to that of the chlorophyll *a* triplet state in organic solvents ($\tau \approx 1.4$ ms (70%)/7 ms (30%) at 5 K and $\tau \approx 1.4$ ms at $T \geq 80$ K). The samples were excited with saturating flashes of about 15 μs in duration from a Xe flash lamp filtered by a colored glass filter (Corning model CS 4–96).

Calculation of Optical Difference Spectra. The calculation of optical difference spectra involving reduced Q_A and oxidized Car and Chlz were performed as described in ref *10*. In short, we use an exciton model that takes into account the pigment–protein coupling by including the dynamic and static modulation of pigment transition energies by the protein. The former is described by a spectral density extracted from independent optical spectra (*39*), and the latter is taken into account by assuming different mean transition energies, the so-called site energies, and a Gaussian distribution function of a certain width (fwhm) for the site energies. An average over the distribution of site energies is performed by a Monte Carlo method. The same (mean) site energies are assumed as in our earlier calculation of optical spectra of D1-D2-cyt *b*559 complexes (*10*), except for a 4 nm red shift of the site energy of Chl_{D1} and assuming a distribution function for the site energy of this pigment narrower by 70% than for the remaining RC pigments. The excitonic couplings are obtained from transition monopole charges obtained by our ab initio TrEsp method (*41*), and the coupling in the special pair is corrected by taking into account exchange contributions as described in detail in a forthcoming publication (*40*). The value for the special pair coupling is 150 cm^{-1}. The remaining nearest neighbor couplings are smaller by roughly a factor of 3. In the calculation of the difference spectra, the absorbance is calculated twice, first for the RC without any charges and second by taking into account site energy shifts by explicit charges (on Q_A, Car_{D1}, Car_{D2}, $Chlz_{D1}$, or $Chlz_{D2}$). The electrochromic shifts are obtained by considering the Coulomb coupling between these charges and the difference in permanent dipole moments of the RC pigments, using an effective dielectric constant of 2 for the screening of the Coulomb coupling. A detailed description of the calculations is given elsewhere (*40*).

RESULTS

Absorbance Spectra. Figure 2 shows the absorption spectra of the PS II core complexes isolated from WT and mutants of *Synechocystis* sp. PCC 6803 in the Q_Y region at 78 K. The WT spectrum appears almost identical to those reported earlier (*23–25*). It shows peaks at 671 and 677 nm and shows a shoulder around 682 nm. The PS II core preparations from the mutants differ in the amplitude of the 677 nm peak and show less absorption around 682 nm.

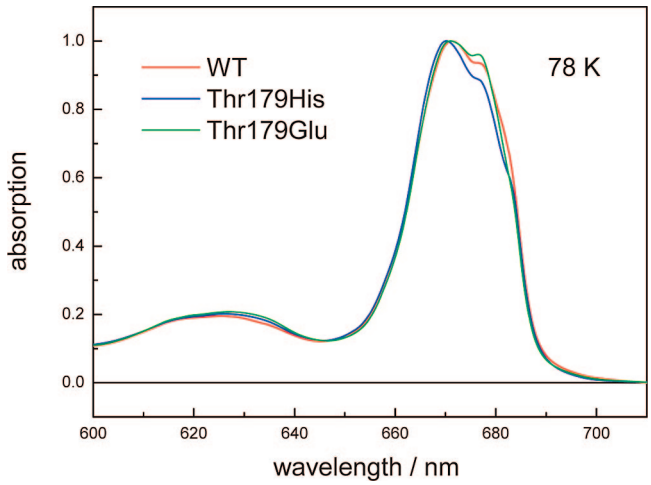

FIGURE 2: Absorbance spectra of PS II core complexes isolated from wild type (red line) and the D1-Thr179His (blue line) and D1-Thr179Glu (green line) mutants of *Synechocystis* sp. PCC 6803 measured at 78 K. The spectra are normalized at the λ_{max} in the Q_Y region.

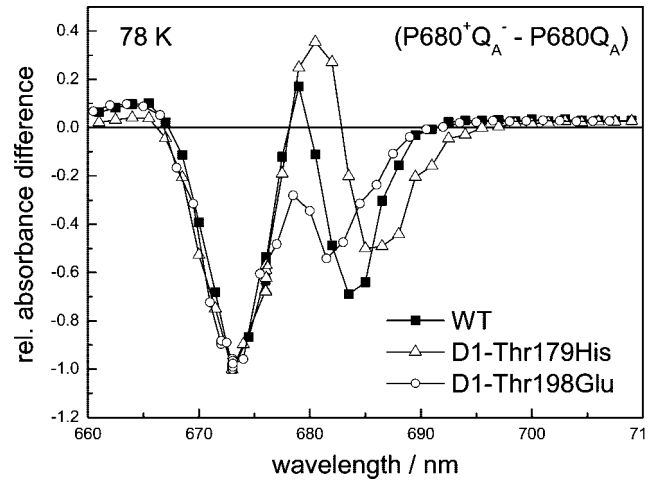

FIGURE 3: Flash-induced ($P680^+Q_A^-$ − $P680Q_A$) absorbance difference spectra of PS II complexes from wild type (filled squares) and the D1-Thr179His (open triangles) and D1-Thr179Glu (open circles) mutants measured at 78 K. The spectra are normalized to 1 at the bleaching minimum to allow better comparison. The PS II core complexes were diluted in a glycerol/buffer mixture (pH 6.5) (65:35; v/v). The OD at the maximum in the Q_Y region was ~1 for a 1 cm optical path length. $K_3Fe(CN)_6$ (3 mM) was added to preoxidize cyt *b*559. The absorbance change at the bleaching minimum was about -3×10^{-2} for the wild type and the mutant difference spectra.

($P^+Q_A^-$− PQ_A) absorbance difference spectra. Flash-induced absorbance changes at 77 K attributed to the formation and the decay of $P680^+Q_A^-$ were measured in the presence of ferricyanide to preoxidize cytochrome *b559*. Preoxidation of cyt *b*559 prevents the accumulation of Q_A^-cyt $b559^+$, which is irreversibly formed with low quantum yield at low temperature (*18, 42*). Under these conditions, the recombination of $P680^+Q_A^-$ takes place within a few milliseconds. A satisfactory fit of the decay in WT PS II is obtained using two exponentials with half-lives of 1.8 ms (47%) and 6.3 ms (53%) plus a constant (1%). The $P680^+Q_A^-$ recombination kinetics in PS II core complexes from the mutants are slightly faster, by about a factor of 1.7 in the D1-Thr179His mutant and by about a factor of 1.2 in the D1-Thr179Glu mutant (not shown).

To obtain the absorbance difference spectra associated with the formation of the secondary radical $P680^+Q_A^-$ pair in PS II core complexes isolated from wild type and mutants, the amplitude of the flash-induced absorbance changes was measured as a function of the wavelength. Figure 3 shows the initial amplitude of the flash-induced absorbance changes for the red region between 660 and 710 nm at 78 K. Upon repeated measurements at the same wavelength, a decrease of the signal amplitude was observed, and the difference spectra have been corrected for the signal loss. This decrease can be explained by an electron transfer from secondary donors, most probably the carotenoid on the D2 side (*43*), to $P680^+$ that occurs with low quantum yield in competition with the recombination of $P680^+Q_A^-$ (*18*). In this wavelength range, absorbance changes due to the reduction of Q_A are much smaller than those due to the oxidation of P680.

The difference spectrum observed with PS II from WT (filled squares) shows bleaching bands at about 673 and 683 nm and a positive peak at 679 nm. The shape of the spectrum has been explained by the bleaching of an absorbance band at 673 nm, associated with the oxidation of P680, and an electrochromic shift of the absorbance band of a neighboring chlorophyll induced by the positive charge located on $P680^+$ and the negative charge located on Q_A^-. It has been proposed that it is the Q_Y band of the accessory chlorophyll Chl_{D1} that is electrochromically shifted to the blue (*9*). Upon replacing the closest residue to the accessory chlorophyll Chl_{D1}, D1-Thr179, with His or Glu, the main bleaching band is unaffected by the mutation, whereas the spectral feature, attributed to the electrochromic band shift, is displaced by about 2 nm to the red in the D1-Thr179His mutant (open triangles) and is slightly shifted (~1 nm) to the blue in the D1-Thr179Glu mutant (open circles). These results confirm the assignment of the electrochromic band shift in ($P^+Q_A^-$ − PQ_A) to Chl_{D1} as the mutations that displace the electrochromic band shift are specifically constructed at Chl_{D1}, producing a modification of its Q_Y transition energy and, consequently, of its absorbance spectrum.

($^3P - {}^1P$) Absorbance Difference Spectra. To generate the RC triplet state ^3P, formed by charge recombination of $P680^+Pheo^-$, PS II complexes were illuminated under conditions in which the primary quinone acceptor Q_A is doubly reduced (see MATERIALS AND METHODS section) and the electron transfer from $Pheo_{D1}^-$ to Q_A is blocked. Figure 4, panels A and B, shows the flash-induced ($^3P - {}^1P$) absorbance difference spectra in PS II core complexes isolated from WT and the D1-Thr179His mutant measured at 5 (Figure 4A) and 78 K (Figure 4B). The difference spectrum observed with PS II from WT (filled squares) shows the main bleaching band at about 684 nm, in good agreement with earlier work (*9*). The difference spectrum above 700 nm exhibits a weak positive, very broad absorbance increase due to triplet–triplet absorption, which is present over the entire wavelength region (*44*). The small features on the short wavelength side of the main bleaching band (positive peak at 674 nm, small negative band around 669 nm) may reflect excitonic interactions between the six RC chlorins altered by triplet formation (*10, 45, 46*).

Replacement of D1-Thr179 by histidine leads to a displacement of the main bleaching band by about 2.5 nm to the red (see Figure 4A, open circles). This result provides direct evidence that the RC triplet state is localized on the

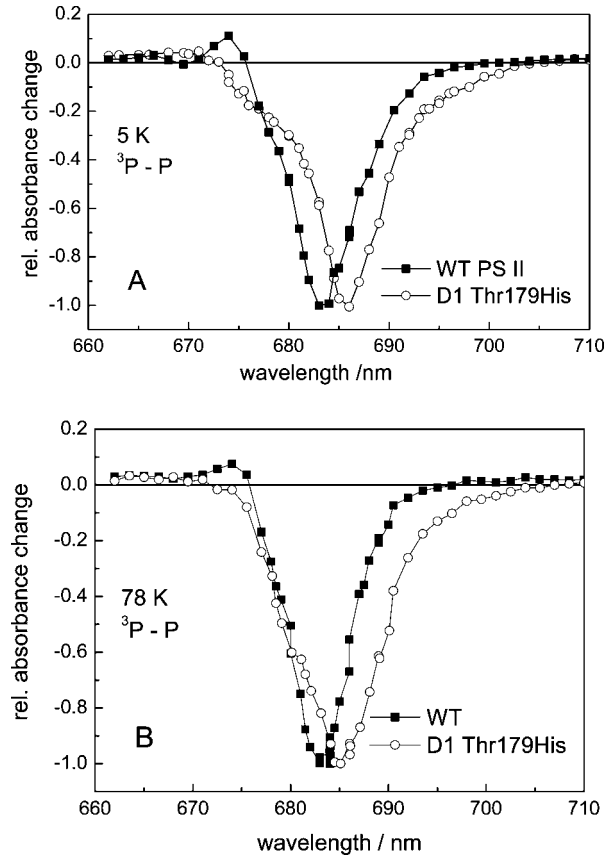

FIGURE 4: Flash-induced (^{3}P − ^{1}P) absorbance difference spectra of PS II complexes from the WT (filled squares) and the D1-Thr179His mutant (open circles) measured at 5 (A) and 78 K (B). The PS II core complexes were suspended in 100 mM MES, pH 6.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.02% β- dodecylmaltoside, and 65% glycerol. The OD at the maximum in the Q_Y region was ~1 with an optical path length of 1 cm. Dithionite (10 mM) was added, and the sample was preilluminated to doubly reduce Q_A. Top panel: The decay of the flash-induced absorbance changes was fit with two exponentials at 5 K ($\tau \approx 1.4$ ms and $\tau \approx 7$ ms). The sum of the amplitudes is depicted as a function of the wavelength. The spectra were normalized to the bleaching minimum, which corresponds to a ΔA value of −0.006 for the WT and −0.004 for the D1-Thr179His mutant. Bottom panel: (^{3}P − ^{1}P) difference spectra measured at 78 K. At 78 K the kinetics were fit to one exponential with $\tau \approx 1.4$ ms. The conditions were the same as for the 5 K spectra. The spectra were normalized to the bleaching minimum, which corresponds to a ΔA value of −0.006 for the WT and −0.0023 for the D1-Thr179His mutant.

accessory chlorophyll Chl_{D1} at low temperature. The (^{3}P − ^{1}P) spectrum in PS II core complexes isolated from the D1-Thr179His mutant exhibits a shoulder on the short wavelength side of the main band and lacks the small positive band. Upon increasing the temperature to 78 K, the main bleaching band in the (^{3}P − ^{1}P) spectrum of PS II core complexes isolated from the D1-Thr179His mutant becomes broader toward shorter wavelengths (see Figure 4B, open circles). It is likely that the bleaching, giving rise to the shoulder in the 5 K spectrum, has become more intense. In addition, the bleaching minimum of both the WT and the mutant shifts by about 1 nm to the blue relative to the difference spectrum at 5 K. This shift is similar to the one (0.5 nm) inferred from our exciton calculations of the temperature dependence of the absorbance spectrum of D1-D2-cyt *b*559 reaction centers (*10*).

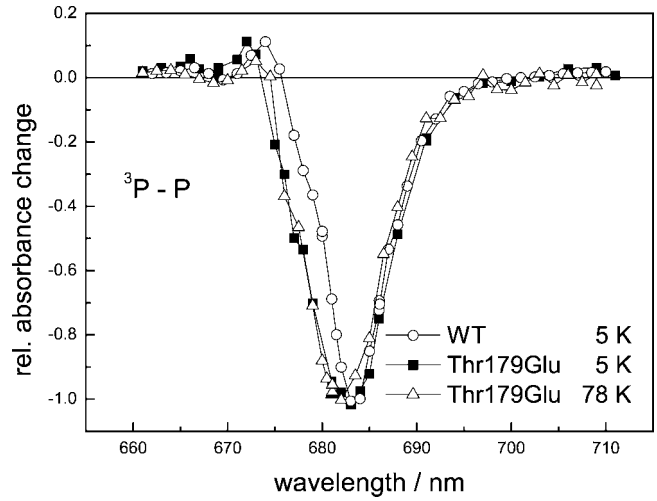

FIGURE 5: Flash-induced (^{3}P − ^{1}P) absorbance difference spectra of PS II core complexes from wild type (open circles) and the D1-Thr179Glu mutant measured at 5 (filled squares) and 78 K (open triangles). The conditions were the same as for the spectra shown in Figure 4. The spectra were normalized to the bleaching minimum, which corresponds to a ΔA value of −0.006 for the WT and −0.006 at 5 K and −0.0004 at 78 K for the D1-Thr179Glu mutant.

Figure 5 shows the flash-induced (^{3}P − ^{1}P) absorbance difference spectra in PS II core complexes isolated from the D1-Thr179Glu mutant measured at 5 (filled squares) and 78 K (open triangles). For comparison, the (^{3}P − ^{1}P) absorbance difference spectrum of WT (open circles) is also depicted. Replacement of D1-Thr179 by glutamate leads to a slight displacement of the main bleaching band to the blue. The major change is, however, the broadening of the bleaching band at both temperatures. As in Figure 4, there is an approximate 1 nm shift of the bleaching minimum to the blue in raising the temperature from 5 to 78 K.

Long-lived Absorbance Changes upon Low Temperature Illumination: Light-Minus-Dark Absorbance Difference Spectra. Low temperature illumination of PS II leads to the formation of long-lived charge-separated states (*18, 19, 23, 47*). The absorbance difference spectra associated with the formation of these states can be obtained by subtracting the absorbance spectrum of PS II in the dark-adapted state from that after illumination with continuous white light for about 30 s. The spectra were recorded at the same temperature at which the illumination was performed. Figure 6 shows absorbance difference spectra obtained by this procedure at 5 K with PS II core complexes isolated from the WT and mutants.

The predominant feature of the absorbance difference spectrum observed with the WT PS II core complexes from *Synechocystis* sp. PCC 6803 looks like the electrochromic blue shift of an absorbance band giving rise to a positive peak at 681 nm and a negative peak at 684 nm (*23, 24*). In addition, there is negative band at 676 nm and a broad bleaching around 667 nm. $K_3(Fe(CN)_6$ (3 mM) was added to the samples to preoxidize cyt *b*559 before freezing the sample to 5 K in the dark. The extent of reduction of Q_A was monitored by the electrochromic shift of the Q_X band of Pheo *a* (see the inset of Figure 6B). The difference spectra in Figure 6, panels A and B, are normalized to this so-called C550 signal. The formation of a carotenoid cation was monitored by the appearance of an absorbance band around

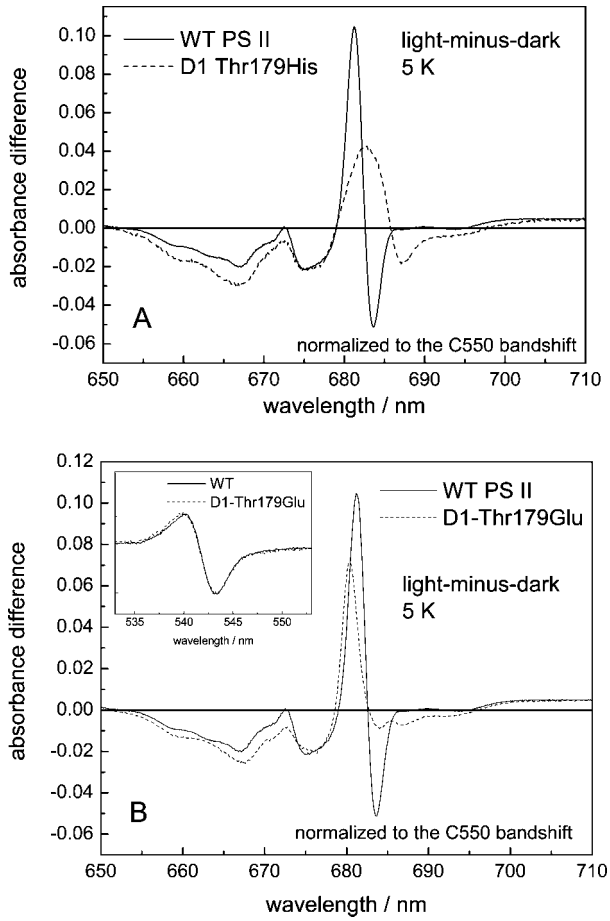

FIGURE 6: Light-minus-dark difference spectra of isolated PS II core complexes from the WT (solid line) and the D1-Thr179His (dashed line) (A) and D1-Thr179Glu (dashed line) (B) mutants of *Synechocystis* sp. PCC 6803 measured at 5 K. The curves were obtained by subtracting the absorbance spectra in the dark-adapted state from those after illumination. Preoxidation of cyt *b*559 was achieved with 3 mM $K_3Fe(CN)_6$. For details see the MATERIALS AND METHODS section. The spectra of WT and mutant were normalized to the C550 signal (see inset in panel B) indicating the amount of reduced Q_A.

1000 nm (not shown). The broad bleaching band in the Q_Y region around 667 nm is assigned to the oxidation of a chlorophyll, most probably Chl_Z (*18, 48*). The electrochromic shifts induced by the low-temperature illumination are therefore caused by the negative charge located on Q_A and by the positive charge located on the secondary donor (either Car or Chl).

Replacement of D1-Thr179 with histidine produces a displacement by about 2 nm to the red of the predominant band shift (see Figure 6A). In addition, the amplitude of the band shift signal is significantly decreased, and the broad bleaching around 667 nm is somewhat enhanced.

Replacement of D1-Thr179 by glutamate produces a small displacement to the blue of the predominant band shift (see Figure 6B). Also in this case, the amplitude of the band shift signal is significantly decreased, and the broad bleaching around 667 nm is somewhat enhanced.

DISCUSSION

To understand the photochemistry occurring in the RC of PS II core complexes, it is of crucial importance (1) to elucidate the electronic (excitonic) states of the RC chlorins, in particular, the nature of the excited state that drives the primary charge separation and (2) to establish where the cation radical of the primary donor and the triplet state formed by charge recombination of the primary radical pair are localized.

To address these issues, we have employed time-resolved optical absorbance spectroscopy to characterize the spectral properties of the transient functional states with high spectral resolution and have constructed site-directed mutants with amino acid replacements in close proximity (e.g., axial ligands) to one of the chlorophylls (P_{D1}, P_{D2}, Chl_{D1}, and Chl_{D2}) in the RC. This work focuses on mutations of D1-Thr179, which is the closest residue to the accessory chlorophyll Chl_{D1} (the D1-Thr179Oγ is 4.8 Å from the central Mg^{2+} of Chl_{D1} (*6*)). The results are discussed with the help of an exciton model previously introduced on the basis of calculations of optical spectra of D1-D2-cyt *b*559 complexes (*10*) that we have recently extended to PS II core complexes (*40*).

Assignment of the Bands in the Absorbance Difference Spectra. Figure 7 compares the observations obtained with the D1-Thr179His mutant presented in this work with those using the D1-His198Gln mutant (data taken from ref *9*). The special pair chlorophyll P_{D1} is directly coordinated by D1-His198, whereas the accessory chlorophyll Chl_{D1} is probably coordinated by a water molecule hydrogen-bonded to D1-Thr179Oγ (*6*). Figure 7 shows the ($P680^+Q_A^-$ − $P680Q_A$) (panels A and B) and the T−S (panels C and D) absorbance difference spectra of PS II core complexes from the WT and the two mutant strains of *Synechocystis* sp. PCC 6803.

Replacement of D1-His198 with glutamine affects the ($P680^+Q_A^-$ − $P680Q_A$) absorbance difference spectrum (Figure 7A). The main bleaching band at 673 nm is blue-shifted by about 3 nm, whereas the spectral features attributed to an electrochromic band shift are not altered. The D1-His198Gln mutation has little effect on the T−S absorbance-difference spectrum (Figure 7C). The inverse effect is observed for the D1-Thr179His mutant. The main bleaching in the T−S absorbance-difference spectrum recorded at 5K (Figure 7D) and the electrochromic band shift in the ($P680^+Q_A^-$-$P680Q_A$) absorbance-difference spectrum (Figure 7B) are both red-shifted in the D1-Thr179His mutant compared to WT, whereas the bleaching maximum upon $P680^+$ formation is not altered (Figure 7B). The absorbance minimum at ∼673 nm has to be assigned as a first approximation to the bleaching of the absorbance band of P680 due to its oxidation or, expressed more properly, as the disappearance of the low-energy exciton band of the special pair as in the bacterial RCs or in PSI. We would like to make it clear that the position of the bleaching band at 673 nm does not reflect the transition energy (site energy) of P_{D1} alone but corresponds to the transition from the ground-state to an exciton state dominated by the special pair chlorophylls, P_{D1} and P_{D2} (*10*).

ENDOR (*14, 15*) and FTIR (*16*) measurements have been interpreted as indicating a very asymmetric charge distribution for $P680^+$, without indicating on which of the P_{D1} and P_{D2} chlorophylls the cation is localized. We have modeled the ($P680^+Q_A^-$ − $P680Q_A$) difference spectrum in wild-type PS II core complexes in which the cation is alternatively placed on P_{D1}, P_{D2}, and Chl_{D1} and equally shared by P_{D1} and P_{D2} (*40*). Only the localization of the cation on P_{D1}

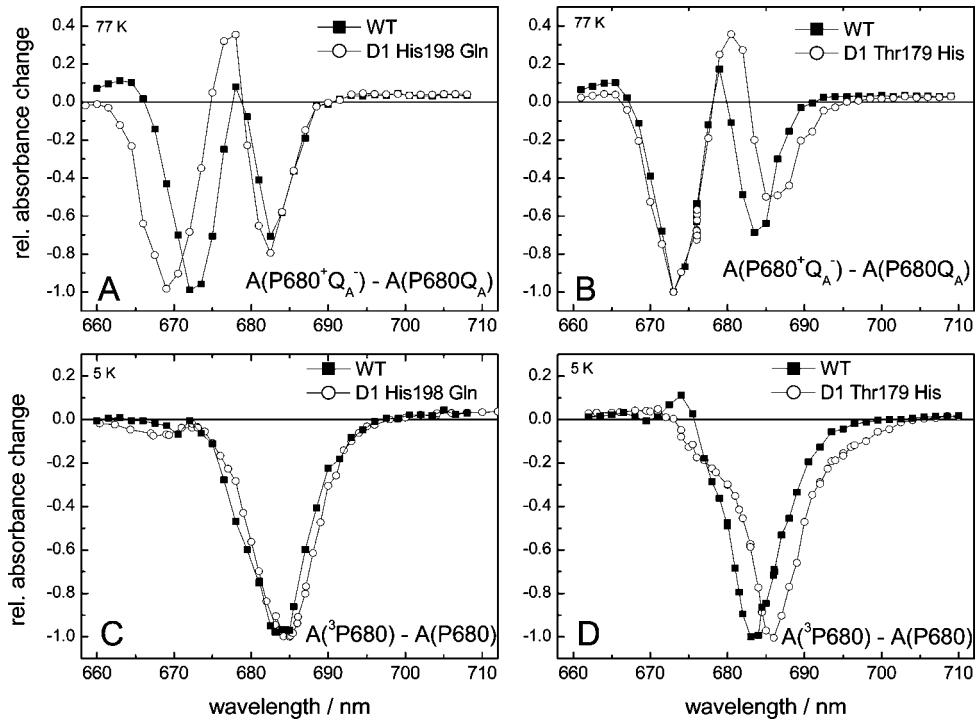

FIGURE 7: Comparison of low temperature ($P680^+Q_A^- - P680Q_A$, measured at 77 K) and ($^3P - {}^1P$, measured at 5 K) absorbance difference spectra of WT (filled squares) and mutant (open circles) PS II core complexes from *Synechocystis* sp. PCC 6803.

produces a wavelength localization and relative amplitudes of the bleaching band and the electrochromic band shifts that closely match the experimental difference spectrum. The experimental results combined with the calculated fits therefore provide direct evidence that the $P680^+$ cation is primarily localized on P_{D1}, as suggested earlier (*9*). We note also that a distribution of the cation between P_{D1} and Chl_{D1} in the calculations provides significantly worse agreement with the experimental data. In addition, such a distribution would be in disagreement with the distance of 27.4 ± 0.3 Å between Q_A^- and the cation determined from pulsed EPR measurements (*49*).

The D1-Thr179 mutants clearly show that the RC triplet state is localized on the accessory Chl_{D1} at low temperature and that the electrochromic band shift observed in the ($P680^+Q_A^- - P680Q_A$) difference spectrum has to be attributed to an electrochromic effect of Q_A^- and P_{D1}^+ on Chl_{D1}, the accessory chlorophyll of the active branch. Interestingly, these results show that the lowest exciton transition is not localized on the special pair in the PS II RC as it is in the bacterial RCs or in PS I. It is instead Chl_{D1} that has the lowest excitation energy. Therefore, we have proposed (*9, 10*), based on the Chl_{D1} transition energy and in agreement with other arguments made earlier in the literature (*8, 50–52*), that the charge separation in PS II starts from the excited-state of Chl_{D1}, at least at low temperature. More recent reports from two groups (*11, 12*) provide evidence that the reduction of $Pheo_{D1}$ occurs, even at ambient temperature, prior to the oxidation of P_{D1}/P_{D2}, implying that Chl_{D1} is most likely the primary electron donor.

We see in Figure 7 that the D1-His198Gln mutation only changes the bleaching band around 673 nm and that the D1-Thr179Glu mutation only changes the band around 683 nm. Because the pigments in the PS-II RC are excitonically coupled, one has to ask the question: why does a local change of the transition energy of a pigment cause the shift of only one band in the spectrum?

The answer is obtained from our exciton model (*10*). As previously discussed in this model, the lowest excited-state is strongly localized on Chl_{D1}, providing the explanation for why only the lowest state shifts in the D1-Thr179Glu mutation. In the case of P_{D1}, the situation is slightly more complicated. In the exciton model, the low-energy exciton state of the special pair contains most of the oscillator strength of the two pigments; therefore, the effect of a site energy shift of P_{D1} is strongest on this band. Hence, we conclude that (a) the 683 nm transition has to be assigned to the lowest exciton state in the RC that is mainly localized on Chl_{D1} and (b) that the 673 nm transition has to be assigned to the low energy exciton state formed mainly by $P_{D1}$and P_{D2}. A quantitative comparison of the present data with exciton calculations shows that the data can be described by the parameters of the model previously reported (except for a 4 nm red shift of the site energy of Chl_{D1}) (*10*). A manuscript devoted to the calculations has been recently submitted (*40*). In addition, theoretical predictions made concerning the effect of the mutations on the ($D^+Q_A^- - DQ_A$) absorbance difference spectra with D ≡ Car or Chl_Z have now been confirmed experimentally (see below).

Explanation of the Mutation-Induced Shift of the Transition Energy of Chl_{D1}. D1-Thr179 is the closest residue to the accessory chlorophyll Chl_{D1}, situated above the Mg^{2+} coordinated by the macrocycle. However, D1-Thr179 cannot directly coordinate the Mg^{2+} of the accessory chlorophyll, as it is too far away (4.8 Å). It has been suggested that Chl_{D1} is more likely coordinated by a water molecule H-bonded to D1-Thr179Oγ (*5, 6*). In this work, Thr was replaced by His and Glu. We used the program Swisspdbviewer to replace D1-Thr179 with histidine and glutamate in the PS II structure of *T. elongatus* and determined possible coor-

dination distances for different orientations of the residues. It does appear that neither His and Glu can function as a direct ligand to the Mg^{2+} of the accessory Chl. However, the strength of the indirect coordination through a water molecule will likely change upon replacement of D1-Thr179 with His or Glu. Differences in Mg^{2+}-ligation, differences in the dielectric constant of the local environment, and mutations that perturb a hydrogen bond to the 13^1-keto group of a particular chlorophyll have been shown to influence the transition (site) energy of chlorophylls (*9, 25, 33, 53*). The loss of histidine as the axial ligand in the D1-His198Gln mutant and the replacement of Thr with His, in the case of the D1-Thr179His mutant, produce 2–3 nm displacements of P_{D1} to the blue and of Chl_{D1} to the red, respectively. These observations can be understood in terms of the electronic polarizability of the π-system of the imidazole moiety of histidine that stabilizes the excited-state of nearby chromophores by dispersive interactions, shifting the absorbance spectrum of the pigment to the red whenever it is present. The shift toward the blue in the case of the D1-Thr179Glu mutant may also be a reflection of the negative charge on the amino acid residue, assuming a standard protonation state of this residue. We note that although one might expect a more drastic change of the site energy of Chl_{D1} by a close charge, the actual amount of the shift depends on the position of this charge relative to the difference potential of the charge density between the excited and the ground-state of Chl_{D1}. The difference potential of chlorophyll has strong negative and strong positive regions, but it also has regions where it is close to zero (*41*). Therefore, an independent quantification of this shift has to await a calculation of the protonation probability of the Glu at D1–179 and its charge density coupling with the excited and ground-state of Chl_{D1}. For this purpose, a quantum chemical/ electrostatic method has been developed and recently successfully tested on the FMO-protein of green sulfur bacteria (*54*).

Electrochromic Band Shifts in PS II. The low temperature ($P680^+Q_A^-$ − $P680Q_A$) absorbance difference spectrum exhibits (a) an electrochromic blue shift of the Q_X band of $Pheo_{D1}$ (C550) centered at 542 nm (see Figure 6B inset), which is induced mainly by the negative charge on Q_A^- (*18, 26*), and (b) an electrochromic blue shift of the Q_Y band of the accessory chlorophyll Chl_{D1} in the Q_Y region (see Figure 3) as discussed in detail above. These observations are similar to those described in the bacterial RCs. The electrochromic shift of the accessory bacteriochlorophyll in the active branch B_A is also to the blue, whereas the Q_Y band of the bacteriopheophytin Ph_A is shifted to the red upon formation of P^+Q^- (*55, 56*). Our calculations on the basis of the X-ray structure of PS II with a resolution of 3.0 Å (*6*) show that this behavior is the same in the RC of PS II (see below). Comparing the (Q_A^- − Q_A) absorbance difference spectra of PS II (*25*) and bacterial RCs (*55*), it is remarkable that in PS II the blue shift of the accessory chlorophyll Chl_{D1} is the predominant feature, whereas in bacterial RCs the red shift of $Pheo_A$ is the predominant feature. The reason for the former effect is two-fold; (1) the site energy of $Pheo_{D1}$ is in the central range of site energies determined for the RC pigments in PS II (*10*). The different electrochromic shifts of the RC pigments largely overlap in this spectral region. Nevertheless, $Pheo_{D1}$ experiences the largest red shift (-47 cm^{-1}) by Q_A^- of all RC pigments and therefore contributes to the positive band around 680 nm in the difference spectra in Figure 6. (2) Chl_{D1} has the lowest site energy in the RC and, in addition, a much smaller inhomogeneous width than the other RC pigments. Both factors lead to a dominant contribution of this pigment in the difference spectra at long wavelengths, despite the fact that its electrochromic blue shift is smaller in magnitude by a factor of 4 than the red shift of $Pheo_{D1}$. We note that an alternative interpretation of the wild type difference spectra in Figure 6 was presented by Krausz and co-workers (*23*). The long wavelength signal was interpreted in terms of an electrochromic blue shift of $Pheo_{D1}$ that, in our case, is calculated to shift to the red. To explain the blue shift, a protein induced 90° rotation of the $\Delta\mu$ vector, representing the difference in permanent dipole moments between the excited and the ground-state of $Pheo_{D1}$, compared to the orientation of this vector in solution (see the discussion given in ref *23*) was proposed. To choose between these two alternatives to explain the spectrum, discussed above, we have performed experiments on mutants where the local environment of $Chl_{D1}$was changed. In fact, calculations of the mutant spectra were even performed before the experiment. In these calculations, only the site energy of Chl_{D1} was shifted by an amount taken from calculations of the ($P680^+Q_A^-$ − $P680Q_A$) and (^3P − ^1P) absorbance difference spectra in PS II complexes from the same mutant (*40*). The experimental mutant difference spectra shown in Figure 6 perfectly confirmed the predictions of the calculations, concerning the band positions, but resulted in a somewhat larger broadening of the bands. This result led to the assumption of a larger inhomogeneous distribution of the site energy of Chl_{D1} in the mutant, which resulted in quantitative agreement between the calculated spectra (see Figure 9) and the experimental spectra (see Figure 6). These calculations demonstrate the predictive power of our exciton model and provide compelling evidence for the present interpretation.

The light-minus-dark absorption difference spectra measured at 5 K (see Figure 6) show electrochromic band shifts induced by the negative charge located on Q_A and the positive charge located on the secondary donor (either Car or Chl). The most dominant feature clearly is the electrochromic blue shift of the accessory chlorophyll Chl_{D1}. The site-specific nature of the mutations gives us considerable confidence in this assignment (see Figure 6).

What conclusions are possible concerning the localization of the positive charge? Figure 8A shows calculations of the ($Car_{D1}^+Q_A^-$ − $Car_{D1}Q_A$) and ($Car_{D2}^+Q_A^-$ − $Car_{D2}Q_A$) absorbance difference spectra, whereas Figure 8B shows calculations of the ($Chl_{ZD1}^+Q_A^-$ − $Chl_{ZD1}Q_A$) and ($Chl_{ZD2}^+Q_A^-$ − $Chl_{ZD2}Q_A$) absorbance difference spectra. All of the calculated spectra shown in Figure 8 contain the contributions due to Q_A^- formation: (a) the blue shift of the Q_Y band of Chl_{D1} and (b) the red shift of the Q_Y band of $Pheo_{D1}$. Interestingly, band shifts in opposite directions are calculated when placing the positive charge on the D1 or on the D2 side. The position of the positive charge relative to the difference vector of the permanent dipole moments of the excited and ground states of Chl_{D1} determines the direction of the electrochromic shift: Car_{D1}^+ and Chl_{ZD1}^+ induce a red shift and Car_{D2}^+ and Chl_{ZD2}^+a blue shift of the Q_Y band of Chl_{D1}. There is a 11 cm^{-1} blue shift of the site energy of $Chl_{D1}$by Q_A^-. This blue shift is overcompensated by a 17

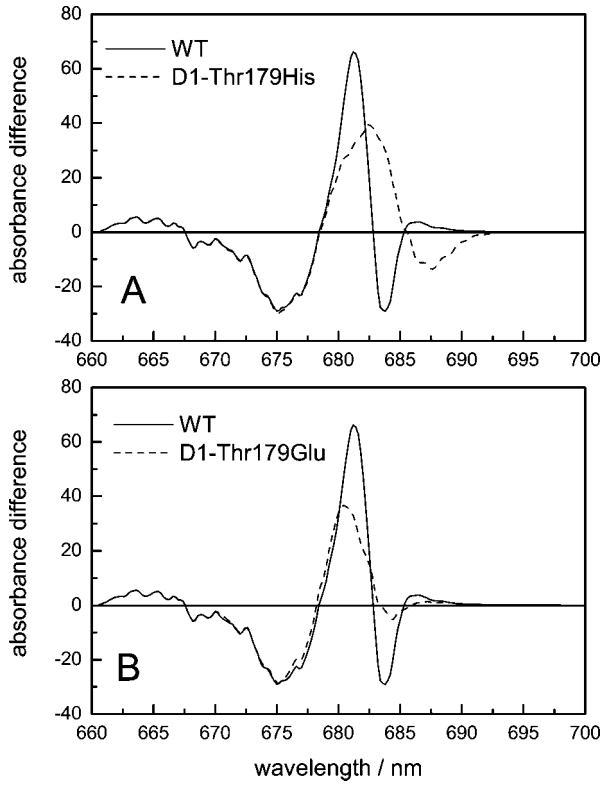

FIGURE 8: Calculation of the ($Car_{D2}^{+}Q_A^{-}$ − $Car_{D2}Q_A$) absorbance difference spectra of PS II core complexes from WT (solids line) and mutants (dashed line). Panel A shows a comparison of WT and the D1-Thr179His mutant. Panel B shows a comparison of WT and the D1-Thr179Glu mutant. For the D1-Thr179His mutant, the wavelength corresponding to the site energy of Chl_{D1} was shifted by 2.5 nm to the red relative to 679.5 nm in WT. Additionally, the inhomogeneous width (fwhm of a Gaussian distribution for the site energies) was increased by about a factor of 2 in the mutant compared to WT. For the D1-Thr179Glu mutant the wavelength corresponding to the site energy was shifted by 0.5 nm to the blue, and the width was increased by about a factor of 1.7, both relative to WT.

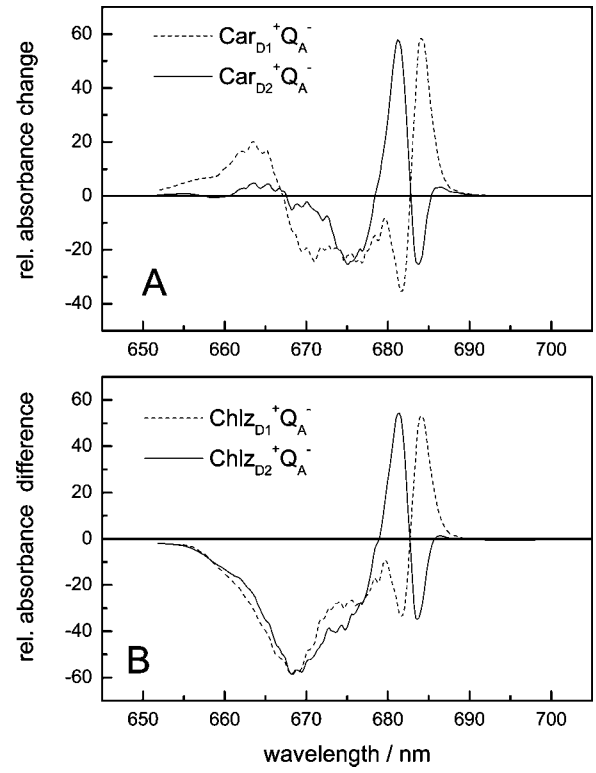

FIGURE 9: Calculation of absorption difference spectra due to the reduction of Q_A and the oxidation of a secondary donor (either Car or Chl). The calculated ($Car_{D1}^{+}Q_A^{-}$ − $Car_{D1}Q_A$, dashed line) and ($Car_{D2}^{+}Q_A^{-}$ − $Car_{D2}Q_A$, solid line) absorbance difference spectra are depicted in Figure 8A, whereas Figure 8B shows calculations of the ($Chl_{ZD1}^{+}Q_A^{-}$ − $Chl_{ZD1}Q_A$, dashed line) and ($Chl_{ZD2}^{+}Q_A^{-}$ − $Chl_{ZD2}Q_A$, solid line) absorbance difference spectra.

cm^{-1} red shift caused by Car_{D1}^{+} or Chl_{ZD1}^{+}. In contrast, Car_{D2}^{+} and Chl_{ZD2}^{+} lead to a blue shift of the site energy of Chl_{D1} by 6 and 5 cm^{-1}, respectively, increasing the blue shift of this site energy, caused by Q_A^{-}. The comparison between the measured light-minus-dark absorption difference spectra (see Figure 6) and the calculated spectra clearly shows that the positive charge has to be localized on the D2 side.

The ($Chl_{ZD2}^{+}Q_A^{-}$ − $Chl_{ZD2}Q_A$) absorbance difference spectrum additionally exhibits a bleaching band around 668 nm due to the oxidation of Chl_{ZD2}, which in the experiments of Figure 6 is less pronounced relative to the amplitude of the band shift signal than is shown in the simulation of Figure 8. Therefore, we conclude that it is mainly the $Car_{D2}^{+}Q_A^{-}$ state that is formed by the low-temperature illumination. The linear dichroism of the Car cation absorption band around 1000 nm supports the conclusion that the Car oriented parallel to the membrane (D2-side) is the one that becomes oxidized (*48*). Figure 9 shows the calculation of the electrochromic band shifts in PS II from the wild type and the D1-Thr179 mutants. The effects of the mutations on the absorbance difference spectrum ($Car_{D2}^{+}Q_A^{-}$ − $Car_{D2}Q_A$) are almost perfectly reproduced using the identical parameters for WT and the mutants except that the site energy and the bandwidth of the accessory chlorophyll Chl_{D1} have been changed in the mutants. For the D1-Thr179His mutant the wavelength corresponding to the site energy was shifted by 2.5 nm to the red, and the bandwidth was increased by a factor of 2. For the D1-Thr179Glu mutant the wavelength corresponding to the site energy was shifted by 0.5 nm to the blue, and the bandwidth was increased by a factor of 1.7. The changes of the spectroscopic properties of the Chl_{D1} upon replacement of D1-Thr179 by His and Glu derived from the calculations are in perfect agreement with the effects of the mutations observed in the ($P680^{+}Q_A^{-}$ − $P680Q_A$) and the (^3P − ^1P) difference spectra shown in Figures 3−5.

There are reports in the literature (*19, 57*) that both carotenoids, Car_{D1} and Car_{D2}, and possibly also both Chl_Z molecules (*19, 58–60*) can be oxidized by low-temperature illumination. Some of these data have been obtained either with D1-D2-cyt *b*559 complexes or with Mn-depleted PS II membranes (*19, 57*) in the presence of ferricyanide and silicomolybdate. In our opinion, it is possible that in these experiments different pathways of silicomolybdate reduction coupled to Chl and/or Car oxidation exist. Other differences are based on the temperature at which the illumination is performed and on the duration and intensity of the illumination.

Triplet Localization. When electron transfer to the first PS II quinone acceptor (Q_A) is blocked, the RC triplet state ^3P is formed with high yield at low temperature by charge recombination of $P680^{+}Pheo_{D1}^{-}$ after singlet–triplet mixing in the radical pair state (*30*). In the presence of singly reduced Q_A, the triplet state decay is 2 or 3 orders of magnitude faster than when Q_A is doubly reduced (*30, 61, 62*). Double reduction of Q_A can be achieved by illumination of PS II

core complexes in the presence of dithonite (*31*). When Q_A is absent or doubly reduced, the lifetime of ^3P is similar to that of the chlorophyll *a* triplet state in solvents ($\tau \approx 1.4$ ms (70%)/7 ms (30%) at 5 K and $\tau \approx 1.4$ ms at $T \geq 80$ K). Under these conditions ^{3}P680 can be detected by its characteristic spin-polarized EPR spectrum (*31, 63*). As noted at the beginning of this paper, orientation studies by Rutherford and co-workers (*31*) indicated that the triplet state was localized on a chlorophyll, the macrocycle ring plane of which was oriented at 30° with respect to the membrane plane. This orientation is consistent with the localization of the triplet state on either Chl_{D1} or Chl_{D2} but the two could not distinguished. The triplet is expected to be localized on the chlorophyll with the lowest triplet energy, and not necessarily on the one on which it was initially formed. A comparison of the ($^3P - {}^1P$) absorbance difference spectra in the WT and D1-His198Gln strains of *Synechocystis* sp. PCC 6803 at 80 and at 5 K (see Figure 7 and ref *9*.) indicated practically no effect of the mutation, which is consistent with the localization of the triplet state on a chlorophyll other than P_{D1}.

The effects of the mutations at D1-Thr179 on the ($^3P - {}^1P$) spectrum in PS II (see Figures 3 and 4) unambiguously demonstrate that the triplet state is localized on the accessory chlorophyll, Chl_{D1}. The ($^3P - {}^1P$) difference spectra were recorded in the presence of doubly reduced Q_A on the millisecond time scale. The clear localization of the triplet energy on Chl_{D1} indicates that at 1 ms the concentration of the Chl triplet states in the CP43 and CP47 antenna complexes is negligible. Presumably, any triplet generated in CP43 and CP47 disappears on a much more rapid time scale ($\ll$1 ms), quenched by antenna carotenoids.

A significant broadening of the bleached band is observed in the mutant ($^3P - {}^1P$) difference spectra relative to WT. This broadening might indicate that the triplet excitation is partially delocalized. However, an analysis of the electrochromic band shifts revealed that the Q_Y band of the accessory chlorophyll Chl_{D1} becomes broader by a factor of 2 upon the replacement of D1-Thr179 by histidine or glutamate. An increased disorder in the surrounding of Chl_{D1} could be one explanation. Even the shoulder in the ($^3P - {}^1P$) difference spectrum of PS II from the D1-Thr179His mutant at 5 K could be explained by a second spectral form of Chl_{D1}, as well as by a partial delocalization, even at 5 K, of the triplet state onto another chlorophyll. It has also been reported that ($^3P - {}^1P$) spectra measured in D1-D2-cyt *b*559 complexes exhibit two spectral forms: the main bleaching band at about 680 nm and a smaller bleaching located at 684 similar to the more intact PS II core preparations (*64*). This observation was also explained by a heterogeneity of the spectral properties of the accessory chlorophyll induced by the preparation of the PS II RCs.

We have used a combination of (1) photoinduction of excited and radical pair states of the RC redox components and (2) perturbation of the site energies of specific RC pigments by site-specific mutation, with (3) study of the recent X-ray crystallographic structures and with (4) analysis using a new exciton model for spectroscopic simulations (*40*) to identify the key players contributing to long-lived (>1 ms) excited and radical pair states. The spectroscopic tags generated and characterized by this synergy are now providing a means to study the mechanism of charge separation using ultrafast time-resolved spectroscopic methods. We expect that these will provide new insights into the mechanism of the most primary process of photosynthetic charge separation in PS II in the near future.

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