Pigment Organization and Their Interactions in Reaction Centers of Photosystem II: Optical Spectroscopy at 6 K of Reaction Centers with Modified Pheophytin Composition†

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ABSTRACT: Photosystem II reaction centers (RC) with selectively exchanged pheophytin (Pheo) molecules as described in [Germano, M., Shkuropatov, A. Ya., Permentier, H., Khatypov, R. A., Shuvalov, V. A., Hoff, A. J., and van Gorkom, H. J. (2000) *Photosynth. Res. 64*, 189−198] were studied by low-temperature absorption, linear and circular dichroism, and triplet-minus-singlet absorption-difference spectroscopy. The ratio of extinction coefficients $\epsilon_{Pheo}/\epsilon_{Chl}$ for Q_Y absorption in the RC is ∼0.40 at 6 K and ∼0.45 at room temperature. The presence of 2 β-carotenes, one parallel and one perpendicular to the membrane plane, is confirmed. Absorption at 670 nm is due to the perpendicular Q_Y transitions of the two peripheral chlorophylls (Chl) and not to either Pheo. The "core" pigments, two Pheo and four Chl absorb in the 676−685 nm range. Delocalized excited states as predicted by the "multimer model" are seen in the active branch. The inactive Pheo and the nearby Chl, however, mainly contribute localized transitions at 676 and 680 nm, respectively, although large CD changes indicate that exciton interactions are present on both branches. Replacement of the active Pheo prevents triplet formation, causes an LD increase at 676 and 681 nm, a blue-shift of 680 nm absorbance, and a bleach of the 685 nm exciton band. The triplet state is mainly localized on the Chl corresponding to B_A in purple bacteria. Both Pheo Q_Y transitions are oriented out of the membrane plane. Their Q_X transitions are parallel to that plane, so that the Pheos in PSII are structurally similar to their homologues in purple bacteria.

Photosystem II (PSII)[1] efficiently converts light energy into the electrochemical energy required for the oxidation of water, the ultimate source of electrons and oxygen for life. The energy conversion takes place in the PS II reaction center (RC), a protein complex embedded in the thylakoid membrane.

The core of the PSII RC consists of the D1 and D2 proteins and their cofactors that confer it its photochemical activity, in a stoichiometry of 6 chlorophyll *a* (Chl), 2 pheophytin *a* (Pheo), and 1−2 β-carotene (β-car) molecules per RC. In its native form, the RC also contains two plastoquinone molecules (Q_A and Q_B) and the oxygen evolving 4-Mn cluster, but they are lost during isolation of the RC complex. One or two copies of cytochrome b_{559} and a few other small polypeptides are also an integral part of the isolated PS II RC (*1*). Charge separation occurs by electron transfer from the singlet excited state of the primary donor, P680, to a Pheo molecule (*2*). In the isolated RC, secondary electron transfer pathways are inactivated and the charge pair is lost by recombination, largely to the triplet state of P680.

Although the isolated PSII RC binds a minimum number of cofactors, its spectroscopy is not clearly understood (*3*, *4*) and its function (energy transfer between pigments and the kinetics and mechanism of the primary reaction) is a subject of debate (*5*−*8*). The present controversy is to some extent caused by the spectral overlap in the Q_Y region, where the six Chls and both Pheos all absorb within approximately 400 cm^{-1}. At low temperatures, the Q_Y absorption appears as two only partially resolved bands at around 670 and 680 nm, with a shoulder of variable amplitude (depending on the preparation) at ∼684 nm (*9*). The Pheo Q_X transitions are well resolved from those of Chls, peaking at ∼543 nm. The superposition of the Q_Y absorption bands results in circular dichroism (CD) and linear dichroism (LD) spectra

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[1] Abbreviations: PSII, Photosystem II; RC, reaction center; Pheo, pheophytin *a*; 13^1-OH-Pheo, 13^1-deoxo-13^1-hydroxy-pheophytin *a*; Chl, chlorophyll *a*; β-car, β-carotene; BPheo, bacteriopheophytin *a*; BChl, bacteriochorophyll *a*; P680, primary donor of Photosystem II; DM, n-dodecyl-β-D-maltoside; CD, circular dichroism; LD, linear dichroism; T − S, triplet-minus-singlet absorption difference; deltaA, absorption difference.

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with little structure, which cannot be interpreted unambiguously (*9*).

One consequence of the overlap of electronic transitions in the red-most region of the absorption spectrum is that it is difficult to determine the nature and strength of excitonic interactions between the RC cofactors. There is no evidence for strong coupling, as the Q_Y absorption bands are not significantly red-shifted with respect to the absorption maxima of monomeric Chl or Pheo. At low temperature (1.2 K), the triplet state (after charge recombination from $P680^+Pheo^-$) seems to be localized on a monomeric Chl (*10*, *11*). However, the positive charge in $P680^+$ at 150 K appears to be delocalized to a certain extent over two Chls (*12*), and at this temperature the triplet state is also delocalized (*11*). Time-resolved spectroscopy at room temperature clearly shows delocalization of the singlet excited state over at least two Chls (*13*, *14*). In an attempt to describe the electronic structure in the Q_Y region, a model has been put forward (*15*) that assumes weak coupling between a "core" of pigments in the RC, including the two Pheos but excluding two of the Chls, which are thought to be located too far from the central cofactors to be involved in any significant interactions.

The optical properties of the Pheo molecules in PSII (both the active — the one that functions as primary electron acceptor — and the inactive Pheo) in the Q_Y spectral region are not clearly understood. While results obtained by several methods (*9*, *10*, *16*−*23*) point to an absorption maximum close to 680 nm for the active Pheo, the location of the inactive Pheo in the congested Q_Y band is controversial (*23*−*25*).

Selective modification of the chromophores in the RC is a valuable tool to study pigment−pigment and pigment−protein interactions, and has been applied extensively to various components of the purple bacteria photosystem (*26*). The most studied PSII RC preparation with altered cofactor composition is the one described by Vacha et al. (*27*), which contains only five Chl molecules per two Pheos. The missing Chl was shown to be involved in slow energy transfer and its removal does not affect the rate of charge separation at room temperature (*27*). It contributes to the absorption at ~670 nm (*27*, *28*) and, by comparison of its absorption and triplet-minus-singlet (T − S) spectra at 4 K with those of a common 6-Chl preparation, a narrowing and red-shift of the 684 nm shoulder is observed in the 5-Chl RC (*28*). This effect is reflected in the fluorescence emission spectrum (*28*). The Chl molecule removed in the 5-Chl preparation is thought to be one of the two peripheral Chls (*27*). On the other hand, Eijckelhoff et al. (*28*) concluded that the absorption at 684 nm originates from the primary donor. It remains unclear though how the removal of a Chl molecule distant from P680 can significantly affect its absorption properties.

Gall et al. (*29*) have described a method for Chl exchange in PSII RCs, and partially characterized a preparation where [3-acetyl]-chlorophyll *a* replaces approximately one Chl molecule per RC. The exchanged Chl absorbs at 670 nm, but its function and location (distant or near P680) cannot be determined based on the spectroscopic characterization presented (*29*).

Recently, a method for exchange of the Pheo molecules has been described (*16*, *17*). The modified pigment introduced is 13^1-hydroxy-13^1-deoxo-pheophytin *a* (13^1-OH-Pheo). This molecule differs from Pheo in that the 13^1-C═O group of Pheo is 13^1-C(H)OH in 13^1-OH-Pheo (Figure 1). 13^1-OH-Pheo is spectrally distinguishable from Pheo, both in the Q_Y and in the Q_X region (Figure 1). Its reduction potential in solution is estimated to be about 300 mV more negative than that of Pheo (*16*), a property that (if conserved in the protein environment) should prevent its photoreduction in the PSII RC. Due to these two characteristics of 13^1-OH-Pheo, PSII RC preparations containing this pigment are very suitable for characterizing the properties and functioning of the Pheo molecules in the RC, through analysis of the effects of their replacement.

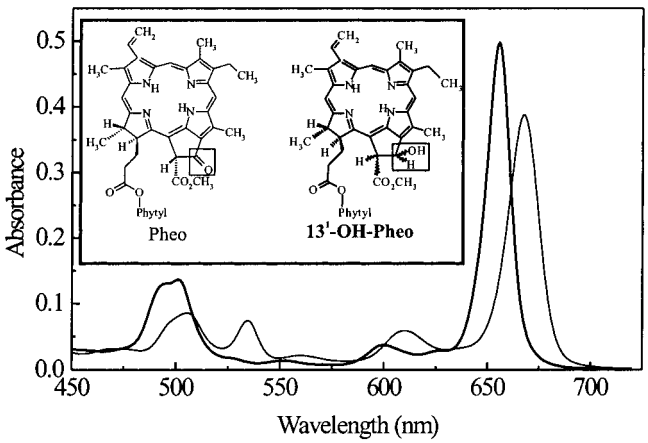

FIGURE 1: Absorption spectra at room temperature of Pheo (thin line) and 13^1-OH-Pheo (heavy line) in diethyl ether. The inset shows the molecular structure of the two molecules.

In a previous paper (*17*), we have shown that the pigment exchange procedure described by Shkuropatov et al. (*16*) does not affect the RC cofactor stoichiometry. By applying the exchange procedure once, a preparation was obtained (RC_{1x}) where approximately one Pheo molecule was replaced by one molecule of 13^1-OH-Pheo per RC complex. By subjecting RC_{1x} to a second exchange procedure, RC_{2x} was prepared. In RC_{2x}, the modified pigment replaces about 50% of the remaining Pheo. This preparation is heterogeneous in the sense that it contains about 50% of RCs with total replacement of Pheo and 50% of RCs where only one of the Pheos is exchanged. In RC_{1x}, $Pheo^-$ can be photoaccumulated with the same yield as in a nontreated preparation, while in RC_{2x} this yield is decreased by about 50% (*17*). Thus, the difference between the spectra of RC_{1x} and a nontreated (or a control) RC reflects the spectroscopic properties of the Pheo molecule of which the anion cannot be photoaccumulated, presumably the one in the inactive branch (H_B). The difference between RC_{2x} and RC_{1x} reflects the properties of the second Pheo molecule, presumably in the active branch (H_A).

Here, we further characterize the modified RCs described in ref *17* by low-temperature CD, LD and triplet-minus-singlet absorption difference (T − S) spectroscopy, and compare these results to the previously reported absorption spectra at 6 K (*17*). The low-temperature absorption spectrum changes in a complex way upon Pheo replacement (ref *17* and Figure 2), suggesting that excitonic interactions in the RC may include Pheo Q_Y transitions. If the CD spectrum of the PSII RC originates from such interactions, it should be

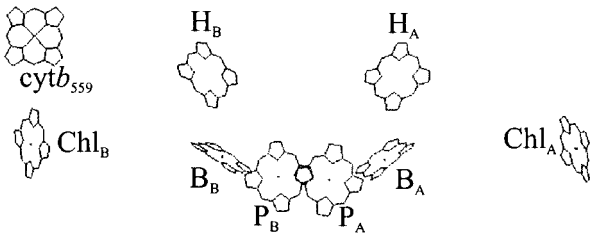

FIGURE 2: Pigment arrangement in the PSII RC (after ref *31*). Cofactors are named by analogy to the nomenclature generally used for purple non-sulfur bacteria RCs.

possible to identify them by comparing the CD spectra of the two modified preparations to that of a control.

Recent progress in elucidation of the structure of the PSII RC points to a pigment organization very similar to what is observed in the RC of purple bacteria (*30*, *31*), except that the two Chl molecules corresponding to the special pair are at a larger center-to-center distance (*31*). The nearest-neighbor distances are approximately the same for all the central cofactors, about 11 Å (*30*). The nearest cofactor to either Pheo is a Chl molecule oriented in the same way as in the purple bacteria RC (Figure 2), that is, close to the magic angle relative to the normal to the membrane plane (*31*). Pheo exchange is expected to affect mainly the spectroscopic properties of these two symmetry related Chls (B_B for exchange of H_B, in the "inactive" branch, and B_A for exchange of H_A).

In the PSII RC, the triplet state is, at low temperatures, largely localized on a monomeric Chl with the plane of the macrocycle oriented at an angle of 30° with respect to the membrane plane (*32*). Since triplets are formed predominantly via charge recombination (presumably from the state $P680^+H_A^-$), and since there is no evidence for triplet transfer at $T < 20$ K (*11*, *33*), the Chl on which the triplet state is mainly localized is most likely the one closer to H_A, taking into account that it probably assumes the same orientation with respect to the membrane plane as that reported by van Mieghem et al. (*32*). The absorption spectrum of this molecule (B_A) can be determined from the T − S spectrum of the RC. To establish if it is altered as a consequence of Pheo exchange, we compare the T − S spectra of RC_{1x} and RC_{2x} to that of a preparation with native Pheo composition.

The structure recently published by Zouni et al. (*31*) has a limited resolution, of 3.8 Å. The positions and orientations of the ring planes of the chlorin pigments are well-defined, but the orientations of the optical transitions of each pigment remain undetermined. In the absence of detailed structural information, LD spectroscopy is a valuable tool to determine the orientations of optical transitions of chlorins embedded in a membrane protein (*34*). However, because of the superposition of the Q_Y absorption bands, the LD spectrum of the PSII RC cannot be easily interpreted (*9*). Breton (*18*) and Ganago et al. (*19*) have shown, by analysis of the linear dichroism of H_A^- photoaccumulation, that the Q_Y transition of H_A is nearly perpendicular to the membrane plane, but there is no information on the orientation of H_B. Van Gorkom and Schelvis (*35*) have suggested that H_A might have opposite LD to that of H_B, if the transition(s) of P680 is (are) oriented close to the magic angle with respect to the membrane plane and if no other pigment absorbs around 680 nm. The multimer model (*15*, *36*) predicts two degenerate exciton states, at 680−684 nm whose transition dipoles are nearly orthogonal to each other. Since these states are predicted to be localized on average on opposite "arms" of the RC, each of them should contain a contribution from either H_B or H_A, and the effects on the LD spectrum of replacing either of these cofactors can be used to critically analyze the calculations based on the multimer model.

MATERIALS AND METHODS

PSII RCs were prepared from Tris-washed BBY particles (*37*) by the method described by van Leeuwen et al. (*38*). 13^1-OH-Pheo was prepared from Pheo (purified from nettle) as in Shkuropatov et al. (*16*). Modified PSII RCs with 40% exchange of Pheo (RC_{1x}) or 75% exchange (RC_{2x}) were obtained as described by Germano et al. (*17*). A "control" sample was prepared by subjecting PSII RCs once to the exchange conditions, in the absence of extraneous pigment, as described previously (*17*).

Low-temperature LD and CD spectra were measured in a home-built setup described by Otte (*39*), equipped with a photoelastic modulator (PEM FS-4, Hinds International Inc.) and a lock-in amplifier. For CD, the samples were diluted in a buffer containing 20 mM Bis-Tris, pH 6.5, 12.5 mM $MgSO_4$, 0.03% (w/v) *n*-dodecyl-*β*-D-maltoside (DM) and 67% (w/v) glycerol (BT buffer). Oriented samples for LD measurements were obtained by biaxial compression (stretching factor of 5.8) of a gelatin gel (6.4% (w/v) of gelatin powder) containing the RC preparations in BT buffer. The optical density at the Q_Y maximum was about 0.8 for CD and for LD, in a 2.5 mm cuvette. The spectral resolution was 2 nm for CD and 1.5 nm for LD. A skewed baseline (originating from the optical components of the setup and from the wavelength-dependent sensitivity of the photomultiplier) was subtracted from all spectra.

Laser flash-induced kinetics in the millisecond time scale were measured in a home-built setup described by Franken (*40*). Excitation was provided by an Nd:YAG laser (532 nm, 15 ns pulse duration, 10 Hz repetition rate, <9 mJ/pulse), and measuring light by a 250 W tungsten−iodine lamp. The instrument response time was set to 10 μs by an electronic filter. Spectral resolution was 1.5 nm. The samples were diluted in BT buffer to an OD of approximately 1/mm, and cooled to 10 K in a helium-bath cryostat (Utreks-LSO, Estonia). T − S spectra were constructed from global analysis of the kinetic traces measured at single wavelengths in the Q_Y region. Fits to the experimental data did not take into account the first 0.1 ms after time zero, in order not to include the flash artifact (mainly due to fluorescence decay).

RESULTS

In Figure 3, we have plotted the absorption spectra of RC_{1x} and RC_{2x}, together with that of a control preparation, taken from ref *17*. They are normalized at 624 nm, an isosbestic point of the reaction by which Pheo is transformed into 13^1-OH-Pheo in the RC (*41*). The percentages of Pheo exchange — determined by HPLC — in these particular modified preparations are 40 and 75% (*17*). The amplitude decrease of the absorption band at 543 nm upon Pheo exchange corresponds well with the decrease in Pheo content measured by HPLC, and serves as an internal control for the assumption that the in vitro extinction coefficients at the Q_Y maximum of Pheo and 13^1-OH-Pheo are the same (*17*).

Figure 3 shows that the presence of Pheo in the RC results in absorption between 675 and 685 nm, as this is the

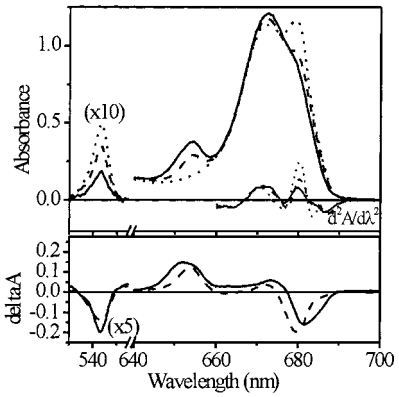

FIGURE 3: (A) Absorption spectra at 6 K (heavy lines) and second derivative of absorption spectra (thin lines) of a control RC preparation (dotted), RC_{1x} (dashed) and RC_{2x} (solid), normalized at 624 nm. The Pheo Q_X region of the spectra was multiplied by 10. (B) Absorption-difference spectra RC_{1x}-minus-control (dashed) and 1.6 × (RC_{2x}-minus-RC_{1x}) (solid), calculated from the absorption spectra in A. The difference-spectrum RC_{2x}-minus-RC_{1x} is multiplied by a factor of 1.6 in order to represent the replacement of the same percentage of Pheo molecules as that in RC_{1x}-minus-control. (The Pheo replacement in RC_{1x} is 40% and in RC_{2x} it is 75%.) The difference spectra were smoothed.

wavelength region where an absorption decrease is observed upon Pheo exchange (*16*, *17*). We consider the difference spectra in Figure 3B as representing the absorption changes due to the exchange of H_B (RC_{1x} minus control) and H_A [1.6 × (RC_{2x} − RC_{1x})]. This assumption is not entirely correct, since only 80% of H_B is exchanged in RC_{1x} (*17*). However, the absorption-difference spectrum representing the exchange of this cofactor contains features, which indicate that not only the absorption of H_B is decreased, but that the absorption of other cofactors in the RC is altered. Thus, the absorption-difference spectrum for the second exchange cannot be corrected, in a straightforward way, to represent solely the exchange of H_A.

Extinction Coefficient of Pheo in Vivo. The total area of the Q_Y absorption in the spectra of Figure 3 (integrated between 640 and 700 nm) does not vary between the control and the two types of modified RCs. Since we have previously determined that the ratio of Chl to total Pheo (Pheo + 13^1-OH-Pheo) is also not significantly affected by pigment exchange (*17*), the invariance of the total Q_Y oscillator strength means that the Q_Y extinction coefficients in the RC (determined from the integrated area of the absorption peak) of 13^1-OH-Pheo and Pheo are the same. (We have observed − see above − that this is also true in solution.) The Q_Y transition of 13^1-OH-Pheo in the PSII RC is, at 6 K, well resolved from those of the other RC pigments, peaking at 653−654 nm (Figure 2) and can be used as a measure of the extinction coefficient of Pheo. Since the extinction coefficient of 13^1-OH-Pheo ($\epsilon_{13(1)-OH-Pheo}$) is equal to that of Pheo (ϵ_{Pheo}), the ratio between the area of the Q_Y absorption of the new pigment [$A_{13(1)-OH-Pheo}$] and the total area of the Q_Y absorption (A_{total}) is related to the ratio $\epsilon_{Pheo}/\epsilon_{Chl}$ according to

$$\frac{A_{total}}{A_{13(1)-OH-Pheo}} = 1 + \frac{n_{Chl}}{n_{13(1)-OH-Pheo}} \times \frac{\epsilon_{Chl}}{\epsilon_{Pheo}} + \frac{n_{Pheo}}{n_{13(1)-OH-Pheo}} \quad (1)$$

where n_X is the number of molecules of "X" per RC. The ratios in (1) that concern pigment content can be determined by HPLC analysis of the RC's pigment extract. We have calculated $A_{13(1)-OH-Pheo}$ in RC_{1x} and in RC_{2x} by integrating the absorption-difference spectra (deltaA) RC_{1x}-minus-control (Figure 2B) and RC_{2x}-minus-control (not shown) between 640 and 660 nm. A_{total} is the total area of the absorption spectrum of RC_{1x} or RC_{2x} between 640 and 700 nm, corrected for the presence of vibrational sidebands in this spectral region (assumed to represent about 28% of the total area, in agreement with what is usually found by Gaussian fitting of the Q_Y absorption band of PSII RCs—see, for example, ref *28*). Taking the values we have reported in ref *17* for the ratios between pigments and the spectral areas determined in this way, we obtain (for both RC_{1x} and RC_{2x}) $\epsilon_{Pheo}/\epsilon_{Chl}$ = 0.43 at 6 K and ∼0.45 at room temperature (from analysis of the spectra of the same samples measured at room temperature, not shown).

The ratio $\epsilon_{Pheo}/\epsilon_{Chl}$ can also be determined by spectroscopic analysis alone, avoiding possible errors in pigment content introduced by HPLC analysis, if we assume that each RC binds 6 Chls and 2 Pheos. This stoichiometry is now generally accepted for the purified PSII RC (*42*). We have confirmed by analytical gel-filtration chromatography (not shown) and by SDS−PAGE (*17*) that the RC preparation from which all the samples analyzed in this report are derived, is highly purified. The ratios $n_{13(1)-OH-Pheo}/n_{Pheo}$ can be determined from the areas of the Pheo Q_X absorption (corrected for the flat underlying absorption due to other pigments) in RC_{1x} and in RC_{2x}, and the ratios $n_{Chl}/n_{13(1)-OH-Pheo}$ using the assumption that $n_{Chl}/(n_{Pheo} + n_{13(1)-OH-Pheo})$ is always equal to 3. In this way, with the same spectroscopic analysis of the Q_Y band as described above, $\epsilon_{Pheo}/\epsilon_{Chl}$ at 6 K is 0.39 for RC_{1x} and 0.41 for RC_{2x}. The difference between these values and those obtained with the combined HPLC and spectroscopic analysis is probably due to an overestimation of the Chl content determined by HPLC analysis of the pigment extract.

Circular Dichroism. Replacement of Pheo with 13^1-OH-Pheo in PSII RCs results in absorption changes at 6 K in the Q_Y region (ref *17* and Figure 3) that can be understood by assuming some extent of electronic coupling between the Q_Y transitions of both Pheos and transitions of (nearby) Chls. Excitonic coupling gives rise to a conservative CD spectrum, with negative and positive bands at the positions of the split electronic transitions (*34*). Thus, if the interactions between cofactors are altered by pigment exchange, this should be reflected in the CD spectrum.

CD spectra measured at 6 K of the two types of modified RCs (RC_{1x} and RC_{2x}) are compared to that of a "control" (see Materials and Methods) in Figure 4. They are normalized to the same RC concentration, which was determined from the absorption of the sample at 624 nm at room temperature (see above).

In the Q_Y region, the CD spectrum of the control preparation peaks at 667 nm (negative chirality) and at 681 nm (positive chirality), with a shoulder at 674 nm, comparable to what was previously reported for nontreated RCs (*9*). Pheo exchange altered the CD spectrum considerably. For both types of modified RCs, the amplitudes of the negative peak at 667 nm and of the positive peak at 681 nm decrease with respect to the spectrum of the control. In RC_{1x}

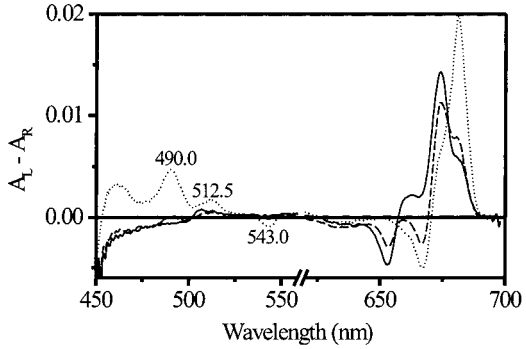

FIGURE 4: CD spectra measured at 6 K of a control RC preparation (dotted), RC_{1x} (dashed) and RC_{2x} (solid). Spectra are normalized on the total RC concentration, determined from the room-temperature absorption spectrum of the corresponding sample.

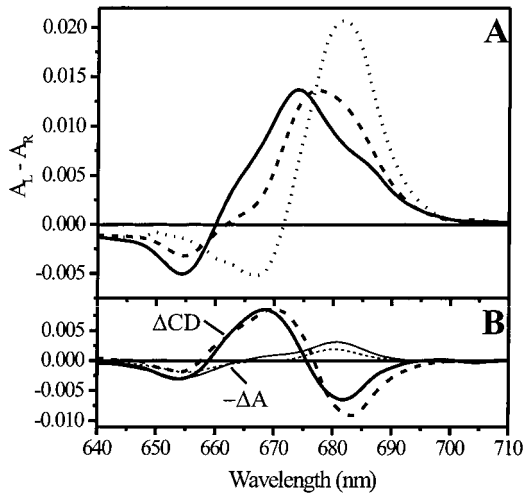

FIGURE 5: (A) CD spectra measured at room temperature of a control RC preparation (dotted), RC_{1x} (dashed), and RC_{2x} (solid). Spectra are normalized on the total RC concentration, determined from the room-temperature absorption spectrum of the corresponding sample. (B) DeltaCD spectra RC_{1x}-minus-control (dashed) and RC_{2x}-minus-RC_{1x} (solid), calculated from the CD spectra in panel A; thinner lines are the corresponding absorption-difference spectra, taken from ref *17*.

and in RC_{2x} the maximum positive CD is at 674 nm, the spectral position of the positive shoulder in the control preparation. Both modified samples exhibit negative chirality at 653.5 nm, approximately the wavelength of maximum Q_Y absorption of 13^1-OH-Pheo. CD and absorption (cf. Figure 3 and Figure 4) of this pigment have also nearly the same shape (CD is nonconservative). The CD decrease at 681 nm and increase at 674 nm correspond roughly to wavelengths at which absorption changes are observed following Pheo replacement − absorption decreases at 680−681 nm and increases between 670 and 675 nm (Figure 3). No absorption changes are observed at 667 nm that could explain the large decrease of negative CD at this wavelength.

The CD spectra of the same samples measured at room temperature change in a similar way (Figure 5A). The peak of positive chirality, at 681.5 nm in the control preparation, shifts to 677 and 674 nm in RC_{1x} and RC_{2x} respectively. The negative peak at 667 nm is not resolved in the CD spectra at room temperature of the two types of modified preparations. In Figure 5B the -deltaA (from ref *17*) and deltaCD spectra (calculated from the spectra in Figure 5A) are plotted. The room-temperature deltaA spectra associated

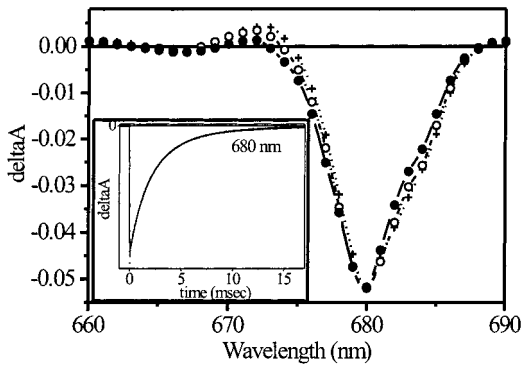

FIGURE 6: T-S spectra at 10 K of the control RC (crosses, dotted line), RC_{1x} (open circles, dash−dot line) and RC_{2x} (filled circles, solid line). The inset shows the kinetics at 680 nm for the control RC and the corresponding fit to a biexponential recovery.

with Pheo exchange apparently indicate simply the disappearance of absorption of Pheo, at ~680 nm (for both types of modified preparations) and the appearance of absorption of 13^1-OH-Pheo at ~654 nm. The negative CD signal at ~654 nm has approximately the shape of the absorption band at this wavelength, and can be interpreted as molecular CD, not originating from excitonic interactions. Between 660 and 675 nm, the increase in positive CD has no counterpart in the absorption-difference spectra and the decrease of the positive CD signal, peaking at ~682 nm, is red-shifted compared to the absorption decrease in this wavelength region. This discrepancy between absorption and CD changes may indicate that both Pheos in the PSII RC take part in excitonic interactions with other cofactors, which are removed by replacing either Pheo with 13^1-OH-Pheo.

The positive peaks at 490 and 512 nm observed at 6 K (Figure 4) probably arise from the β-car present in the RC, which contributes to the absorption at these wavelengths. It is not clear to what extent the 510−515 nm transition of Pheo (see Figure 1) contributes to the CD signal at this wavelength, since it overlaps with the transition of β-car at 508 nm. The Pheo Q_X transition at 543 nm shows nonconservative negative dichroism. After one Pheo exchange, the peaks at 490, 512 and 543 nm practically disappear.

Triplet-minus-Singlet. Laser flash-excitation of PSII RCs produces the charge-separated state $P680^+H_A^-$ that, at low temperatures, recombines with a high yield to the triplet state ($P680^T$) in ~100 ns (*43*). $P680^T$ decays to the ground-state in milliseconds (*43*).

Figure 6 displays the T − S spectra of a control preparation (dotted line, crosses), RC_{1x} (dashed−dotted line, open circles) and RC_{2x} (solid line, filled circles), scaled so that the maximum bleaching is the same for the three preparations. We note that the experimental conditions in which we have measured these spectra do not allow for the determination of triplet quantum yields. The T − S spectrum of the control is similar to those previously reported (*10*, *43*, *44*), with a maximum bleaching at 680 nm, a shoulder at 684 nm, and a small positive peak between 670 and 674 nm. The T − S spectra of the modified samples are very similar and also have a maximum bleaching at 680 nm. As the percentage of Pheo exchange increases, the relative contribution of the 684 nm shoulder decreases slightly and the bleaching progressively broadens to the blue of 680 nm. The amplitude of the positive peak around 673 nm decreases.

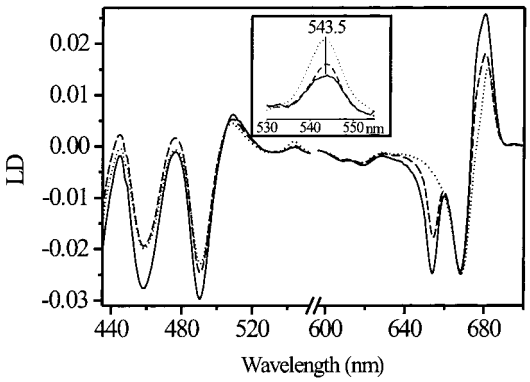

FIGURE 7: LD spectra at 6 K of the control RC preparation (dotted), RC_{1x} (dashed) and RC_{2x} (solid). The inset displays the Pheo Q_X spectral region in an expanded scale.

The T − S spectra were constructed from global analysis of kinetic traces measured in the millisecond time scale at the corresponding wavelengths in the Q_Y region. The inset of Figure 6 displays a characteristic kinetic trace (at 680 nm) and its fit to a biexponential recovery of the initial bleaching. The bleaching at $t = 0$ is due to the formation of $P680^T$ within the instrument response time. All traces could be fit with two time constants, of 1.7 and 4.5 ms, which do not vary significantly between the three samples. A biphasic triplet decay at low temperatures has been observed before in optical measurements in PSII RCs (*44*). The rate constants correspond reasonably well to those observed by millisecond time-resolved EPR (*45*) and ADMR (*46*) for depopulation of the individual triplet sublevels, which are expected to be resolved optically at $T < 30$ K, taking into account that the spin relaxation is probably very slow at these temperatures (*11*). The absorption-difference (deltaA) spectra associated with the two kinetic components are identical, but the amplitudes of the slower component are about half of those of the fast component (not shown). The deltaA spectra at $t = 0.1$ ms are identical to the T − S spectra (not shown). This observation confirms the assignment of the decay-associated spectra to the triplet state in the RC, since this is formed with a 100 ns time constant.

Linear Dichroism. Compression of suspensions of PSII RCs in gelatin gels results in orientation of the RCs with the membrane plane parallel to the direction of expansion, similar to what is observed in larger PSII particles (*18*). LD was measured as the difference $A_{||} - A_{\perp}$ with respect to the direction of gel expansion. Thus, transitions oriented at an angle smaller than 54.7° with respect to the normal to the membrane plane will show negative LD, and those oriented more close to parallel to the membrane plane (at an angle larger than the magic angle) will show positive LD (*34*).

Figure 7 displays the LD spectra of the control sample (dotted line), RC_{1x} (dashed line) and RC_{2x} (solid line), measured at 6 K. They are normalized at 608 nm, also an isosbestic point of pigment exchange (*17*). The spectrum of the control is similar to those previously reported for PSII RCs (*9,18*).

The Pheo Q_X transition has positive LD, peaking at the same wavelength as the corresponding absorption maximum (543 nm). The LD spectrum at this wavelength has the shape of the absorption band (cf. Figures 3 and 7). Alternating positive and negative bands are observed in the spectral region where β-car absorbs: 444 nm (+), 458 nm (−), 475.5 nm (+), 490 nm (−), and 508 nm (+). The positions of these bands are not affected by Pheo exchange, although their intensities slightly are. The positive LD at 543 nm decreases upon Pheo exchange, in a manner similar to what is observed in the absorption spectrum (cf. Figure 3 and Figure 7).

In the Q_Y region, the LD spectrum of the control shows positive and negative bands at 681.5 and 667.5 nm, respectively, and a positive shoulder at ~677 nm, and is comparable to previously reported spectra (*9, 18*). In RC_{1x} and in RC_{2x}, the Q_Y transition of 13^1-OH-Pheo shows negative LD, and the amplitude of this peak is higher for RC_{2x} than for RC_{1x}. In the spectral region where the native RC pigments absorb, the positive LD increases at the wavelengths where absorption changes are also observed (cf. Figure 3 and Figure 7). The amplitude of the negative signal at 667.5 nm is practically unaffected by Pheo replacement. The maximum positive LD, at 681.5 nm in the control, shifts to 680.0 nm in the samples with modified Pheo composition and its amplitude increases, probably reflecting the disappearance of transitions with negative LD following exchange of both H_B and H_A.

DISCUSSION

In a previous report (*17*), we have shown that both Pheos in the PSII RC contribute to the absorption spectrum at wavelengths around 680 nm, and we suggested that they might be involved to some extent in excitonic interactions with Chls. These two conclusions were based on the effects on the 6 K absorption spectrum of the replacement of Pheo with 13^1-OH-Pheo, both in the active and in the inactive site. Here we present results from other optical spectroscopies that provide a much more complete characterization of the modified RCs. Firm conclusions can now be drawn on the interactions of the natural Pheos with the protein environment and with the other RC cofactors, and on the orientations of their optical transitions with respect to the photosynthetic membrane plane.

Extinction Coefficient of Pheo in Vivo. We have determined that in the PSII RC the extinction coefficient of the Q_Y absorption of Pheo is 40−45% of that of Chl. In solution, values of $\epsilon_{Pheo}/\epsilon_{Chl}$ between 0.50 and 0.64, depending on the solvent, were reported when the Pheo spectrum was measured in the presence of small amounts of HCl (*47*), and Eijckelhoff and Dekker (*42*) measured a ratio of 0.68 in a neutral solvent. It should be noted that although the ratio in the RC was calculated from integration of the absorption peak while the ratio in solution was calculated from the absorbance at the peak maximum, the comparison is valid since the peak widths in solution are the same for Pheo and Chl (*42*).

In the RC of the purple bacterium *Rhodobacter sphaeroides*, the Q_Y absorption of both BPheos is decreased relative to what is determined in solution (see, for example, ref *48*). This effect is also observed for bacteriochlorophyll *a* (BChl), when this molecule is incorporated in the position of the photoactive BPheo (*48*). On the other hand, when BPheo occupies the binding site of the accessory BChl in the active branch, its Q_Y absorption relative to that of BChl is again comparable to what is observed in solution (*49*). Apparently, the decrease in the Q_Y oscillator strength is protein-induced and specific for the BPheo binding pockets, although its

origin is not known (*49*). Franken et al. (*50*) have suggested that in *R. sphaeroides* RCs containing Pheo instead of BPheo the macrocycle of the molecule incorporated in the pocket of the primary electron acceptor might be distorted from planarity. Such a distortion decreases the amplitude of the vibrational sideband at ~511 nm, and might cause a decrease of the $Q_Y(0-0)$ band also. But this effect can have other causes, for example, interactions of the pigment's conjugated π-system with amino acid side chains of the protein.

Similar pigment−protein interactions in the binding pockets of Pheo in the PSII RC may be expected. The model based on X-ray crystallography at 3.8 Å resolution that was recently published by Zouni et al. (*31*) places both Pheos at the same positions as those of the BPheos in the RC of *Rhodopseudomonas viridis*, and the glutamic acid residue − GluL104 − that is hydrogen bonded to the 13^1-C═O group of BPheo is conserved in the amino acid sequence of the D1 protein, as D1-Glu130 (*51*). The decreased Pheo Q_Y absorption in the PSII RC is therefore not unexpected, in view of the structural analogy between the RCs of purple bacteria and PSII.

Interactions between Cofactors. The nature and strength of the electronic interactions of either Pheo with the other RC pigments have not been established, although Tetenkin et al. (*21*) have suggested that the active Pheo might take part in electronic interactions with the remaining five "core" pigments and, possibly, with β-car. Both Pheos and the four "core" Chls have been assumed to form a weakly coupled "multimer", in a model that attempts to explain the Q_Y absorption properties of the RC (*15*, *36*).

The CD signal from the chlorin molecules in the RC is either protein-induced (nonconservative) or results from excitonic interactions between pigments (conservative), since isolated Chl in solution is practically achiral (*52*). However, the origin of the CD spectrum of the PSII RC is still unclear, in part due to the indetermination introduced by the overlap of electronic transitions in the red-most region. The CD spectrum of PSII RC has previously been interpreted as resulting from a dimeric nature of P680 (with weak coupling) (*20*). Tetenkin et al. (*21*) measured the dependence of the CD spectrum on the redox state of H_A, and concluded that at least this molecule has a contribution to the CD at 680 and 667 nm that results from excitonic interactions with other RC pigments. Otte et al. (*9*) noted that the shape of the CD spectrum suggests conservative contributions by the main absorption bands in the Q_Y region, although the overall spectrum shows a net positive chirality. The latter may also point to some delocalization of excitonic states.

Excitonic CD is sensitive to the relative orientations and distances between the transition dipole moments of the chromophores. On the other hand, pigment−protein interactions may be responsible for molecular CD, which originates from a protein-induced chirality of the chromophore itself and is nonconservative. Alteration of the protein structure due to the pigment exchange procedure could be responsible for the different CD spectra of the modified preparations. However, we have verified, by comparing a nontreated preparation to a control (not shown), that no significant changes occur in the CD spectrum when PSII RC is subjected to the same treatment as that applied for Pheo exchange, but in the absence of the extraneous

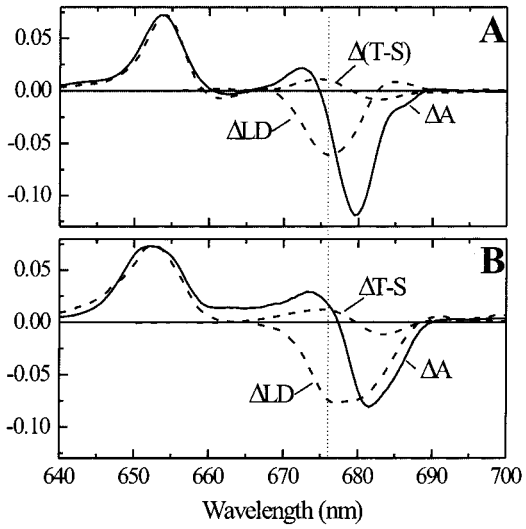

FIGURE 8: Comparison of the deltaA (solid line), deltaLD (dotted line), and delta(T − S) (dashed line) spectra associated with the replacement of $Pheo_1$ (A) and $Pheo_2$ (B). See text for details on the scaling procedure.

pigment. The main difference that seems to result from that treatment is that the intensity of the signal in the blue-green region is higher in the spectrum of the control, paralleling the previously observed higher relative absorption in that region (*17*). Similarly, the LD spectrum of the control does not differ significantly from that of a nontreated preparation (not shown), indicating that the orientations of the Q_Y transitions of the cofactors do not change as a consequence of the pigment exchange procedure.

(i) 13^1-OH-Pheo. In solution, CD of 13^1-OH-Pheo (not shown) has comparable intensity to that of Chl, much smaller than what is generally measured in Chl-protein complexes. In RC_{1x} and RC_{2x}, both at room temperature and at 6 K, 13^1-OH-Pheo displays a negative, nonconservative, band at 653−654 nm. This optical activity can be interpreted as arising from interactions of the new pigment with the protein environment. However, 13^1-OH-Pheo lacks the 13^1-C═O functional group, which can assume hydrogen-bonding interactions. Even if it is incorporated in the same position as the Pheo molecule it replaces, it must interact with the protein in a slightly different manner than Pheo. Thus, the CD properties of 13^1-OH-Pheo allow no conclusions on the optical activity of the original Pheo molecules.

The Q_Y transition of 13^1-OH-Pheo is expected to be localized on this pigment and not delocalized on Chls, since its site energy is considerably different from that of the Chls (654 vs 668 nm or longer wavelengths). The nonconservative CD signal from the new pigment (Figures 3 and 4) confirms this expectation, and suggests that 13^1-OH-Pheo incorporated in the RC does not take part in significant excitonic interactions with other cofactors. However, we cannot exclude that the positive feature that appears at ~662 nm in the CD spectra of the modified samples is not related to the new pigment.

(ii) 670-nm-Absorbing Pigments. Figure 2 (see also ref *17* and Figures 7 and 8) clearly shows that the absorption at 670 nm is not affected by Pheo exchange. The Chls that absorb at this wavelength do not interact with either of the Pheos. They probably show no CD, and their Q_Y transitions

are oriented close to perpendicular to the membrane plane (Figure 7, see also below). The large amplitude of the absorption at 670 nm indicates that at least 2 Chl molecules must absorb at this wavelength. These are probably the "distant" Chls, bound to His118 on D1 and D2 (*13*) — $Chla_A$ and Chl_B in Figure 2.

(iii) 680-nm-Absorbing Pigments. Replacement of Pheo with 13^1-OH-Pheo leads to complex changes in the CD spectrum in the Q_Y wavelength range: both the positive band at 681 nm and the negative band at 668 nm have decreased intensity in RC_{1x} and in RC_{2x} (Figures 4 and 5), and the deltaCD spectra at room temperature show features that are not observed in the deltaA spectra (Figure 5B). These observations cannot be interpreted as originating from the disappearance of nonconservative contributions from the Pheo molecules. Thus, at least in part, the optical activity of both Pheos results from excitonic interactions with other RC cofactors.

The CD and absorption spectra of the two types of modified RCs show that the transitions resulting from electronic interactions involving Pheo molecules peak at 681 and 668−670 nm, the latter carrying very little oscillator strength, since no absorption decrease is observed at this wavelength following Pheo exchange (ref *17* and Figure 3). After Pheo exchange, the increase in CD and absorbance around 674 nm can be understood as resulting from an adjustment of the Q_Y electronic structure to account for the "missing" electronic transition(s). Interestingly, replacement of BPheo with Pheo in *R. sphaeroides* R-26 RCs also results in complex CD changes in the Q_Y region (*50*). However, the negative contributions to the CD spectrum from both BPheos are at approximately their respective absorption positions. The replacement of BPheo in these RCs induces a blue shift of the absorption of the accessory BChls (B_A and B_B), which is also seen in the CD spectrum, but it does not strongly affect the CD bands related to the low and high energy excitonic transitions of the primary donor (*50*).

The disappearance of CD in the blue-green spectral region that results from the exchange of H_B suggests that this molecule interacts with β-car. However, some caution should be exerted, since we have also observed that the CD spectrum in this region of the nontreated preparation is much less intense than that of the control, an indication that the pigment exchange procedure may affect the spectroscopic and/or structural properties of the β-car in the RC. The ratio of Chl to β-car, determined by HPLC analysis (Table 1 in ref *17*) is not significantly affected by the exchange of H_B, although we do observe a slight increase in the relative amount of β-car in RC_{2x}, which could explain the increased negative amplitude of the LD spectrum of this preparation in the region between 440 and 490 nm.

Effect of Pheo Exchange on Triplet States. It has been shown by absorbance-detected magnetic resonance (ADMR) spectroscopy at low temperatures that there are at least three different populations of triplet-carrying Chl molecules in the PSII RC, absorbing between 677 and 685 nm (*10*, *53*). Freezing in different conformations of the same molecule may induce this heterogeneity and/or it may represent intrinsically different possibilities for the location of the triplet state in energetically different Chl molecules. The major contribution to the T − S spectrum is from (a) Chl spectral form(s) absorbing at ∼680 nm. This Q_Y transition moment was shown to be oriented close to the magic angle relative to the normal to the membrane plane (*10*), and the plane of the molecule's macrocycle makes an angle of 30° with the membrane plane (*32*). It was suggested that this Chl is equivalent to one of the accessory BChls in the purple bacteria RC (*32*). Since the structure of PSII RC is comparable to that of the purple bacteria RC (*30*, *31*, *51*), then a Pheo molecule will be close to the triplet-carrying molecule. We have shown (see above and ref *17*) that both Pheos interact with nearby Chls and that their replacement with 13^1-OH-Pheo results in a modification of the absorption spectrum of Chls. It is then expected that the T − S spectra of the modified RC preparations will reflect the modified spectral properties of the Chl(s) that carries the triplet state.

The reduction potential of 13^1-OH-Pheo in solution is estimated to be ∼300 mV more negative than that of Pheo (*16*). Thus, it is expected that the energy level of $(P680)^+(13^1\text{-OH-Pheo})^-$ will be above P680* and this radical pair will not be formed. Hence, no triplet formation via recombination from that radical pair will occur. This prediction is consistent with the observed absence of features at ∼654 nm in the T − S spectra of the modified RCs (not shown).

The T − S spectra of RC_{1x} and RC_{2x} are both very similar to that of the control (and of a nontreated) preparation (Figure 6). Small differences are observed at ∼684 nm and at ∼674 nm. It has been previously reported that the 684-685 nm feature in the T − S spectrum is sensitive to the pigment composition of the RC: the absorption at 4 K and relative contribution to the T − S spectrum at 77 K and at 1.2 K are more pronounced in a 5-Chl RC (*28*, *53*). Replacement of H_B with 13^1-OH-Pheo affects the intensity of this transition in the opposite way. However, the oscillator strength of the absorption at 684 nm is too low to represent one Chl molecule (see, for example, ref *28*), so that this transition probably results from excitonic interactions.

The triplet state of P680 in RC_{1x} has also been studied by direct-detection EPR (I. Proskuryakov, personal communication) at 30 K. A similar electron spin polarization pattern and zero-field splitting parameters indistinguishable from those measured in nontreated RCs were observed, indicating that $P680^T$ is formed by recombination from the same radical pair as in nontreated RCs and that the nature and structure of P680 are not influenced by the exchange of H_B.

In summary, H_B exchange does not affect significantly the absorption spectrum of the Chl molecule where the triplet state is mainly localized, confirming that this is B_A rather than B_B. The small differences between the T − S spectra, however, suggest that the distribution of intensities of the electronic transitions in the 680 nm-absorbing pigment pool is altered upon Pheo exchange. Recent results from time-resolved infrared spectroscopy are in agreement with the location of the triplet state in B_A (*54*).

Orientation of Optical Transitions. (i) 13^1-OH-Pheo. It is evident from Figure 6 that the Q_Y transitions of 13^1-OH-Pheo — incorporated either in the inactive (RC_{1x}) or in the active (RC_{2x}) site — are close to perpendicular to the membrane plane. The orientations of their Q_X transitions, at 490−500 nm, are difficult to determine, since they overlap with transitions of β-car. Shkuropatov et al. (*41*) have observed in situ transformation (by addition of sodium borohydride) of Pheo in 13^1-OH-Pheo in PSII RCs (limited

to the inactive site). This result suggests that the new pigment can indeed occupy the Pheo position in a similar orientation as that of the molecule it replaces. Thus, the LD of 13^1-OH-Pheo indicates that the (noninteracting) Q_Y transitions of Pheo in the RC are close to perpendicular to the membrane plane.

(ii) Peripheral Chls. Breton (*18*) ascribed the negative LD at ~668 nm to Chl molecules that have lost their native orientation due to effects of the detergent used for purification of the RC. However, the amplitude of this negative LD peak in the two modified preparations does not differ significantly from that of the control. If the LD at 668 nm would be related to Chl molecules in nonnative orientations, then its amplitude would be expected to vary between preparations. The invariability of the LD at 668 nm upon Pheo exchange can best be explained by assigning it to Chl molecules that do not interact significantly with Pheo and absorb at ~670 nm. [No absorption changes occur at this wavelength upon Pheo exchange (see ref *17* and Figure 3).] These Chls are probably the two "distant" Chls, bound to His118 of D1 and D2 (see above), and we can conclude that their Q_Y transitions are oriented close to perpendicular to the membrane plane. This orientation is different from what is generally observed for antenna pigments (*34*). However, it has been shown (*13*) that Chls absorbing at ~670 nm transfer energy to the 680 nm pigment pool, with a time constant of ~30 ps. This energy transfer process is very efficient, as the fluorescence excitation spectrum (with detection to the red of 680 nm) matches the 1-transmittance spectrum (*55*). Since energy transfer is inefficient when the transition moments of the two states involved are perpendicular to each other, it follows that the most probable acceptors in the energy transfer process from the 670 nm absorbing pigments are more likely Pheo rather than the Chls responsible for the positive LD at 680 nm.

(iii) β-Carotene. The intensities of the β-car LD peaks, between 440 and 510 nm, seem to be slightly affected by the replacement of Pheo with 13^1-OH-Pheo, although the increased amplitude of the LD signal in this spectral region after the second Pheo exchange treatment is probably due to the slightly increased β-car content in RC_{2x} (*17*). The pattern of alternating positive and negative peaks in this wavelength region is remarkable, considering the absorption spectrum of β-car in the RC, with a peak at 490 nm and shoulders at ~508 nm and ~458 nm at low temperatures (see, for example, ref *17*). The positive and negative LD peaks have been interpreted as either arising from excitonic interactions (*56*) or reflecting the presence of two spectrally and structurally distinct β-car molecules, with opposite LD (*9*, *18*, *57*). The positions of the absorption peaks (*17*) and of the peaks in the LD spectra (Figure 7) are not affected by Pheo exchange (*17*). If the alternating positive and negative bands in the latter spectra would arise from excitonic interactions of β-car with Pheo, then more drastic changes would be expected following pigment exchange. Furthermore, the energy differences between the three positive peaks and the two negative peaks are all in the order of 1300−1400 cm^{-1}, corresponding to the vibrational pattern expected for the absorption of a noninteracting all-trans β-car molecule in this spectral region (*58*). Thus, the LD spectrum between 440 and 510 nm probably arises from *two* spectrally distinct forms of β-car, in agreement with the conclusions in (*9*, *18*, *57*). The transition moments of the two β-car have opposite LD, with the molecule that absorbs at 490 nm close to perpendicular to the membrane plane. By measuring the anisotropy of fluorescence excitation, Kwa et al. (*55*) have determined that this β-car molecule is also close to perpendicular to the Q_Y transition(s) of the emitting pigments, which are tilted only slightly out of the membrane plane (*55*, *57*). We note that such a carotenoid has no counterpart in the RC of purple bacteria.

(iv) Pheophytins. Results from LD of $Pheo^-$ photoaccumulation suggest that H_A contributes to the absorption at 680 nm and that its Q_Y transition is oriented close to perpendicular to the membrane plane (*18*, *19*). Since the orientation of the Pheo Q_X transition is close to parallel to the membrane plane, it was concluded that the macrocycle plane of the molecule is approximately perpendicular to the membrane plane (*18*, *19*), analogous to the position of $BPheo_A$ in the structure of the purple bacteria RC and in agreement with the present knowledge, from X-ray and electron crystallography, of the PSII RC structure (*30*, *31*). A similar orientation was recently reported by Dorlet et al. (*59*), who measured high-field EPR of the H_A^- anion radical in oriented BBY particles. The angle between the ring plane of H_A and the membrane plane was determined to be 70°, with the Q_X transition probably at an angle of about 2° with respect to the membrane plane (*59*). Ganago et al. (*19*) observed that H_A^- formation induces a blue-shift of a transition at 680 nm oriented close to parallel to the membrane plane.

By comparison of the LD spectrum of RC_{1x} and that of the control preparation (Figure 7) it is evident that H_B (the "inactive" Pheo) has a Q_X transition close to parallel to the membrane plane and contributes to a Q_Y transition oriented close to perpendicular. Thus, the plane of its macrocycle is close to perpendicular to the membrane plane. The differences between the LD of RC_{2x} and of RC_{1x}, which result from replacement of the "active" Pheo, indicate that H_A is also close to perpendicular to the membrane plane. These observations are in agreement with the conclusions from the optical photoaccumulation experiments (*18*, *19*) and from the EPR measurements (*59*).

The changes in the LD spectrum upon Pheo replacement can be better visualized by computing the deltaLD. In Figure 8, we have plotted the difference spectra associated with the exchange of H_B (Figure 8A) and H_A (Figure 8B) in the Q_Y spectral region. The deltaA spectra (solid lines) are taken from Figure 3, and are normalized to represent each the exchange of one Pheo molecule per RC (as explained in ref *17*, and see also the legend to Figure 3). The deltaLD spectra (dotted lines), calculated from the LD spectra in Figure 7, are mutually normalized in the same way, multiplied by −1, and scaled so that the peak at 654 nm in the upper panel has the same amplitude as the deltaA spectrum (both deltaLD spectra were multiplied by the same factor in this scaling). The delta(T − S) spectra (dashed lines) are calculated from the T − S spectra in Figure 6, after normalization to the deltaA spectra such that the area of their main bleaching, centered at 680 nm, represents the absorption of one Chl molecule (1/0.4 times the integrated absorption of 13^1-OH-Pheo).

(v) H_B. The deltaLD spectrum in Figure 8A strongly suggests that the new negative LD band of 13^1-OH-Pheo at 654 nm simply replaces a band of similar shape at 676 nm due to H_B. The latter band may well be present in the deltaA spectrum, if superimposed on a blue-shift of a Chl Q_Y

transition at 680 nm that has little or slightly positive LD. Since no corresponding shift is seen in the T − S spectrum and H_B substitution does not affect photoaccumulation of H_A^-, the Chl whose absorption undergoes a blue-shift is most likely B_B, corresponding to the accessory BChl in the inactive branch of the bacterial RC. This interpretation suggests that the triplet state is localized on B_A, corresponding to the bacterial "B_A" in the active branch. Other than the small blue-shift of the 685 nm transition (*17*), the deltaA and deltaLD spectra do not show much evidence for involvement of the inactive Pheo in excitonic interactions. However, the deltaCD spectra appear to contain a substantial contribution from conservative CD. In fact, no absorption changes are observed at ∼670 nm, while the CD signal changes from negative to positive at this wavelength.

(vi) H_A. The deltaLD spectrum in Figure 8B associated with replacement of H_A is more complicated. Its shape in the wavelength region where the native pigments absorb suggests contributions by two transitions with maxima near 676 and near 681 nm. Their combined area is obviously larger than that under the 13^1-OH-Pheo peak at 652 nm. The deltaA spectrum is similar to that in Figure 8A, suggesting an analogous composition of a bleaching of H_A Q_Y absorbance and a blue-shift most likely dominated by the "B_A" Chl, which appears at about 2 nm longer wavelengths than in the deltaA spectrum of H_B replacement. The absence of a corresponding shift in the T − S spectrum in this case may be attributed to absence of charge separation and triplet formation in those centers where H_A has been exchanged.

The fact that the LD in the 670−685 nm region increases more than can be explained by the removal of the negative H_A contribution suggests that the absorption shift attributed to the "B_A" Chl involves a rotation of its transition moment toward the membrane plane. This interpretation implies that the absorption is associated with an exciton state at 681 nm containing a significant contribution by the perpendicular H_A, and is replaced by an exciton state containing a larger contribution at ∼675 nm by the parallel transition moment of one or both of the "special pair" Chls. The bleaching of the 685 nm transition in the deltaA spectrum, observed in the T − S spectrum as well, points to a change in excitonic interactions since this band is much too small to represent a localized Chl Q_Y transition. Thus, it appears that excitonic interactions play a more significant role in the active than in the inactive branch of the PSII RC. This asymmetry indicates that the assumption of equal site energies, equal distances and equal interaction energies between the pigments in the active branch and those in the inactive branch in the "multimer model" proposed by Durrant and co-workers (*15*, *36*, *60*) is an oversimplification.

The wavelengths at which H_A contributes to the absorption are in agreement with previous results obtained by other methods (*61*, *62*).

The net LD of all pigments contributing to the 680 nm absorption band of the PS II RC is positive. Also in uniaxially pressed gels a predominantly in-plane orientation of 680 nm absorption (and emission) is observed (*57*). Since the contribution of both Pheos is perpendicular and that of both "accessory" Chls is near the magic angle, the contribution of the Chls that correspond to the bacterial special pair must be responsible for the net in-plane orientation of 680 nm absorbance. This conclusion is consistent with the current view that the pigment arrangement and orientations in the PSII RC is similar to that in purple bacteria, apart from a larger separation of the "special pair" Chls.

CONCLUSIONS

We present a characterization of the two Pheo molecules in RCs of PSII made possible by selective sequential replacement of Pheo with 13^1-OH-Pheo. Their properties and interactions with the other cofactors in the PSII RC are summarized as follows:

(i) The protein environment of both Pheos causes a decrease of their Q_Y absorption (relative to the absorption of the Chls) in comparison to that in solution. The decrease is analogous to that observed for the BPheos in the purple bacteria RC (*48*,*49*). Its origin is not known.

(ii) The presence of two spectrally different β-car molecules with opposite LD is confirmed: their LD does not depend on interactions with Pheo.

(iii) Pheo exchange does not affect the spectroscopic properties of the RC around 670 nm. This result rules out suggestions that the inactive Pheo absorbs in this wavelength region (*23*−*25*, *61*).

(iv) The Chl molecules that absorb at 670 nm have Q_Y transitions oriented close to perpendicular to the membrane plane, and are probably the two "distant" Chls, bound to His118 in D1 and D2, that transfer energy in 30 ps to the 680 nm pigment pool (*13*).

(v) Replacement of either Pheo disturbs the excitonic interactions between the RC's central cofactors, as indicated by large changes of the CD spectrum, but the absorption and LD spectra clearly reflect that perturbation only in the case of the active Pheo.

(vi) Replacement of the *inactive* Pheo removes a single Q_Y transition at 676 nm with perpendicular orientation and causes a blue-shift of a ∼680 nm transition oriented near the magic angle, attributed to the adjacent "accessory" Chl, B_B, in the inactive branch.

(vii) Replacement of the *active* Pheo prevents triplet formation and H_A^- photoaccumulation, causes an LD increase at 676 and 681 nm, a blue-shift of ∼680 nm absorption, and a bleach of the 685 nm exciton band. Disappearance of the negative Pheo contribution cannot account for the total LD increase, indicating that the remaining absorption becomes oriented more in-plane.

(viii) $P680^T$ is localized on the "accessory" Chl in the active branch, B_A.

(ix) The orientation of the Q_Y transition(s) of one or both "special pair" Chls absorbing at ∼680 nm is close to the membrane plane. It is little affected by Pheo substitution.

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