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Pheophytin–Protein Interactions in Photosystem II Studied by Resonance Raman Spectroscopy of Modified Reaction Centers[†]

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ABSTRACT: Soret-excited resonance Raman spectra of two types of pheophytin-exchanged photosystem II RCs are reported. The cofactor composition of the reaction centers was modified by exchanging pheophytin *a* for 13¹-deoxo-13¹-hydroxypheophytin *a*, yielding one preparation with selective replacement of the photochemically inactive pheophytin (H_B) and a second one exhibiting total replacement of H_B and 40% replacement of H_A, the primary electron acceptor. Resonance Raman spectra indicate that the other bound cofactors present are not significantly perturbed by Pheo substitution. The resonance Raman contributions from H_A and H_B in the carbonyl stretching region are identified at 1679 and 1675 cm⁻¹, respectively, indicating that both pheophytin molecules in the photosystem II reaction center have hydrogen-bonded keto-carbonyl groups. This conclusion differs from what is observed in the functionally related RCs of purple non-sulfur bacteria, where the keto-carbonyl group of H_B is not hydrogen bonded, but confirms predictions from models based on protein sequence alignments.

In photosystem II (PSII)¹ water is converted to molecular oxygen, the ultimate oxidized product of a series of electron-transfer reactions that take place in the PSII reaction center (RC). Primary electron transfer occurs between the singlet excited primary electron donor, P680, and the primary electron acceptor, a pheophytin *a* (Pheo) molecule (see ref 1 for a review on PSII function). The PSII RC is a transmembrane pigment-protein complex with a cofactor composition of six chlorophyll *a* (Chl) molecules, two Pheo molecules (of which only one is photochemically active (2)), two β -carotene (β -car) molecules, and at least one copy of cytochrome *b*₅₅₉ (cyt *b*₅₅₉) (2, 3).

The recently determined structure of a PSII core complex from the cyanobacterium *Synechococcus elongatus* (3) has revealed an arrangement of the cofactors in the RC very similar to that in RCs of purple non-sulfur bacteria. However, at the limited resolution of 3.8 Å the structure does not provide information on the orientations of the chlorins' electronic transitions or on the interactions of the cofactors with the protein. In addition, the β -car molecules could not be resolved (3). Interactions between protein side chains and pigment functional groups that are part of the conjugated double bond system are expected to play a role in modulating the electronic and redox properties of the cofactors (4), critically important factors that can determine their different functions in the protein.

In the absence of detailed structural information, several approaches have been used to probe the pigment-protein interactions in PSII. Models based on the crystallographic structure of RCs from purple non-sulfur bacteria (5–7) are now supported by the work of Zouni et al. (3). All models predict hydrogen-bonding interactions between the protein and several functional groups on both the active and the inactive Pheo; in particular, the keto-carbonyl groups of these two molecules are suggested to be hydrogen-bonded to, respectively, D1-E130 and D2-Q130 in spinach (6). The residues lining the binding pockets of the Chl's corresponding to the primary donor in bacterial RCs (P_A and P_B) are predicted to be hydrophobic and nonpolar (6, 7). No hydrogen-bonding interactions are expected between the protein and keto-carbonyl groups of either P_A or P_B (6).

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¹ Abbreviations: PSII, photosystem II; RC, reaction center; Pheo, pheophytin *a*; 13¹-OH-Pheo, 13¹-deoxo-13¹-hydroxy-pheophytin *a*; Chl, chlorophyll *a*; β -car, β -carotene; cyt *b*₅₅₉, cytochrome *b*₅₅₉; P680, primary donor of photosystem II; H_A, photochemically active pheophytin; H_B, photochemically inactive pheophytin; P_A, "primary donor" Chl bound to the D1 protein; P_B, "primary donor" Chl bound to the D2 protein.

The binding-site properties of the photochemically active Pheo (H_A) have been studied by various spectroscopic approaches (8–10), all of which provide evidence for the existence of a hydrogen-bonded 13^1-C=O group in this cofactor. Giorgi et al. (11) have performed site-directed mutagenesis of the D1-130 residue in *Synechocystis* 6803, and showed that the Q_X absorption maximum of H_A and the yield of the primary charge separation reaction are modulated by the polarity of the amino acid side chain at that position (11). Recently, Dorlet et al. (12), using a combination of high-field EPR and site-directed mutagenesis in the green alga *Chlamydomonas reinhardtii*, have obtained convincing evidence that the hydrogen bond of 13^1-C=O in H_A is to the residue at D1-130. In a strain of cyanobacteria (*Synechococcus* sp. PCC 7942) there are two genes encoding for the D1 protein (13), which differ at 25 amino acids including the one at position D1-130, and the two different forms of D1 are correlated with differences in photochemical yield (14). These observations stress the importance of this Pheo-protein interaction in optimizing PSII function.

Direct experimental information on the binding of the inactive Pheo (H_B) is unavailable. In ref 9 it was suggested, by analogy to the RCs of purple bacteria, that the keto-carbonyl group of H_B is not hydrogen bonded to the protein. It has been demonstrated that in purple bacterial RCs the asymmetry in the interactions between the keto-carbonyl of the (bacterio)Pheo's and the protein results in different Q_X absorption bands, although these pigment-protein interactions play no direct role in the unidirectionality of electron transfer (15). In PSII RCs there is no splitting of the Pheo Q_X absorption, even at low temperatures (16). In addition, both the currently available structural models (5–7) and the protein sequence alignments on which they are partly based (e.g., ref 17) predict that, in PSII, the keto-carbonyl group of H_B is in fact hydrogen bonded to the residue at D2-130 (a glutamic acid in spinach (6, 17)).

The spectroscopic approaches used so far to study pigment-protein interactions in the PSII RC only allow the observation of reaction-induced changes in spectroscopic properties. For example, the binding of H_A has been studied by photoaccumulation of the H_A^- radical, and either looking at its properties directly using EPR methods (8, 12) or analyzing the difference FTIR or Raman spectrum ($H_A^- - H_A$) (9, 10). One way of probing photochemically inactive cofactors is by their selective exchange for another pigment with different, known properties (for a review on pigment exchange in photosynthetic systems see ref 18). We have recently described a method for selective sequential replacement of the Pheo's in the PSII RC (19). This method allowed us to obtain information on the contributions of each Pheo to the optical spectra of the RC (20). The pigment used to replace Pheo in the modified RCs was $13^1\text{-deoxy-}13^1\text{-hydroxypheophytin } a$ (13^1-OH-Pheo), which is spectrally distinguishable from Pheo in the Q_Y , Q_X , and Soret absorbance regions (Figure 1), differing by the absence of the keto-carbonyl group at position 13^1 in ring V (see the inset in Figure 1).

High-resolution structural information about pigment-protein complexes can be obtained by vibrational spectroscopy. In particular, resonance Raman can be used to probe selectively the binding of cofactors that have specific electronic transitions (see ref 21 for a review on the

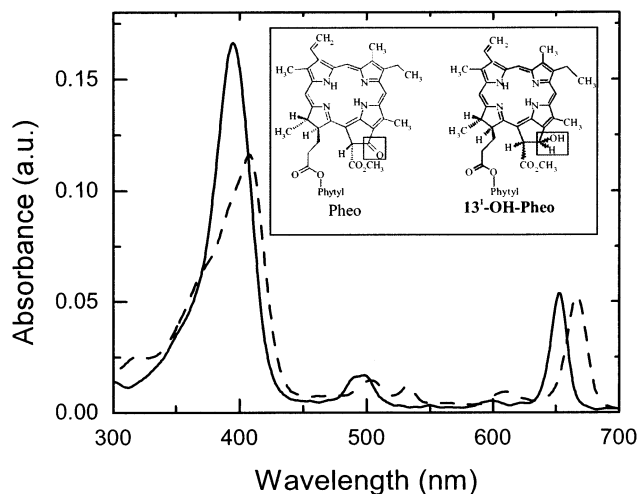


FIGURE 1: Room-temperature absorption spectra of Pheo (dashed line) and 13^1-OH-Pheo (solid line) in diethyl ether. The inset shows the molecular structure of each pigment.

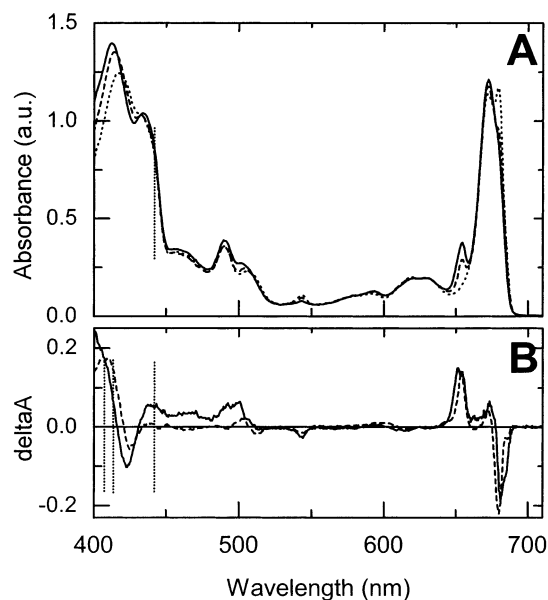


FIGURE 2: (A) Absorption spectra at 6 K of nontreated RC (dotted), RC_{1x} (dashed), and RC_{2x} (solid), normalized at 624 nm. (B) Absorption difference spectra associated with the exchange of H_B (dashed) and of H_A (solid), calculated from the spectra in (A) (adapted from ref 20). The vertical dotted lines are at the wavelengths used for obtaining the resonance Raman spectra.

applications of resonance Raman spectroscopy in photosynthesis). Carbonyl groups that are part of the conjugated double bond system in chlorin cofactors (13^1-C=O in Chl and Pheo) give rise to resonance Raman bands with frequencies that are very sensitive to the nature of the molecule's local environment.

In this work we present a characterization by resonance Raman spectroscopy of two types of pheophytin-exchanged PSII RCs. In the first type, $RC_{1x}\text{-}H_B$ has been replaced selectively with 13^1-OH-Pheo . In the second type $RC_{2x}\text{-}H_B$ and a substantial percentage of H_A have been exchanged for the same pigment. We make use of the different optical (Figures 1 and 2) and molecular (Figure 1, inset) properties of Pheo and 13^1-OH-Pheo to assign the resonance Raman contributions of H_A and H_B in the PSII RC.

MATERIALS AND METHODS

PSII RCs were purified according to van Leeuwen et al. (22) from Tris-washed spinach "BBY" grana membranes (23). ^{13}C -OH-Pheo was purified from nettle as in Shkuropatov et al. (24). RC_{1x} and RC_{2x} were prepared as described previously (19). Pheo exchange was monitored by the decrease of the Pheo Q_x absorption band at 543 nm and the appearance of a peak around 654 nm, which both indicate incorporation of ^{13}C -OH-Pheo (Figure 2). The pigment composition of the modified RCs was determined by HPLC analysis of an acetone/methanol extract, as described previously (19). The total yields of Pheo exchange were 41% for RC_{1x} (corresponding to 82% exchange of H_B) and 70% for RC_{2x} (corresponding to 100% exchange of H_B and 40% exchange of H_A). The β -car content was unaltered by the exchange procedure. All preparations (untreated RC, RC_{1x}, and RC_{2x}) contained about 1.6 β -car's per 2 Pheo's. Samples were kept in a buffer consisting of 20 mM Bis-Tris, pH 6.5, 0.03% (w/v) *n*-dodecyl- β -D-maltoside, and 200 mM sucrose. For resonance Raman measurements, they were concentrated over 30 kDa membrane filters to an optical density of about 70 cm⁻¹ (0.5–1.0 mg of Chl/mL) at the Q_y absorption maximum.

Resonance Raman spectra were measured at 77 K as described previously (25, 26). The spectra were excited using the 413.1 and 406.7 nm lines of a Kr⁺ laser (Innova 90), the 441.6 nm line of a Liconix He-Cd laser, and the 488.0 and 514.5 nm lines of an Ar⁺ laser (Innova 100). Actinic effects of the measuring beam (i.e., photoaccumulation) were avoided by defocusing the laser beam before it passed through the cryostat.

RESULTS AND DISCUSSION

The Soret absorption bands of the six Chl's and two Pheo's in the PSII RC strongly overlap, appearing as a peak around 416 nm with a shoulder at 435 nm (Figure 2A). Oxidized cyt *b*₅₅₉ contributes at ~416 nm (27). Pheo transitions occur between 415 and 420 nm, as is apparent from the absorption difference spectra associated with the exchange of H_B and H_A (Figure 2B). The Soret transitions of Chl molecules in vitro are at 436 nm (B_x) and 430 nm (B_x, B_y) (28, 29). The primary donor Chl's (bound at the positions corresponding to the primary donor bacterio-Chl's in purple bacteria RCs) have been proposed to have Soret maxima at 433 nm (P_A) and 436 nm (P_B) (30). β -Car transitions contribute between 400 and 510 nm (31). Consequently, resonance Raman spectra with excitation in the Soret region will include contributions from all the chlorins and from β -car. However, some degree of selectivity for Pheo relative to Chl is expected when the laser excitation is moved to the blue of 430 nm, and similarly, Chl's are expected to have enhanced contributions in spectra collected with excitation to the red of that wavelength. Nevertheless, it should be kept in mind that the absorption spectrum of the PSII RC in the Soret region is complicated and not completely understood, since it is not clear how the maxima of these transitions are affected by pigment-protein (or pigment-pigment) interactions. Indeed, experiments conducted with bacterial reaction centers have clearly shown that when excitation is done in the Soret region of bacteriochlorophyll and bacteriopheophytin molecules, resonance contributions are extremely difficult to predict,

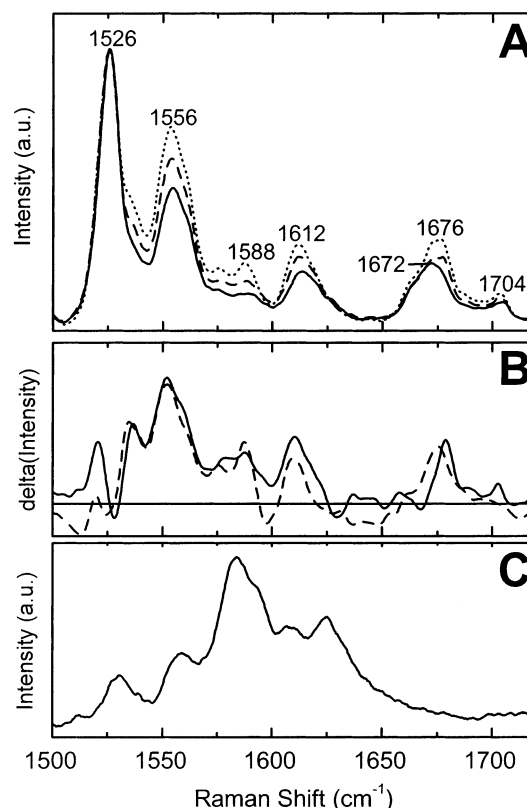


FIGURE 3: (A) Resonance Raman spectra at 77 K of nontreated RC (dotted), RC_{1x} (dashed), and RC_{2x} (solid) for excitation at 413.1 nm. (B) Difference resonance Raman spectra associated with the exchange of H_B (dashed) and of H_A (solid), calculated from the spectra in (A) (nontreated minus RC_{1x} and RC_{1x} minus RC_{2x}, respectively). (C) Resonance Raman spectrum at 77 K of ^{13}C -OH-Pheo in THF. Excitation was at 406.7 nm.

being tightly dependent on both pigment-protein and pigment-pigment interactions (32).

The Soret transition of ^{13}C -OH-Pheo peaks at ~400 nm in vitro (Figure 1) and at 400–410 nm when incorporated in the PSII RC (Figure 2). Considering the positions of the Soret absorption maxima only, the resonance coefficients of Pheo and ^{13}C -OH-Pheo are expected to be roughly the same for excitation at 413.1 nm. However, excitation at this wavelength enhances Pheo contributions relative to those from Chl.

Control of Pigment Exchange and Binding. Carotenoid resonance Raman spectra excited throughout the Soret region (not shown, although see Figure 3A for the ν_1 position) were identical to those reported previously (33, 34), consistent with an all-trans conformation of these cofactors (35). The spectra indicate that the exchange procedure did not result in any alteration in the β -car configuration. In particular the specific features of the two, nonequivalent β -car's, selectively excited at 488.0 and 514.5 nm (Telfer, A., Frolov, D., Barber, J., Robert, B., and Pascal, A., unpublished experiments), were observed in all three preparations (data not shown). Spectra obtained at 413.1 nm excitation are presented in Figure 3A for RC, RC_{1x}, and RC_{2x}. They are normalized on the intensity of the ν_1 band of β -car, at 1526 cm⁻¹. The resonance Raman spectrum of untreated RC (Figure 3, dotted line) is comparable to previously published data obtained under similar experimental conditions (9). Methine bridge stretching modes from both Pheo and Chl appear between 1550 and 1650

cm^{-1} . Their positions at 1556 and 1612 cm^{-1} in the spectra in Figure 3A indicate pentacoordination of the Chl's in all three preparations (36). These positions and the bandwidths in the chemically modified RCs are unchanged with respect to those of untreated RC. This is a strong indication that no drastic modifications occur in the electronic and molecular structure of the Chl macrocycles upon Pheo substitution. In addition, the resonance Raman spectra of untreated RC and RC_{2x} acquired with excitation at 441.6 nm (which enhances Chl modes) are not significantly different (data not shown). These observations can be taken as evidence that the binding sites of the Chl and β -car molecules have not experienced any significant changes upon Pheo exchange.

Figure 3C displays the resonance Raman spectrum of 13¹-OH-Pheo in THF, for excitation at 406.7 nm. As expected, no bands are observed in the C=O stretching region (1650–1720 cm^{-1}), since there is no carbonyl group conjugated with the molecule's macrocycle. The strongest band observed is at 1584 cm^{-1} (close to the 1588 cm^{-1} marker band of Pheo (9, 37)) with less intense bands at 1531, 1559, 1609, and 1625 cm^{-1} .

The replacement of Pheo with 13¹-OH-Pheo is accompanied by a decrease in intensity of several resonance Raman bands (Figure 3A) that can be assigned to Pheo, including the C–C stretching modes of this pigment (1530, 1556, 1588, and 1612 cm^{-1}) (37). In particular, the intensity of the band at 1588 cm^{-1} , which is a marker of Pheo contributions (9, 36) and is also the strongest mode for 13¹-OH-Pheo (Figure 3C), decreases in the spectra of RC_{1x} and RC_{2x}. This decrease matches the reduction in Pheo content in the modified RCs as a result of pigment exchange, indicating that excitation at 413.1 nm results in stronger resonance of Pheo than of the modified cofactor. This result is unexpected, considering the electronic absorption properties of the two pigments (Figure 2). However, it is evident from the spectra in Figure 3 that the resonance coefficient of 13¹-OH-Pheo in the RC environment upon excitation at 413.1 nm must be at least 2 times lower than that of Pheo. This observation suggests that the coupling of most vibrational bands of 13¹-OH-Pheo to the Soret electronic transition and/or the intrinsic strength of its Raman modes are weaker than in Pheo. This could be a general property of 13¹-OH-Pheo, since its delocalized electron system is more symmetric than in Pheo (due to the absence of the double bond at the isocyclic ring).

The exchange-associated difference spectra "RC minus RC_{1x}" and "RC_{1x} minus RC_{2x}" (Figure 3B) reveal that the methine bridge stretching frequencies of H_A and H_B are 1552 and 1610 cm^{-1} , slightly downshifted with respect to those of the bound Chl molecules.

C=O Stretching Region. The bands observed in the higher frequency region (1650–1750 cm^{-1}) of the spectra in Figure 3A are due to stretching modes of carbonyl groups. They are a probe for the molecule's microenvironment, since the vibrational frequency of a carbonyl group is sensitive to the nature and strength of the intermolecular interactions (for example, hydrogen bonds) in which it is involved, and to the dielectric constant of its local environment (38). In particular, for Pheo and Chl in an apolar environment and in the absence of intermolecular interactions, bands are expected around 1700 and 1695 cm^{-1} , respectively, at room temperature. In the presence of hydrogen bonds to the 13¹-

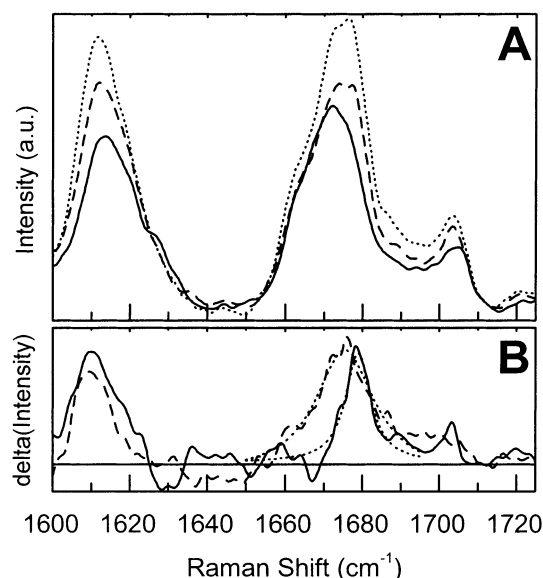


FIGURE 4: Carbonyl stretching region of the resonance Raman spectra from parts A and B, respectively, of Figure 3 plotted on an expanded scale. The dotted lines in (B) represent the fits of the difference spectra to Lorentzian bands.

C=O group, these frequencies are downshifted by up to 40 cm^{-1} , the extent of the shift being dependent on the strength of the intermolecular interaction (37, 38). An increase in the dielectric constant (i.e., an increase in polarity) of the immediate environment causes downshifts of 5–10 cm^{-1} (see also ref 25 and references therein).

The high-frequency region of the spectra in Figure 3A is plotted on an expanded scale in Figure 4A. The untreated RC exhibits a broad band peaking at 1676 cm^{-1} , with a shoulder at 1665 cm^{-1} . A smaller band is observed at 1704 cm^{-1} . These bands arise from the 13¹-keto-carbonyl groups of all six Chl's and two Pheo's. Upon Pheo substitution the contributions from H_B (for RC_{1x}) and from H_B and H_A (for RC_{2x}) are decreased due to the incorporation of 13¹-OH-Pheo at their binding sites. This molecule has no keto-carbonyl group in ring V, and consequently, its resonance Raman spectrum shows no features in the frequency region between 1650 and 1750 cm^{-1} (see Figure 3C). As a result, the exchange-associated difference spectra "RC minus RC_{1x}" and RC_{1x} minus RC_{2x} represent only the contributions from the Pheo molecule that has been replaced-H_B and H_A, respectively. These difference spectra are shown in Figures 3B and 4B.

Binding of H_B. In the carbonyl stretching region, the difference spectrum for exchange of H_B (dashed line in Figure 4B) represents a broad decrease in intensity, with a maximum at 1675 cm^{-1} and an fwhm of about 15 cm^{-1} . The frequency of this stretching mode indicates that the 13¹-keto-carbonyl group of H_B is involved in a moderately strong hydrogen bond, and is consistent with predictions from structural models (5–7). Svensson et al. (6) have predicted that the 13¹-C=O of H_B may participate in two hydrogen-bonding interactions, namely, with D2-Gln130 and with D2-Asn143. The frequency observed for the stretching mode of the keto-carbonyl group of H_B is not consistent with the existence of two strong hydrogen bonds between this group and two adjacent residues. However, the absence of two such strong hydrogen bonds does not preclude the presence of

these residues in the vicinity of the 13¹-keto group, if one of them is in a geometry unfavorable for the formation of a hydrogen bond.

Binding of H_A. The difference spectrum associated with the second exchange (Figure 4B, solid line) resembles that reported by Moënné-Loccoz et al. (9) for photoaccumulation of Pheo⁻. The main feature is a positive peak with a maximum at 1679 cm⁻¹ (fwhm = 8 cm⁻¹), but it is also apparent that the intensity of the band at 1704 cm⁻¹ is decreased upon H_A exchange. The similarity between the difference spectra associated with Pheo⁻ photoaccumulation and with the second Pheo exchange is consistent with our previous observation that this exchange procedure is selective, and that the difference spectrum associated with the second Pheo exchange can be assigned to H_A (19). The frequency of the main band in the H_A-associated difference spectrum also corresponds well with that determined by photoaccumulation-induced difference FTIR spectroscopy (1677–1678 cm⁻¹) (10, 39), and indicates that the keto-carbonyl group of this molecule is involved in a medium-strength hydrogen-bonding interaction with the protein. Note that the bandwidth of this feature (Figure 4B) is unusually low (8 cm⁻¹ fwhm). While the significance of this observation is difficult to determine, it may be due to an overlapping, negative contribution at lower frequencies (see below).

Tentative Assignment of the Chl Carbonyl Bands. At least three populations of Chl contribute in the carbonyl stretching region, as can be observed by examination of the resonance Raman spectrum of RC_{2x} (Figure 4A). The main peak is at 1672 cm⁻¹, with a shoulder at 1664 cm⁻¹. There is also a small band at 1704 cm⁻¹, whose intensity is somewhat diminished for RC_{2x} in comparison to untreated RC (Figure 4A).

Chemical exchange of H_A (this work) and the photoaccumulation of its anion (9) are accompanied by a decrease in intensity of the weak band at 1704 cm⁻¹. This band had been tentatively assigned to H_B (9) by analogy to the structure of purple bacterial RCs (40), where the bacterio-Pheo in the inactive branch is free from interactions with the protein. However, models constructed on the basis of that structure predict both Pheo's in PSII to be involved in hydrogen-bonding interactions (5–7). We have shown above that H_B exchange does not result in a decrease in intensity of this band, and that H_B contributes at 1675 cm⁻¹. By a combination of mutations to the D2-Gln130 amino acid residue and resonance Raman spectroscopy, a similar frequency was recently assigned to the keto-carbonyl group of H_B (Force, D. A., Pascal, A., Robert, B., and Diner, B. A., unpublished experiments). Moreover, the band at 1704 cm⁻¹ should be absent in RC_{2x} if it arises from H_B, since this cofactor is fully replaced with 13¹-OH-Pheo in this preparation. The spectra in Figure 4 clearly show that the decrease in intensity of the 1704 cm⁻¹ band is associated with the substitution of H_A. In addition, it has been demonstrated by a number of different spectroscopic methods (8–12) that the keto-carbonyl group of H_A is hydrogen bonded to the protein, more specifically to D1-Gln130. The main feature in the H_A exchange-associated difference spectrum (Figure 4B) is a contribution at 1679 cm⁻¹, which we assign to the keto-carbonyl group of H_A. These observations leave open two possibilities to explain the presence of the weak band at 1704 cm⁻¹. It could reflect heterogeneity in the preparation, that

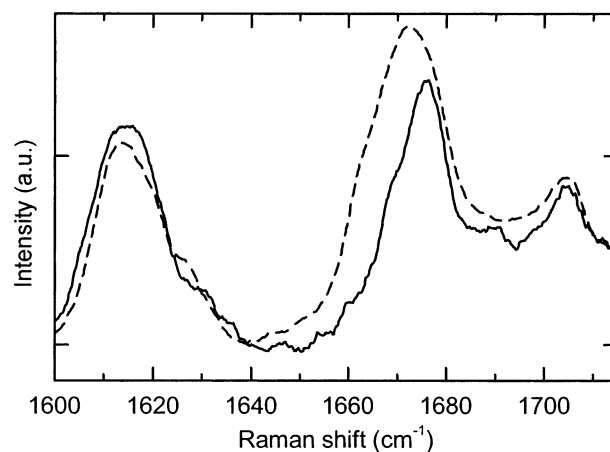


FIGURE 5: Carbonyl stretching region of the 77 K resonance Raman spectra of RC_{2x} with excitation at 413.1 nm (dashed) and 406.7 nm (solid).

is, that in a subpopulation of the RCs H_A is free from interactions. Alternatively, it may arise from a Chl molecule that is perturbed by exchange of H_A (and by the photoaccumulation of its anion; see ref 9), such that its structure and/or its electronic properties in the Soret region are altered. From the results presented here the assignment of this 1704 cm⁻¹ band to H_B can be ruled out.

If the 1704 cm⁻¹ mode is from a Chl molecule in the RC, then its keto-carbonyl group exhibits an unusually high frequency, and must be free from intermolecular interactions and in a highly apolar environment. The shape of the difference spectrum (Figure 4B, solid line) could indicate a broad, negative feature in the 1665–1670 cm⁻¹ region, superimposed on the positive contribution at 1679 cm⁻¹. This overlap could explain the unusually low apparent bandwidth of the latter band; see above. A negative feature would represent an increase in contributions in this region for RC_{2x}, which may in turn correspond to newly formed interactions with the C=O group previously at 1704 cm⁻¹. The apparent downshift in frequency of this band in RC_{2x} may thus be due to a structural change in some of the RCs, by which the C=O group of one pigment becomes more exposed to the hydrophilic surface of the protein. Indeed, C=O groups in the presence of water are seldom found free from interactions when exposed to water (see ref 41 and references therein). Interestingly, the RC pigments closer to the hydrophilic surface in PSII are those corresponding to the primary donor in RCs of purple bacteria, P_A and P_B (3), and their binding pockets are predicted in structural models to be hydrophobic and nonpolar (6, 7). Moreover, FTIR difference spectra for P⁺-P and for Y_Z-Y_Z (39, 42–44) show a shift attributed to a keto-carbonyl stretch at 1704–1707 cm⁻¹. All these observations suggest that the 1704 cm⁻¹ band in the resonance Raman spectrum could arise from one of the “P” Chl's, possibly P_A (since this is the closest to Y_Z (3)).

The shoulder at 1660–1670 cm⁻¹ in the untreated RC represents carbonyl stretching modes from hydrogen-bonded Chl molecules, since both Pheo's contribute at 1675–1680 cm⁻¹. Figure 5 displays the dependence of the resonance Raman spectra of RC_{2x} on the wavelength of excitation. Moving the excitation to 406 nm results in enhancement of the higher frequency modes in the carbonyl region, and in the disappearance of the 1664 cm⁻¹ shoulder. The Chl's

whose 13^1-C=O groups give rise to these modes thus have Soret transitions that are red-shifted with respect to those of Pheo's, corresponding to the spectral characteristics of the pigments in vitro (28, 29).

The stretching frequencies of Chl's in the PSII RC have been studied by FTIR difference spectroscopy associated with accumulation of the triplet state or of the primary donor cation (39, 42, 45). Both Noguchi et al. (39, 42) and Breton et al. (45) concluded that the triplet state is localized on a pigment whose keto-carbonyl stretching frequency is 1670 cm^{-1} ; carbonyl stretching bands associated with P680^+ were found at 1679 and 1704 cm^{-1} (42, 45). Note that care should be taken when these frequencies are considered; difference FTIR does not yield the absolute frequencies of stretching modes of the studied pigments. In a difference spectrum, signals may peak at positions significantly shifted from the positions of the bands from which they arise. However, these experiments suggest that the triplet state in the PSII RC is localized on a pigment (Chl) that has a hydrogen-bonded keto-carbonyl group.

Finally, the vibrational properties of the low-temperature "trap" of excitation energy (the most red-shifted RC pigment) have been studied by fluorescence line-narrowing spectroscopy (46). It was found that this molecule has a keto-carbonyl stretching frequency of 1669 cm^{-1} , corresponding to a moderate to strong hydrogen-bonding interaction with the protein. The assignments made here of the stretching frequencies of the Pheo keto-carbonyl groups in the PSII RC are not consistent with either the triplet state or the "trapped" excitation energy being localized on a Pheo molecule. This result is thus in agreement with the conclusions of Peterman et al. (46) that the lowest energy electronic transition in the PSII RC is localized on a Chl molecule.

CONCLUSIONS

We have combined resonance Raman spectroscopy and selective pigment exchange to assign the keto-carbonyl stretching frequencies of the Pheo cofactors in the PSII RC. Both H_A and H_B have hydrogen-bonded 13^1-C=O groups, with stretching frequencies around 1679 and 1675 cm^{-1} , respectively. Similar frequencies have been deduced for these modes through the use of mutants in which the two Pheo's had been exchanged individually for Chl molecules (Force, D. A., Pascal, A., Robert, B., and Diner, B. A., unpublished experiments). The exchange-associated difference Raman spectra confirm our previous conclusion (19) that H_B is the cofactor that is exchanged in $\text{RC}_{1\text{x}}$, and that H_A is only replaced after two successive modification treatments.

After pigment exchange, the binding-site properties of the remaining cofactors in the modified RCs are not significantly different from those in untreated preparations. However, the intensity of the small band at 1704 cm^{-1} assigned to a Chl molecule, is diminished in $\text{RC}_{2\text{x}}$, possibly reflecting an alteration of the environment of a keto-carbonyl group that in the native protein is free from interactions and in an apolar environment.

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