Electric Field Effects on the Chlorophylls, Pheophytins, and β-Carotenes in the Reaction Center of Photosystem II†

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ABSTRACT: We present an electric field modulated absorption spectroscopy (Stark effect) study of isolated photosystem II reaction center complexes, including a preparation in which the inactive pheophytin H_B was exchanged for 13^1-deoxo-13^1-hydroxy-pheophytin. The results reveal that the Stark spectrum of the Q_x and Q_y transitions of the pheophytins has a second-derivative line shape, indicating that the Stark effect is dominated by differences in the dipole moment between the ground and the electronically excited states of these transitions ($\Delta\mu$). The $\Delta\mu$ values for the Q_x and Q_y transitions of H_B are small ($\Delta\mu$ = 0.6−1.0 D f^{-1}), whereas that of the Q_x transition of the active pheophytin H_A is remarkably large ($\Delta\mu$ = 3 D f^{-1}). The Stark spectrum of the red-most absorbing pigments also shows a second-derivative line shape, but this spectrum is considerably red-shifted as compared to the second derivative of the absorption spectrum. This situation is unusual but has been observed before in heterodimer special pair mutants of purple bacterial reaction centers [Moore, L. J., Zhou, H., and Boxer, S. G. (1999) *Biochemistry 38*, 11949−11960]. The red-shifted Stark spectra can be explained by a mixing of exciton states with a charge-transfer state of about equal energy. We conclude that the charge transfer state involves H_A and its immediate chlorophyll neighbor (B_A), and we suggest that this ($B_A^{\delta+}H_A^{\delta-}$) charge transfer state plays a crucial role in the primary charge separation reaction in photosystem II. In contrast to most other carotenes, the two β-carotene molecules of the photosystem II reaction center display a very small $\Delta\mu$, which can most easily be explained by excitonic coupling of both molecules. These results favor a model that locates both β-carotene molecules at the same side of the complex.

Photosynthesis is the process by which various types of prokaryotic and eukaryotic organisms convert solar energy into chemical energy. All photosynthetic organisms contain pigment−protein complexes that absorb light and transfer the excitation energy to a special set of chlorophyll molecules in a membrane-bound complex generally denoted as the reaction center (RC).[1] Excitation of this special set of chlorophylls leads to the ultrafast transfer of an electron to a nearby acceptor molecule. It is now commonly believed that the reaction centers of all photosynthetic organisms contain six chlorins in a horseshoe-like organization (*1*), in which the central two chlorins are usually strongly electronically coupled and give rise to the so-called special pair, of which the lowest excitonic state is clearly red-shifted as compared to the other electronic states in the RC. The major exception is the reaction center of photosystem II (PSII), in which the excitonic coupling between the special pair chlorophylls is significantly smaller and similar to that between the other chlorins (*2*−*4*). The smaller exciton coupling between the special pair chlorophylls probably originates from a larger distance between these molecules (*5*) and is possibly related to the capacity of PSII to use electrons from water to reduce the photooxidized electron donor (*6*).

It has long been assumed that only the special pair functions as the primary electron donor, but recent work from our group has demonstrated that in purple bacteria at least one of the other chlorophylls, the chlorophyll known as B_A, also can initiate ultrafast charge separation events (*7*−*9*). But also in PSII it is possible that primary charge separation is initiated, at least in part, by the electronically excited B_A molecule (*10*−*12*). B_A contributes significantly to the red-most absorption in the PSII RC (*13*, *14*), and a chlorophyll molecule with a similar orientation with respect to the plane of the membrane as B_A has been shown to give rise to the spin-polarized reaction center triplet generated by low-temperature illumination (*15*). The steady-state emission at 6 K may also arise from this molecule (*16*). The charge separation process itself is multiphasic (*17*−*20*), which has been explained by several mechanisms (recently discussed in refs *3*, *10*, *12*, and *21*), including slow equilibration with the peripheral chlorophylls, a small energy difference

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[1] Abbreviations: Car, carotene; Chl, chlorophyll; CT, charge transfer; 13^1-OH-Pheo, 13^1-deoxo-13^1-hydroxy-pheophytin *a*; Pheo, pheophytin; PSII, photosystem II; RC, reaction center.

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between the lowest excited state and the first radical pair state (*22*), slow electron-transfer reactions between B_A and P, and the formation of relaxed radical pair states.

A number of earlier observations have suggested a role for intermediate charge-separated states and/or charge transfer (CT) states in the initial dynamics of charge separation in PSII (*19*, *23*). CT states have been described in detail in a series of site-directed mutants of the related RC from the purple bacterium *Rhodobacter sphaeroides* (*24*). In particular, the so-called heterodimer mutants, in which one of the special pair bacteriochlorophylls was replaced by a bacteriopheophytin, gave rise to a strong CT character.

A powerful tool to investigate the possible presence of CT states is given by Stark spectroscopy (*25*), which monitors the effect of an externally applied electric field on the spectroscopic properties of the chromophores. In this report, we present a Stark spectroscopy study on isolated PSII RC complexes, both in a standard preparation and in a preparation in which the pheophytin molecule of the inactive branch (H_B) was chemically exchanged by 13^1-deoxo-13^1-hydroxy-pheophytin *a* (13^1-OH-Pheo) (*14*, *26*). The results indicate the existence of a CT state involving the B_A and H_A molecules. We suggest that this CT state plays a crucial role in the primary charge separation reaction in photosystem II.

We also monitored the electric field response of the two β-carotene molecules of the PSII RC. The results reveal unusual Stark effects for both carotenes, which can be explained by excitonic coupling between the two carotenes. On the basis of these and other results, we suggest that the two carotenes are both located on the D2 side of the complex.

MATERIALS AND METHODS

Sample Preparation. PSII RC (D1-D2-cyt *b*559) complexes containing six Chl *a* per two Pheo *a* were isolated from spinach by a short Triton X-100 treatment of CP47-RC complexes as described (*19*, *27*). Alternatively, PSII RCs were prepared from Tris-washed PSII membranes as in ref *28* and exchanged with 13^1-OH-Pheo as in ref *26*, resulting in a preparation (RC_{1x}) with 55% Pheo replacement and a complete exchange of H_B. CP47 was isolated and purified as described previously (*29*). The samples were diluted in a buffer containing 20 mM BisTris (pH 6.5), 0.03% *n*-dodecyl-β,D-maltoside (β-DM), and 69% (w/v) glycerol and checked by diode-array assisted gel filtration chromatography (*30*) to monitor the presence of CP47 or other contaminating pigment−protein complexes.

Stark Spectroscopy. Stark and absorption spectra were recorded simultaneously at 77 K in a home-built setup (*31*, *32*) at a spectral resolution of 1 nm, an optical path length of 0.1 mm, and an optical density of 0.4 cm^{-1} at 672 nm. An electric field of 2.77×10^5 V cm^{-1} was applied, and the angle between the electric field and the probing light beam was set at magic angle. The magic angle between probe light and electric field was set by rotating the Stark cell (and thus the electric field) to 45° with respect to the propagation direction of the probe light and by selectively probing with (close to) magic angle polarized light. The polarization was tuned to account for the different refractive indices of liquid nitrogen and glassy glycerol. The correctness of this procedure was checked by performing Stark measurements by rotating the Stark cell with the polarization direction of the probing light perpendicular to the axis of rotation of the cell. The angle between the electric field and the probe light was calculated from the difference in path length with respect to the nonrotated cell (when the angle is 90°). The reliability of this procedure was checked by measuring the Stark effect and calculating the Stark parameters of the purple bacterial RC that has well-documented Stark values (*38*, *39*, *40*, *43*). For randomly oriented and fixed molecules, the Stark line shape is usually described by a sum of the zeroth, first, and second derivatives of the ground-state absorption spectrum (*33*). Within this formalism and with the angle between the measuring light beam and the electric field vector at magic angle, the second derivative contribution scales with the size of the difference in permanent dipole moment $\Delta\mu$ between the excited and the ground states of the molecules. The first derivative component yields the trace (Tr) of the polarizability tensor ($\Delta\alpha$), and $Tr(\Delta\alpha)$ is a measure for the difference in polarizability between the excited and the ground states of the molecule. In principle, this component has to be corrected for contributions of the transition moment polarizability (*33*, *43*). However, especially in the case of no or small zeroth order effects, these contributions can be neglected (*44*). The zeroth derivative is a measure of the field-induced changes of the oscillator strength of the optical transition.

Analysis. The Stark and absorption spectra were fitted simultaneously using a nonlinear least-squares fitting program. The absorption spectra were fitted with a number of (skewed) Gaussian functions, and the Stark spectra were composed of a combination of the zeroth, first, and second derivatives of these functions. Alternatively, the absorption of the chlorophylls was described by a single homogeneously broadened transition, which was then fitted with a polynomial function (spline) and the Stark spectrum with the zeroth, first, and second derivative of this function. Because the local field correction factor f is hard to estimate, all values for $\Delta\mu$ and $Tr(\Delta\alpha)$ are represented in terms of D f^{-1} and $Å^3$ f^{-2}, respectively (1 D = 3.34×10^{-30} C m, 1 $Å^3$ = 1.113×10^{-40} C m^2 V^{-1}).

RESULTS

Figures 1 and 2 show the simultaneously recorded 77 K absorption spectra (panels A), their second derivatives (panels B), and conventional (2ω) Stark spectra (panels C) of the standard and 13^1-OH-Pheo exchanged PSII RC complexes, respectively. In the following, we discuss the electric field responses of the chlorophylls, pheophytins, and β-carotenes in these preparations at their most characteristic absorption wavelengths.

Electric Field Response of the Chlorophylls. The 77 K absorption spectrum of the standard PSII RC preparation (Figure 1A) shows main absorption bands peaking near 678 and 672 nm, in agreement with many earlier reports (*34*). The 672 nm peak arises for a considerable part from the two peripheral chlorophylls known as Chl-Z_{D1} and Chl-Z_{D2}, whereas the 678 nm peak is dominated by contributions from the four central chlorophylls P_A, P_B, B_A, and B_B and the two pheophytins H_A and H_B (*10*, *12*, *14*). In the RC_{1x} sample, part of the ~678 nm absorption is blue-shifted to 655 nm, which is caused by the exchange of Pheo a at the H_B binding

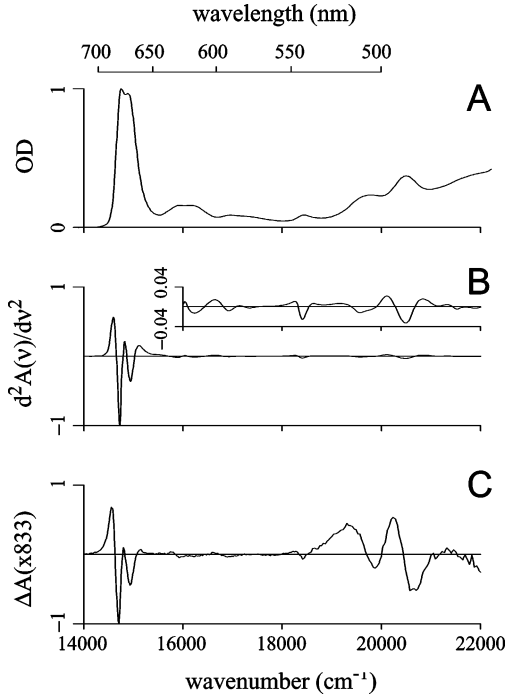

FIGURE 1: Simultaneously recorded 77 K absorption (A), the second derivative of the absorption (B), and Stark spectra (C) of standard PSII RC complexes from spinach. The spectra were normalized to OD = 1 at 678 nm, and the Stark spectra were recorded at magic angle and an electric field strength of 2.77×10^5 V cm^{-1}.

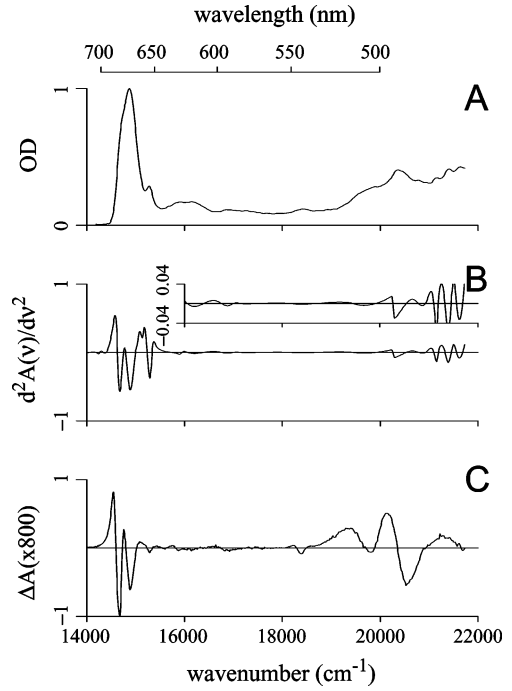

FIGURE 2: Simultaneously recorded 77 K absorption (A), the second derivative of the absorption (B), and Stark spectra (C) of 13^1-OH-Pheo exchanged PSII RC complexes (RC_{1x}). The spectra were normalized to OD = 1 at 673 nm, and the Stark spectra were recorded at magic angle and an electric field strength of 2.77×10^5 V cm^{-1}.

position with 13^1-OH-Pheo (Figure 2A), in agreement with earlier results (*14*, *26*). The broad bands near 630 and 620 nm (Figures 1A and 2A) are dominated by Q_x and vibrational Q_y transitions of the chlorophylls, respectively.

At wavelengths longer than 670 nm, the Stark spectra of the standard (Figure 1C) and exchanged PSII RC complexes

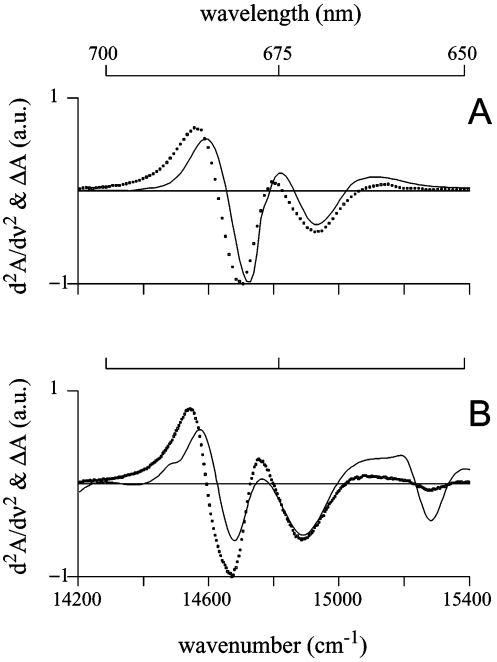

FIGURE 3: Second derivatives of the absorption spectra (full lines) and Stark spectra (dashed lines) of standard (A) and RC_{1x} (B) PSII RC complexes in the Q_y absorption region (data from Figures 1 and 2).

Table 1: Stark Parameters for Different Transitions of Standard and 13^1-OH-Pheo Exchanged PSII RC and CP47 Complexes[a]

prep.	Stark param.	Q_y 1-band	Q_y 2-bands main band	Q_y 2-bands minor band	Q_x Pheo	β-carotene
standard	Dm	1.6	1.4	<2	2.0	<1
standard	Tr(Δα)	0	0	1300	125	1450
RC_{1x}	Dm	1.8	1.6	<1	3.0	<3
RC_{1x}	Tr(Δα)	0	14	1600	<1	1360
CP47	Dm	0−0.4				5.9
CP47	Tr(Δα)	20−60				1540

[a] The values for the difference dipole moment $\Delta\mu$ are expressed in D f^{-1} and those for the difference in polarizability Tr(Δα) in $Å^3$ f^{-2}. The parameters of the Chl Q_y transitions of CP47 were obtained from ref *37*.

(Figure 2C) are very similar and characterized by pronounced negative bands at 670 and 680 nm and a strong positive band at 687 nm (see also Figure 3A,B, dashed lines). Figure 3 also shows that the overall line shape of the Stark spectra (dashed lines) resembles the second derivatives of the absorption spectra (full line), which means that the Stark spectrum is dominated by the difference in permanent dipole moment $\Delta\mu$ between the excited and the ground states of the molecules. An analysis of the size of the Stark and absorption spectra according to the Liptay formalism (*33*) gave values for $\Delta\mu$ of 1.6 and 1.8 D f^{-1} for the standard and RC_{1x} samples, respectively (see also Table 1). These values are slightly larger than the 0.9−1.0 D f^{-1} observed for monomeric chlorophyll *a* (*35*) and also larger than the 1.4 D f^{-1} suggested in an early Stark spectroscopy study of the PSII RC (*36*).

Figure 3A,B show that at wavelengths longer than 675−680 nm, both Stark spectra are red-shifted as compared to the second derivatives of the absorption spectra. This is remarkable and in fact very unusual. Inclusion of a given amount of first-derivative contribution will only result in a blue-shift of the Stark spectrum (see Supporting Information).

A good simultaneous fit of the absorption and Stark spectra according to the Liptay formalism (*33*) could, however, be obtained by introducing a new band at 685 nm with a very small amplitude in the OD spectrum (with an oscillator strength corresponding to that of much less than one chlorophyll per RC) and a huge difference in polarizability ($Tr(\Delta\alpha) = 1300-1600$ Å^3 f^{-2}, Table 1). Simultaneous fits of the absorption and Stark spectra in which the spectra were decomposed into Gaussians also resulted in a red band at about 685 nm with very small amplitude and huge Stark parameters (see Supporting Information). Also, allowing negative first derivatives in the fits (which can arise if the excited state is less polarizable than the ground state) did not avoid the presence of a band with very small amplitude and huge Stark parameters in the red edge of the spectrum.

The question is whether such fits are physically correct. We think that they are not, also because other types of spectroscopy have not indicated very small bands at the red edge of the absorption spectrum. It is more likely that the red-shifted Stark spectrum cannot be explained at all within the framework of the classic Liptay formalism. This theory only takes into account field-induced band-shifts to lower energy (for polarizable molecules) and the broadening of absorption bands (for molecules with a permanent dipole moment) but not the field-induced mixing of new states with different spectral properties than the optically allowed states.

Red-shifted Stark spectra with similar characteristics as in the PSII RC complexes have also been seen in a number of special pair mutants of *R. sphaeroides* (*24*), in particular the LM160H mutant (a mutant in which a hydrogen bond is introduced for the keto-group of P_B) and the heterodimer mutants HL173L and HM202L (mutants in which the P_A or P_B bacteriochlorophyll has been replaced by a bacteriopheophytin). The impossibility to fit the Stark spectrum by a combination of zeroth, first, and second derivatives was in these cases explained by a considerable field-induced mixing of the electronically excited state with one or more CT states at about equal energy (*24*). In wild-type RCs of *R. sphaeroides*, the energy of the CT state is probably much higher than that of the electronically excited state, so that mixing takes place to a lesser extent, and the Stark parameters can largely be explained within the framework of the Liptay formalism, although deviations were found when analyzing higher order (4ω) Stark spectra (*24*).

We note that the red-shift of the Stark spectrum cannot be explained by contamination with the core antenna protein CP47. This is not only because CP47 was not present in our samples (see Materials and Methods) but also because the Stark spectrum of CP47 is relatively small and has its red-most peak at 684 nm (*37*), which is blue-shifted as compared to that of the PSII RC. We conclude that around 680 nm there can be some field-induced mixing of electronically excited and CT states. It is unlikely that H_B and B_B are involved in the CT state, in view of the similarity of Stark responses between the standard and the RC_{1x} PSII RC preparations (H_B is absent, and B_B is shifted to shorter wavelength in RC_{1x}—ref *14*). It is also unlikely that P_A is involved to a significant extent because the site energy of this molecule, at least in cyanobacteria, is more at the blue side of the spectrum (*13*).

Electric Field Response of the Pheophytins. The fact that the Stark spectra of the standard and RC_{1x} PSII RC preparations are very similar in the complete Q_y absorption region (Figure 3) shows that H_B does not contribute significantly to these spectra. Also, in the bacterial RC, chemical exchange of H_B resulted in minor changes in the Stark spectrum (*38*). The Stark signal of the 13^1-OH-Pheo at the H_B binding position has a clear second-derivative line shape around 653 nm (Figure 3B), and fitting this band with the Liptay formula gave a $\Delta\mu$ of about 0.6 D f^{-1} (Table 1), much less than the average value for the other chlorins in the PSII RC (1.6−1.8 D f^{-1}).

The Stark effect of the pheophytin Q_x band is surprisingly strong. The Stark spectrum of the RC_{1x} preparation shows a clear second-derivative type of signal around 543 nm, which in this preparation arises exclusively from the active pheophytin H_A. The amplitude is almost the same as that of 13^1-OH-Pheo at the H_B binding position around 655 nm (Figure 2C), despite a much smaller amplitude in the absorption spectrum. We estimated $\Delta\mu$ values of about 3.0 and 2.0 D f^{-1} for the Pheo Q_x band in the RC_{1x} and standard PSII RC preparations, respectively (Table 1). The value for the standard PSII RC preparation is, however, less reliable because of the overlap with the Stark signal from the β-carotenes. The finding that the $\Delta\mu$ value for the Q_x transition of H_A is higher than that of the averaged $\Delta\mu$ value for all Q_y transitions is noteworthy because in the bacterial reaction center the Stark effects of the pheophytin Q_x transitions are 2−3 times smaller than of the corresponding Q_y transitions (*39*). Bacterial RC complexes with plant Pheo *a* molecules at the H_B or $H_B + H_A$ positions give $\Delta\mu$ values of about 1.5 D f^{-1} for both pheophytins (*40*), which points to a fundamentally different electric field response of the pheophytins in the PSII and purple bacterial RC complexes.

Electric Field Response of the β-Carotenes. The main β-carotene transitions are at 507 and 487 nm (Figure 1A). On the basis of linear dichroism measurements, it was shown that the 507 and 487 nm transitions are oriented parallel and perpendicular to the plane of the membrane, respectively (*34*, *41*, *42*). Figures 1C and 2C show that the carotenes give rise to pronounced Stark effects, with maxima near 517 and 496 nm and minima near 504 and 487 nm.

A simultaneous fit of the absorption and Stark spectra in the main β-carotene absorption region (between 475 and 525 nm) showed that a first-derivative line shape together with a small negative zeroth-derivative contribution gives a very good fit of the spectra (Figure 4). The latter contribution suggests some loss of oscillator strength because of the electric field and has been found for many photosynthetic complexes (*43*, *44*). The first-derivative contribution scaled to $Tr(\Delta\alpha) = 1450$ Å^3 f^{-2}, which is about the same value as found for β-carotene in solution (*45*). Inclusion of a second-derivative contribution resulted in a $\Delta\mu = 0-1$ D f^{-1} (for the standard preparation—see Table 1) so that a small $\Delta\mu$ cannot be excluded.

The large value of the polarizability difference is what one would expect for molecules with long polyenic chains, and at first sight, also the low $\Delta\mu$ values for the β-carotene molecules of the PSII RC are not surprising. β-carotene is in principle a symmetrical molecule in which no difference in permanent dipole is expected. However, β-carotene in

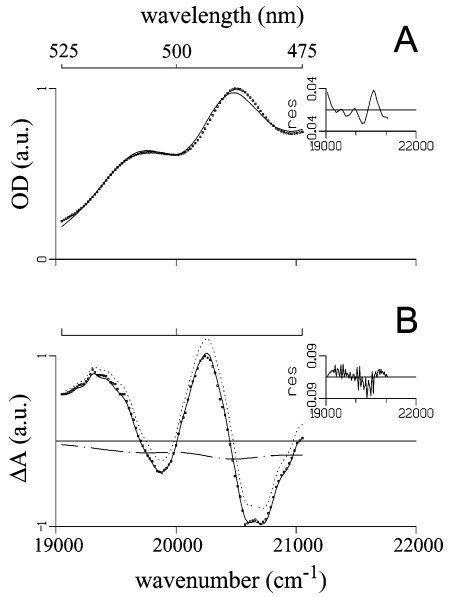

FIGURE 4: Simultaneous fit of the Stark and absorption spectra of the β-carotenes in the standard PSII RC complexes. The absorption spectrum (A) was fitted with a polynomial (line shape) function, whereas the Stark spectrum (B) was fitted with the zeroth, first, and second derivatives of this function. Open squares: data points. Solid lines: fit results. Dotted line: first derivative. Dot−dashed line: zeroth derivative. The inset shows the residuals of the fit.

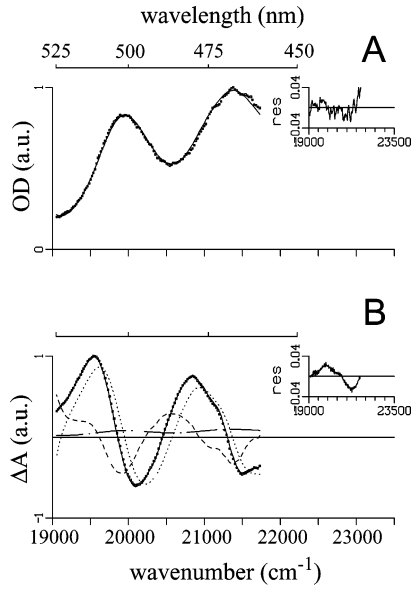

FIGURE 5: Simultaneous fit of the Stark and absorption spectra of the β-carotenes in CP47. The absorption spectrum (A) was fitted with a polynomial (line shape) function, whereas the Stark spectrum (B) was fitted with the zeroth, first, and second derivatives of this function. Open squares: data points. Solid lines: fit results. Dashed line: second derivative. Dotted line: first derivative. Dash−dotted line: zeroth derivative. The inset shows the residuals of the fit. The Stark spectra had been recorded at magic angle and with an electric field strength of 2.3×10^5 V cm^{-1}.

solution (*45*) and in photosystem I (*32*), as well as carotenoids of many other photosynthetic complexes (*45*), all gave relatively large second-derivative contributions that scaled to $\Delta\mu$ values of at least 5 D f^{-1}.

To understand the peculiarity of the Stark response of the two β-carotenes of the PSII RC, we also measured the Stark effect of the β-carotenes of the CP47 core antenna protein of PSII (*37*, *46*). Also, the CP47 complex contains about two β-carotene molecules, and these molecules could therefore serve as a reference for β-carotenes in a PSII environment. Figure 5 shows the Stark response of the β-carotene molecules in CP47. Unlike the two β-carotenes in the PSII RC complex, these carotenes absorb at the same wavelength at 502 nm at 77 K (*46*). In contrast to the situation in the PSII RC complex, the Stark spectrum and the first derivative of the absorption spectrum have clearly different zero-crossing points, and a second derivative contribution is absolutely needed to fit the Stark spectrum (Figure 5). The Stark parameters of the β-carotenes of CP47 converted to $\mathrm{Tr}(\Delta\alpha) = 1540$ Å^3 f^{-2} and $\Delta\mu = 5.9$ D f^{-1} (Table 1). The latter value is much larger than that found for the β-carotenes in the PSII RC complex but similar to that of all other investigated β-carotenes. These results stress the unique behavior of the β-carotenes of the PSII RC in an electric field.

Large values of $\Delta\mu > 5$ D f^{-1} for carotenoids can be explained by a conversion of the polarizability difference to permanent dipole moments by the internal electric field of the surrounding protein (*43*) or by distortions of the molecular geometry caused by asymmetry of the solvent or protein environment (*45*). For the β-carotenes in the PSII RC, highly symmetrical protein surroundings are unlikely, also because recent resonance Raman studies have indicated that at least the 487 nm spectral form adopts a slightly distorted conformation (*47*, *48*). We will discuss below that the most likely explanation for the unusually small $\Delta\mu$ values is given by excitonic coupling between the two β-carotenes.

DISCUSSION

The results in this contribution reveal that, at least at 77 K, almost all pigments of the PSII RC complex reveal unusual Stark effects. The absorption bands at the red side of the spectrum show a strong electric field response that cannot be explained within the framework of the classic Liptay formalism, while the pheophytin at the active side of the complex (H_A) shows a much stronger Stark response than that at the inactive side (H_B), and the β-carotenes show unusually small second-derivative contributions.

Possible ($B_A^{\delta+}H_A^{\delta-}$) CT State in the PSII RC. The only other photosynthetic complexes that are known to show nonclassic behavior of the Stark spectrum similar to that observed in the red part of the spectrum of the PSII RC complex are a number of special pair mutants of *R. sphaeroides* (*24*). The absorption spectra of these special pairs could be described by a model of three states (i.e., a ground state, a pure exciton state, and a vibronically broadened CT state), with an intermediate coupling strength between the latter two states (*49*). The coupling of two states may in principle lead to a variation of the electrooptic parameters across the band, which then can give rise to nonclassic Stark behavior (*50*). The deviations from the classic Liptay behavior were most significant in those mutants that were predicted to have the average energy of the CT state closest in energy to the exciton state (*49*). For this reason, the deviations from the classic Liptay behavior were attributed to the mixing of exciton and CT states of almost equal energy (*24*).

A coupling of two states of about equal energy can also explain the nonclassic Stark spectra of the PSII RC com-

plexes. It is unlikely that these two states are two different exciton states. Exciton calculations have suggested the presence of two nearly degenerate exciton bands, each localized at a different arm of the RC, if the coupling between the two central chlorophylls is very small (*2*). In this case, however, a significant difference is expected between the standard and RC_{1x} preparations since in the latter preparation the exciton bands of the B-arm are blue-shifted and have smaller amplitudes (*14*). In addition, it is unlikely that a coupling of two similarly shaped bands can explain the deviations in the Stark spectrum. The Stark spectra of the standard and RC_{1x} preparations are remarkably similar and thus suggest another explanation.

We suggest that the nonclassic Stark spectra of the PSII RC complexes arise from a coupling between an exciton state and a vibronically broadened CT state, as in the various special pairs of *R. sphaeroides* (*24*). It is very likely that B_A and H_A are involved in this CT state. The Q_y transitions of both molecules contribute significantly to the absorption at 680−683 nm (*13*, *14*), the wavelength region where strong nonclassic Stark effects occur, although exciton interactions will also give some delocalization of the excitation energy over the other chlorins of the PSII RC. Our data show furthermore that the Q_y transition of H_B only has a weak Stark response and that the Q_x transition of H_A has a surprisingly strong response, although it does show a classic Stark response dominated by its permanent dipole moment. A direct mixing with the CT state is, however, unlikely for the 543 nm absorption band. On the other hand, the permanent dipole of the Q_x transition of H_A will probably feel the $(B_A^{\delta+}H_A^{\delta-})$ CT state, just like the Q_x transition of B_A. The Stark spectra in Figures 1C and 2C show indeed some pronounced features caused by the Q_x transition of chlorophylls. The light-induced formation of a CT state on the A-branch (or D1-branch), but not on the B-branch (or D2-branch), will facilitate directed charge separation along the A-branch.

Fast time-resolved absorbance-difference measurements on heterodimer mutants of purple bacteria have shown that the charge separation reaction starting from the heterodimer special pair is slowed as compared to the reaction starting from the wild-type special pair (*51*, *52*) and that in the HM202L heterodimer the charge separation route starting from B_A* occurs to a larger extent than in the wild-type (*52*). The CT state observed by Stark spectroscopy in this mutant is, however, between P_A and P_B, and not between B_A and H_A, as in the PSII RC complex, so there is no reason to suggest that the CT state of the PSII RC will slow the charge separation process. On the contrary, an asymmetry of the excited state, with some negative charge on H_A and some positive charge on B_A, will facilitate a very fast charge separation reaction. Recent time-resolved absorbance-difference measurements suggest that direct excitation of B_A in the RC_{1x} preparation results in charge separation kinetics (at 77 K) of about 0.5 ps (M. Germano, C. C. Gradinaru, A. Ya. Shkuropatov, I. H. M. van Stokkum, V. A. Shuvalov, A. J. Hoff, J. P. Dekker, R. van Grondelle, and H. J. van Gorkom, unpublished observations).

Red-shifted Stark spectra were not observed for the B and H molecules of the purple bacterial RC (*53*), at least not when conventional (2ω) Stark spectroscopy is used. This suggests differences between the two systems regarding the electronic couplings between B_A and H_A and/or the relative energies of the CT states. Higher order (4ω and 6ω) Stark spectroscopy studies on the purple bacterial RC, however, did reveal an unusual Stark effect of B_A and H_A, which was explained (*53*) and modeled (*54*) by a charge resonance interaction between the singlet-excited and charge-separated states of B_A and H_A and which could influence the rate of the charge separation reaction.

In purple bacteria, the $B_A^* \rightarrow B_A^+H_A^-$ charge separation reaction is physiologically not very relevant because the excited-state energy of B_A is about 1000 cm^{-1} higher than that of the core antenna chlorophylls. In PSII, however, the excited-state energy of B_A is similar or even slightly lower that of the core antenna chlorophylls. This about equal energy, and that of the $(B_A^{\delta+}H_A^{\delta-})$ CT state, will favor the $B_A^* \rightarrow B_A^+H_A^-$ charge separation route as the most prominent one in photosystem II.

Are the Two β-Carotenes of the PSII RC Excitonically Coupled? The Stark spectroscopy studies described in this contribution indicate that the Stark response of both β-carotene molecules in the PSII RC is unusual. The response is dominated by first-derivative contributions scaling to normal $\Delta\alpha$ values of 1450 $Å^3$ f^{-2} but does not show significant second-derivative contributions ($\Delta\mu$ is maximally 1 D f^{-1}). To our knowledge, such low difference dipole moment contributions have not been observed before for carotenoids in photosynthetic systems. We stress that both β-carotene molecules of the PSII RC complex display this unusual behavior.

A very large $\Delta\mu$ of about 15 D f^{-1} has been found for the xanthophyll responsible for the red-shifted 508 nm absorption band in trimeric LHCII, whereas normal $\Delta\mu$ values of about 5 D f^{-1} were found for the main absorption bands of the xanthophylls absorbing at 486 and 494 nm (ref *55* and M. A. Palacios, A. V. Ruban, R. N. Frese, C. C. Gradinaru, L. Premvardhan, P. Horton, R. van Grondelle, and H. van Amerongen, unpublished observations). The absorption wavelengths of these xanthophylls resemble those of the β-carotenes in the PSII RC, but the Stark effects are completely different. In the PSII RC, both the red and the blue β-carotene molecules display the same, unusually small $\Delta\mu$. The 508 nm xanthophyll in LHCII has been identified as lutein in a special, twisted conformation (*56*), and the red-shift is most likely caused by pigment−protein interactions induced by trimerization (M. A. Palacios, A. V. Ruban, R. N. Frese, C. C. Gradinaru, L. Premvardhan, P. Horton, R. van Grondelle, and H. van Amerongen, unpublished observations).

The absence of dipole moment contributions to the Stark spectra of the PSII RC can in principle be explained by the absence of distortions of the geometry of the β-carotene molecules (*45*). A total absence of distortions is, however, unlikely, because both carotenes, as well as their protein environment, have to be fully symmetric. Indeed, recent resonance Raman studies have indicated that at least the 487 nm spectral form has a slightly distorted conformation (*47*, *48*), which makes this explanation rather unlikely. A precise cancellation of the effects of a distorted carotenoid geometry and protein dipole moments could theoretically explain a zero-permanent dipole moment, but this seems a highly improbable configuration, also because the two carotenes would both need to have this special configuration. Another

factor that could play a role is the bandwidth of the absorption bands of the carotenes. Krawczyk and Olszowka (*45*) have shown that the second-derivative contributions in the Stark spectra of carotenoids become weaker if the absorption bands become narrower. This correlation was explained by the notion that inhomogeneous broadening of the main absorption bands of β-carotene originates from a flexible geometry of the molecule. A more pronounced flexibility will lead to larger distortions and thus to stronger second-derivative contributions. While this effect could play a role, it seems, however, insufficient to explain the very low second-derivative contributions of the β-carotenes of the PSII RC because the bandwidth of these transitions is comparable to that of β-carotene in some organic solvents and in CP47, for which $\Delta\mu$ values of about 5 D f^{-1} were found (ref *45* and Figure 5).

Another mechanism that could diminish the second-derivative contribution in Stark spectra is excitonic coupling (*57*, *58*). In the most simple case of coupling of two chromophores, the coupling will give rise to the same $\Delta\mu$ values as for the two monomers in the case of a head-to-tail dimer, a zero $\Delta\mu$ value in the case of a head-to-head dimer, and intermediate $\Delta\mu$ values for all other organizations (*58*). In the case of a pure head-to-head dimer, the dipole contributions just cancel. A pure head-to-head dimer is, however, not possible for the β-carotenes of the PSII RC because in this case the high exciton component would not have any oscillator strength. An organization of a dimer in which most of the $\Delta\mu$ cancels and in which considerable oscillator strength occurs in both transitions is, however, very well possible.

Excitonic coupling of the two β-carotene molecules of the PSII RC has been suggested before (*42*, *46*, *59*), but this requires a location of both carotenes at the same side of the complex, which contrasts a widely accepted notion (ref *48*, but see the following). This notion was partly based on work of Tomo et al. (*60*), who treated isolated PSII RC preparations with organic solvents and noted that under certain conditions only the β-carotene absorbing at 487 nm is extracted. This result disagrees with the notion of excitonic coupling because the extraction of one constituent of an exciton-coupled dimer will break the interaction and remove both exciton bands, while at the same time a monomer absorption band should be generated. A problem with the data reported in ref *60*, however, is that the treatment with organic solvents also may have induced bandshifts of the chromophores of the PSII RC. In particular, the proposed connection between the carotene absorbing at 507 nm and the inactive pheophytin H_B is suspicious because H_B was, in ref *60*, thought to have an absorption band at 538 nm, while we now know that H_B, just like H_A, peaks at 542 nm (*14*, *61*) and that a pheophytin peaking at 538 nm is probably disconnected from the PSII RC. We note that the different vibrational characteristics found for the two spectral components (*47*) does not disagree with the idea of excitonic coupling because this coupling involves the electronic states and will not influence the vibrational states of the two molecules.

Recent work has indicated that both β-carotenes can be oxidized (*47*, *62*), although the extent may differ in PSII from different organisms, and that β-carotene mediates the electron transfer from a peripheral chlorophyll (Chl-Z) to $P680^+$. This peripheral chlorophyll is most likely located on D2 (*63*). In the most recent structural models of the PSII RC (reviewed in ref *48*), the β-carotene molecule is positioned between Chl-Z_{D2} and P680 because the distance between these molecules (center-to-center 24.6 Å, ref *5*) may be too long to allow the electron-transfer process. This reaction may thus participate in a cyclic electron-transfer reaction from Q_B^-, via cytochrome *b*559 and Chl-Z_{D2}, to $P680^+$ that was postulated to form a mechanism to minimize photoinhibition (*64*).

Very recently, a structure of the PSII complex from the cyanobacterium *Synechococcus vulcanus* was published at 3.7 Å resolution (*65*). In this structure, both β-carotene molecules are modeled at the D2 side of the complex, in agreement with our interpretation of the Stark and other spectroscopic results. It is not clear, however, how this model of the carotenes can give rise to an exciton band with very significant amplitude and with an orientation perpendicular to the plain of the membrane. Nevertheless, a location of both β-carotenes on the same D2 side of the complex could be functionally relevant if both are needed to facilitate the postulated cycle of electron transfer.

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SUPPORTING INFORMATION AVAILABLE

Two spectra figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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