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# Reaction Center of Photosystem II with No Peripheral Pigments in D2 Allows Secondary Electron Transfer in D1<sup>†</sup>

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**ABSTRACT:** A pigment-deficient reaction center of photosystem II (PSII)—with all the core pigments (two molecules of chlorophyll *a* and one of pheophytin *a* in each D protein) but with only one molecule each of peripheral chlorophyll *a* (Chlz) and  $\beta$ -carotene (Car)—has been investigated by pump-probe spectroscopy. The data imply that Car and Chlz are both bound to D1. The absence of Car and Chlz in D2 allows the unprecedented observation of secondary electron transfer in D1 of PSII reaction centers at room temperature. The absorption band of the Car cation in D1 (Car<sub>D1</sub><sup>+</sup>) peaks around 910 nm (as against 990 nm for Car<sub>D2</sub><sup>+</sup>), and its positive hole is shared by Chlz<sub>D1</sub>, whereas Car<sub>D2</sub><sup>+</sup> can disappear by capturing an electron from Chlz<sub>D2</sub>.

Photosystem II (PSII)<sup>1</sup>—a membrane-embedded pigment-protein complex found in thylakoids of cyanobacteria, algae, and higher plants—harnesses the energy of sunlight and catalyzes the oxidation of water and the reduction of plastoquinone, releasing molecular oxygen to the atmosphere as a byproduct (1). The reaction center (RC) of PSII consists of the heterodimer of D1/D2 proteins, the cytochrome *b*<sub>559</sub>

(Cytb<sub>559</sub>) subunits PsbE and PsbF, and the small subunit named PsbI (2); its most active and stable form binds six chlorophyll (Chl) molecules, two pheophytin (Pheo) molecules, and two  $\beta$ -carotene (Car) molecules (3, 4). Photoexcitation of the special donor P<sub>680</sub> of the PSII RC leads to the transfer of an electron to a Pheo molecule. Charge recombination of the resulting ion pair (P<sub>680</sub><sup>+</sup> Pheo<sup>•−</sup>) is prevented by rapid electron transfer from Pheo<sup>•−</sup> to the plastoquinone Q<sub>A</sub>. After two consecutive steps of reduction of the secondary plastoquinone Q<sub>B</sub>, followed by protonation, the plastoquinol (PQH<sub>2</sub>) leaves the Q<sub>B</sub> site and a new plastoquinone molecule occupies this site. P<sub>680</sub><sup>+</sup>, a species with a high positive redox potential (ca. 1.1 V), is reduced by the tyrosine residue Y<sub>Z</sub> (D1-Tyr161) that subsequently oxidizes the Mn cluster, where catalytic water oxidation takes place (5). In the event that electron transport from the donor site of PSII is impaired and P<sub>680</sub><sup>+</sup> is not reduced by the primary electron-donation pathway, a secondary electron-donation pathway comes into effect and protects PSII from oxidative damage induced by P<sub>680</sub><sup>+</sup> itself. In this case, Cytb<sub>559</sub> and oxidizable Chl and Car molecules of RC act as secondary electron donors and reduce P<sub>680</sub><sup>+</sup> (6–8). In isolated membranes or core complexes of PSII, the secondary electron-donation pathway for the reduction of P<sub>680</sub><sup>+</sup> is unable to compete effectively, at room temperature, with the charge recombination of P<sub>680</sub><sup>+</sup> Q<sub>A</sub><sup>•−</sup>. In the presence of reduced, high-potential Cytb<sub>559</sub>, the state Cytb<sub>559</sub><sup>+</sup> Q<sub>A</sub><sup>•−</sup> is formed preferentially with a yield close to unity after illumination at 77 K (9); it has been suggested that in this case Cytb<sub>559</sub> donates an electron to P<sub>680</sub><sup>+</sup> via a Car molecule (7). In contrast, when Cytb<sub>559</sub> is preoxidized and is in its low-potential form, electron donation from Chl (again via a Car molecule) takes place (7, 10, 11); due to the energy barrier for electron donation from Chl to Car, oxidation of Car dominates at temperatures below 20 K, and that of Chl

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<sup>1</sup> Abbreviations: AcN, acetonitrile; Car,  $\beta$ -carotene; Chl, chlorophyll; Chlz, peripheral chlorophyll *a*; Cytb<sub>559</sub>, cytochrome *b*<sub>559</sub>; DM, *n*-dodecyl  $\beta$ -D-maltoside; DmS, doublet-minus-singlet;  $D_w \equiv A_d(\lambda) - A_b(\lambda)$ , difference spectra, where  $w = \lambda/\text{nm}$  and  $A_d(\lambda)$  and  $A_b(\lambda)$  denote the absorbance (at wavelength  $\lambda$ ) after and before photolysis by a cw light source, respectively; FeCy, ferricyanide; LD, linear dichroism; Pheo, pheophytin; PQH<sub>2</sub>, plastoquinol; PSII, photosystem II; PVDF, polyvinylidene difluoride; P<sub>680</sub>, chlorophyll *a* special donor of photosystem II; RC, reaction center; RC-5, five-chlorophyll photosystem II reaction center; RC-6, the most active and stable form of the photosystem II reaction center; Q, plastoquinone; SiMo, silicomolybdate; TmS, triplet-minus-singlet;  $\Delta A(\lambda; t)$ , flash-induced changes in absorbance at wavelength  $\lambda$  and a pump-probe delay time of  $t$ ;  $\Delta @ d$ , abbreviation for  $\Delta A(\lambda; t)$  where  $d \equiv t/\text{ms} \geq 1$ ;  $C(\lambda)$ , absorption spectrum of chlorophyll *a* in 95% acetone;  $Z_1(\lambda)$ , absorption spectrum of Chlz<sub>D1</sub> (Chlz in the D1 protein) modeled by red-shifting  $C(\lambda)$ ;  $\Sigma(\lambda)$ , a smooth fit to the absorption spectrum of Car<sup>+</sup> in AcN;  $M(\lambda)$ , absorption spectrum of Car<sub>D1</sub><sup>+</sup> (the cation of Car in the D1 protein) modeled by blue-shifting  $\Sigma(\lambda)$ ;  $\rho \equiv [\text{Chlz}_{D1}^+]/[\text{Car}_{D1}^+]$ , molar ratio Chlz<sub>D1</sub><sup>+</sup>/Car<sub>D1</sub><sup>+</sup>;  $\epsilon_w^{\text{Abb}}$ , molar absorption coefficient, at wavelength  $\lambda$ , of the chromophore represented by the abbreviation in the superscript (for Car<sup>+</sup>, Abb = Car<sup>+</sup>; for CarD1<sup>+</sup>, Abb = Car<sub>D1</sub><sup>+</sup>; for ChlzD1, Abb = zD1; for Chlz<sub>D1</sub><sup>+</sup>, Abb = zD1+•);  $\Delta_w^{\text{Abb}}$ , the value of  $\Delta A(w \text{ nm}; 30 \text{ ms})$  for the chromophore abbreviated in the superscript.

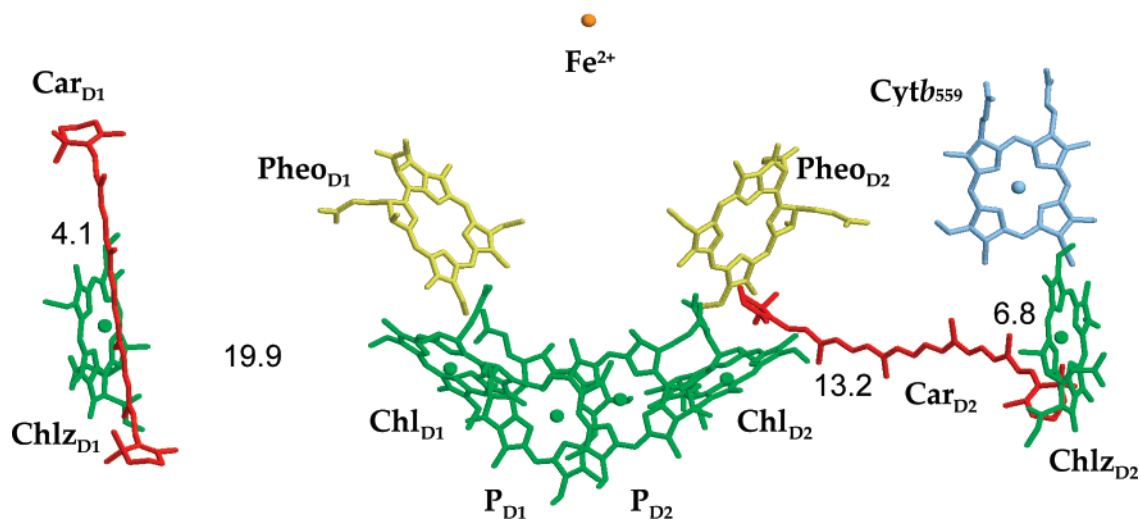


FIGURE 1: Pigment arrangement in the photosystem II reaction center. Relative locations of pigments, non-heme iron, and cytochrome  $b_{559}$  in the most active and stable form of the reaction center of photosystem II are based on the 3.0 Å resolution structure of cyanobacterial photosystem II (PDB code: 2AXT). Relevant  $\pi$ - $\pi$  system distances for this study (Chl $_{D1}$ -to-Car $_{D1}$ -to-Chl $_{D1}$  and Chl $_{D2}$ -to-Car $_{D2}$ -to-Chl $_{D2}$ ) are given in angstroms.

at temperatures above 120 K. A branched pathway, involving Cyth $_{559}$  (which is close to D2), has been put forward; here reduced high-potential Cyth $_{559}$  or Chl (when Cyth $_{559}$  is oxidized) donates an electron to P $_{680}^{+}$  through an intermediary Car (7). However, evidence for the concurrent oxidation of the two nonequivalent Car molecules at 77 K, with different spectroscopic properties, has also been presented (12).

The molecular architecture of PSII, as revealed by the 3.0 Å resolution structure (13), has led to a better understanding of how secondary electron-transfer reactions involving some cofactors in the D2 protein might protect P $_{680}$  in functionally impaired PSII. Due to its favorable location for electron transfer (see Figure 1), the Car in D2 (Car $_{D2}$ ) initially donates an electron to P $_{680}^{+}$  but is rapidly regenerated (at room temperature) through electron transfer from Cyth $_{559}$ , or from the peripheral chlorophyll in D2 (Chl $_{D2}$ ) if the former happens to be already oxidized as described above. Electron transfer from the Car in D1 (Car $_{D1}$ ) to P $_{680}^{+}$ , less likely when Car $_{D2}$  is present and Cyth $_{559}$  is reduced, could come to the fore in the absence of Car $_{D2}$ ; were this to happen, the positive charge would be shared, it has been suggested by Loll and coauthors (13, 14), between Car $_{D1}$  and the peripheral chlorophyll in D1 (Chl $_{D1}$ ). The chief obstacle to investigating side electron transport in the D1 protein is the presence of Car $_{D2}$ ; we show here that the D2 protein in the pigment-deficient RC isolated by means of immobilized metal affinity chromatography is devoid of Car as well as Chl $_{Z}$  (15) and report the exclusive occurrence of side electron transport in D1 of higher plant PSII RC.

## EXPERIMENTAL PROCEDURES

**Five-Chlorophyll and Six-Chlorophyll Reaction Center Purification.** The five-chlorophyll RC (RC-5) was purified from spinach PSII through Cu-immobilized chromatography as described in ref 15. The RC-5 was eluted from the Cu-immobilized column by adding 5 mM imidazole to the washing buffer, 0.02% *n*-dodecyl  $\beta$ -D-maltoside (DM), 50 mM Mes pH 6.5. The concentrated RC-5 samples were passed through a Superdex 200 size exclusion column

(Amersham Pharmacia Biotech., Buckinghamshire, England) equilibrated with 0.02% DM, 20 mM Tris-HCl pH 7.2 (assay buffer) to ensure that the RC-5 samples did not contain traces of free pigments or imidazole. In order to compare the absorption spectrum of RC-5 with that of the most active and stable form of the RC containing approximately six Chl and two Car molecules per two Pheo molecules (RC-6), the latter was purified in accordance with the procedure described by Vacha et al. (15) and references therein.

**Pigment Analysis.** The pigment stoichiometry of RC-5 and RC-6 was analyzed by high-performance liquid chromatography (HPLC). Pigments were extracted by 80% of acetone (v/v), filtered through 0.2  $\mu$ m polyvinylidene difluoride (PVDF) filter (Whatman plc, Maidstone, U.K.), and separated on a Zorbax ODS reversed-phase column (4.6 mm  $\times$  250 mm, 5  $\mu$ m) (Agilent Technologies, Inc., Santa Clara, CA) by isocratic elution with methanol/ethyl acetate, 68:32 (v/v), at a flow rate of 1 mL/min using a Waters HPLC system consisting of Pump Controller 600, Delta 600 injection system, and a PDA 996 detector (Waters Corporation, Milford, MA). Chromatograms of Chl $a$  and Pheo $a$  were detected at 663 nm and Car at 450 nm. The HPLC was calibrated as described by Vacha et al. (15), and the values of 86.9, 49.3, and 135 mM $^{-1}$  cm $^{-1}$  were used for the molar absorption coefficients of Chl $a$ , Pheo $a$ , and Car, respectively, in methanol/ethyl acetate, 68:32 (v/v), at the HPLC recording wavelengths. The pigment composition of RC-5 was also examined by means of the spectrum reconstruction method described in ref 16; the advantages of this method are discussed in ref 17. An aliquot of the RC-5 sample was dissolved in acetone, and the mixture was centrifuged to remove the denatured RC proteins. The final content of water in acetone was 5%. The absorption spectrum of the extracted pigments was recorded by using a commercial spectrophotometer model UV-1601PC (Shimadzu Scientific Instruments, Inc. Columbia, MD). Chl $a$  and  $\beta$ -Car were purchased from Sigma-Aldrich Co. (St. Louis, MO), and Pheo $a$  was obtained by acidifying a solution of Chl $a$  in acetone ( $\sim$ 1 mM HCl). The absorption spectra of the free pigments were recorded in water/acetone (5:95, v/v) to match the content

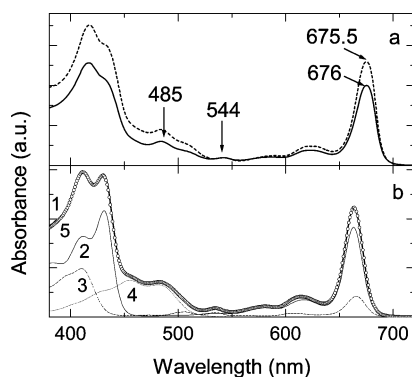


FIGURE 2: (a) Room-temperature absorption spectra of the five-chlorophyll (solid line) and six-chlorophyll (dashed line) reaction centers of photosystem II normalized at the  $Q_X$  band of pheophytin  $a$  at 544 nm. The numbers in nanometers indicate the peak position of the  $\beta$ -carotene, pheophytin  $a$   $Q_X$ , and chlorophyll  $a$  (and pheophytin  $a$ )  $Q_Y$  bands. (b) Spectral fitting, based on the spectrum reconstruction method, of the pigment content of the five-chlorophyll reaction center: absorption spectra of the extracted pigment mixture from the five-chlorophyll reaction center (curve 1, solid line) and of pure chlorophyll  $a$  (curve 2), pheophytin  $a$  (curve 3), and  $\beta$ -carotene (curve 4) in water/acetone (5:95, v/v). The reconstructed spectrum (curve 5, open circles) was fitted by using a least-squares routine to the absorption spectrum of the extracted pigment mixture in the 380–750 nm region.

of water in the solution of the extracted RC-5 pigment mixture. The molar absorption coefficients for Chla, Pheoa, and Car were 81.6, 47.2, and 141  $\text{mM}^{-1} \text{cm}^{-1}$  in water/acetone (5:95, v/v) at 663, 666, and 455 nm, respectively, based on comparative measurements in 100% acetone and published extinction coefficients (18).

**Transient Absorption Spectroscopy.** Transient absorption difference spectra of RC-5,  $\Delta A(\lambda; t) \equiv \tilde{A}(\lambda; t) - A(\lambda)$  where  $A(\lambda)$  and  $\tilde{A}(\lambda; t)$  denote the absorbance (at wavelength  $\lambda$ ) before and at time  $t$  after the firing of the pump source, were recorded by using a home-built multichannel kinetic spectrometer whose components and operation have been described in (19, 20). The shortest delay was 5  $\mu\text{s}$ ; the longest was 30 ms. RC-5 samples were suspended in the assay buffer at an absorbance of 1 at 676 nm in a 1 cm cuvette. Anaerobic conditions were reached by directing, 15 min before and during the measurements, a stream of Ar to the surface of the RC-5 sample in the cuvette. When needed, 0.5 mM silicomolybdate (SiMo) was used as electron acceptor.

## RESULTS AND DISCUSSION

Absorption spectra of the isolated RC-5 and RC-6 are shown in Figure 2a. The broad absorption band below 450 nm corresponds to the Soret bands of Chla, Pheoa, and oxidized  $\text{Cytb}_{559}$ . The Car absorption band shows a prominent peak at 485 nm, and the  $Q_X$  band of Pheoa appears as a small band at 544 nm. The red region of the spectra is characterized by the prominent  $Q_Y$  bands of Chla and Pheoa that form a single band peaking at 676.0 nm in RC-5 and 675.5 nm in RC-6. The two standard procedures to purify RC-5 and RC-6 are known to yield RC with no plastoquinone in the  $Q_A$  (or  $Q_B$ ) site (data not shown). On the basis of the assumption that both RC preparations contain two Pheoa per RC, the spectra were normalized at 544 nm. The normalization shows a decrease in both the absorbance of the  $Q_Y$  and Soret bands of Chla and the absorbance of the visible band of Car for RC-5 when compared to RC-6. Pigment analysis

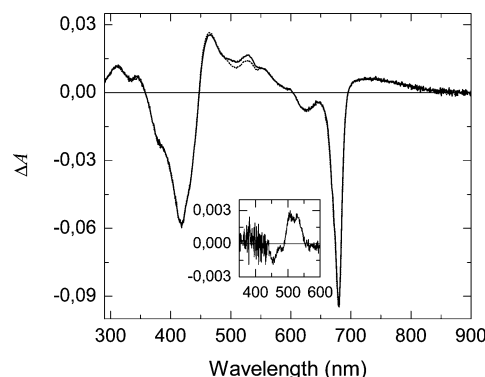


FIGURE 3: Room-temperature photoinduced absorption difference spectra of the five-chlorophyll reaction center of photosystem II under anaerobic conditions at delays of 5  $\mu\text{s}$  (solid line) and 30  $\mu\text{s}$  (dotted line); the two difference spectra are normalized to the same amplitude at 680 nm. Each curve is an average of 120 spectra. Inset: subtraction of the normalized 5 and 30  $\mu\text{s}$  difference spectra in the spectral region where the carotenoid singlet ground state and the lowest triplet state absorb.

by HPLC yielded, in the case of RC-5, the average ratio of  $5.0 \pm 0.1$  Chla and  $1.2 \pm 0.1$  Car per 2 Pheoa; the corresponding values for RC-6 came out to be  $6.2 \pm 0.1$  and  $2.1 \pm 0.1$ , respectively. The outcome of pigment analysis of RC-5 by the spectral reconstruction method (16) coincided with the HPLC data; a spectral display of the results appears in Figure 2b. In other words, RC-5 lacks, when compared with RC-6, a Chl $z$  and a Car. On the basis of arguments that will be presented later, we identify the Car in RC-5 as Car $_{D1}$ . As to which of the two molecules of peripheral Chla is lacking in RC-5, it has been suggested that, since Chl $z_{D2}$  is shielded by  $\text{Cytb}_{559}$  and PsbI subunits of the PSII RC (21), the chromatographic procedure used for isolating RC-5 is more likely to dislodge Chl $z_{D1}$ , but our flash photolysis results, presented below, imply otherwise.

Under anaerobic conditions and in the absence of electron acceptors, the difference spectra of RC-5 turned out to be similar to those of RC-6 (22). Figure 3 shows two difference spectra at room temperature ( $t = 5$  and 30  $\mu\text{s}$ ), which have been normalized to the same amplitude at 680 nm. The 30  $\mu\text{s}$  spectrum can be recognized as the triplet-minus-singlet ( $\text{TmS}$ ) spectrum of  $\text{P}_{680}^+$ , manifesting the formation of  $\text{P}_{680}^+$  as a result of charge recombination between  $\text{P}_{680}^{+\bullet}$  and  $\text{Pheo}_{D1}^{-\bullet}$  through the radical pair mechanism; where  $\text{P}_{680}^{+\bullet}$   $\text{Pheo}_{D1}^{-\bullet}$  decays with a lifetime of about 45 ns at room temperature (not detected under our experimental conditions) (23). Triplet excitation (denoted by the dagger) is believed to be localized mainly on the accessory Chla of  $\text{P}_{680}$  in D1 (Chl $_{D1}$ ) at cryogenic temperature, but in thermal equilibrium ( $\sim 70:30$ ) with the central special-pair Chl molecules ( $\text{P}_{D1}$  and  $\text{P}_{D2}$ ) at room temperature (24). In the 5  $\mu\text{s}$  spectrum, a small contribution around 530 nm, made by a more short-lived species is apparent; its spectrum, isolated by subtracting the two difference spectra, is shown in the inset of Figure 3. On account of its spectral features and rapid decay, the spectrum in the inset can be identified with  $\text{Car}^+$  (23); on the basis of spectral evidence presented later, we will label it as  $\text{Car}_{489}^+$  and place it in D1. Since  $\text{Car}^+$  can only be populated as a result of triplet-triplet transfer from an adjacent triplet donor, we are led to conclude that the donor in this case must be  $\text{Chl}z_{D1}^+$ , which is known to be in van der



Waals contact with Car<sub>D1</sub> (Figure 1). Since the Car<sub>D2</sub>-Chl<sub>D2</sub> separation is appreciably larger, Car<sub>D2</sub> is expected to be a less efficient quencher of Chl<sup>+</sup> (13), and it would be unreasonable, in the absence of a weighty argument to the contrary, to locate the peripheral pigments of RC-5 in D2.

We pause now to summarize some spectroscopic and structural information about the peripheral pigments and to recall some other data that would prove to be pivotal for the interpretation of our own results. Low-temperature linear dichroism (LD) spectroscopy had revealed, before structural data became available, the existence of two spectroscopically distinguishable Car molecules in RC-6: Car<sub>489</sub> (with an absorption peak at 489 nm), oriented nearly perpendicular to the membrane plane, and Car<sub>507</sub> (with an absorption peak at 507 nm), lying nearly parallel to the membrane plane (25, 26). On the basis of the available structural information, Car<sub>489</sub> and Car<sub>507</sub> can now be identified with Car<sub>D1</sub> and Car<sub>D2</sub>, respectively. The absorption spectra of isotropic suspensions of PSII RC, even when recorded at cryogenic temperatures (27), are of little help in establishing the identity of the two Car molecules, or for inferring the presence or absence of a particular Car. Losi et al. (28) did conclude that the Car in RC-5 is Car<sub>489</sub>, but the evidence does not appear to be compelling. In the absence of LD data on RC-5, we will rely on the difference in the absorption spectra of Car<sub>D1</sub><sup>+</sup> and Car<sub>D2</sub><sup>+</sup>.

Charge recombination between P<sub>680</sub><sup>+</sup> and Phe<sub>D1</sub><sup>-</sup>, responsible for the formation of P<sub>680</sub><sup>+</sup>, can be prevented by the addition of a suitable electron acceptor, such as SiMo or ferricyanide (FeCy). Low-temperature irradiation of RC-6 under these conditions leads to the formation of a number of species, including P<sub>680</sub><sup>+</sup> and Car cation(s), which can be easily investigated by spectroscopic means. Noguchi et al. (29) conducted a study of RC-6 at 150 K in the presence of FeCy and reported their results in the form of difference spectra  $D_w \equiv A_a(\lambda) - A_b(\lambda)$ , where  $w = \lambda/\text{nm}$ ,  $A_a(\lambda)$  and  $A_b(\lambda)$  denote the absorbance (at wavelength  $\lambda$ ) after and before photolysis, respectively. They observed a sharp negative peak at 682 nm, due to bleaching of P<sub>680</sub>, and a broad, unstructured positive band peaking at 1004 nm; since negative peaks were also seen (at 508, 476, and 440 nm) where Car<sub>507</sub> (or Car<sub>D2</sub>) is known to absorb (30), the positive band at 1004 nm was assigned to Car<sub>D2</sub><sup>+</sup>; absorption by a species other than Car<sub>D2</sub><sup>+</sup>, particularly P<sub>680</sub><sup>+</sup>, could not be discerned in the 700–1200 nm region, and  $D_{1004}$  was nearly equal to  $|D_{682}|$ . Telfer et al. (12) conducted a similar investigation, using SiMo instead of FeCy and Raman spectroscopy in addition to absorption spectroscopy. They reported that Car<sub>489</sub> is also depleted “but to a lesser extent (~30% of the total carotenoid bleaching)”; they were able to rationalize the excitation profile of the  $\nu_1$  mode (~1490 cm<sup>-1</sup>) by assuming “that the absorption shift [for the two Car cations] is the same as that for their two neutral counterparts, i.e., that Car<sub>507</sub><sup>+</sup> absorbs at longer wavelengths than Car<sub>489</sub><sup>+</sup>”. Since the energy difference between 489 and 507 nm corresponds to about 730 cm<sup>-1</sup> and the absorbance of Car<sub>507</sub><sup>+</sup> peaks around 990 nm, that of Car<sub>489</sub><sup>+</sup> would be expected to occur, if one accepts their assumption, at approximately 923 nm. As for P<sub>680</sub><sup>+</sup>, they stressed: “It is of particular interest to note that no detectable bands could be observed at higher frequencies, where carbonyl vibrations

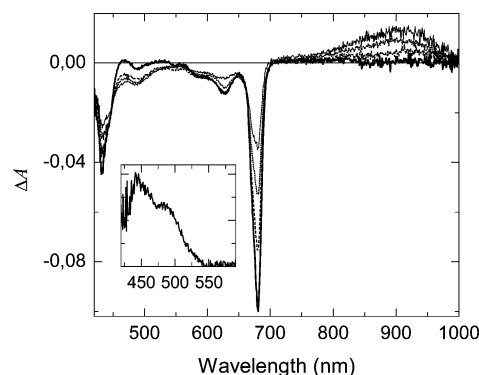


FIGURE 4: Carotenoid cation formation in the five-chlorophyll photosystem II reaction center at room temperature. Photoinduced absorption difference spectra of the five-chlorophyll reaction center of photosystem II in the presence of 0.5 mM silicomolybdate under anaerobic conditions at the following delays: 5  $\mu\text{s}$  (thick solid line), 1 ms (dashed line), 5 ms (dotted line), and 30 ms (thin solid line). Repetition rate: 5 Hz. Each curve is an average of 60 spectra. Inset: subtraction of the 575 nm normalized 5  $\mu\text{s}$  and 30 ms difference spectra in the absorption region of the carotenoid ground state.

of Chl<sup>+</sup> species are expected to contribute”. That the absorption spectrum of P<sub>680</sub><sup>+</sup> differs so markedly from that of Chl<sup>+</sup> species can be understood, according to Okubo et al. (31), by invoking delocalization of the positive charge in a Chl dimer. The stage has now been set for the presentation and interpretation of our own observation.

Flash-induced changes in the absorption spectrum of an RC-5 sample incubated with SiMo are shown in Figure 4. The spectrum at  $t = 5 \mu\text{s}$  agrees with the observations of Noguchi et al. (29): one sees bleaching of P<sub>680</sub> at 681 nm without any clear indication of a positive signal at wavelengths longer than 700 nm, where P<sub>680</sub><sup>+</sup> would be expected to absorb if its absorption spectrum bore any resemblance to that of Chl<sub>D1</sub><sup>+</sup>, which absorbs weakly in the 820–850 nm region (8). As the delay becomes longer ( $d \equiv t/\text{ms} \geq 1$ ), a gradual reduction in the negative signal at 681 nm takes place, accompanied by a growth, at longer wavelengths, of a positive signal, which peaks around 910 nm, and by a negative contribution in the region of absorption by the Car in RC-5. Allowing for the difference in experimental conditions (sample preparation, temperature, acceptor concentration), it is natural to propose that the 910 nm species is Car<sub>D1</sub><sup>+</sup> (or Car<sub>489</sub>) and to place the Car in RC-5 in D1. This means that the transient spectra recorded at long delays portray the recovery of the ground state of P<sub>680</sub> due to electron transfer from Car<sub>D1</sub> to P<sub>680</sub><sup>+</sup>; as suggested by Loll and co-workers (13, 14), the positive hole will be shared between Car<sub>D1</sub> and Chl<sub>D1</sub>. If the broad band in the 700–1000 nm region is indeed a superposition of Car<sub>D1</sub><sup>+</sup> and Chl<sub>D1</sub><sup>+</sup>, the negative peak around 680 nm in a *long-delay* spectrum ( $\Delta@d$ ,  $d \geq 1$ ) must be a superposition of the bleaching signals of P<sub>680</sub> and Chl<sub>D1</sub>, which is believed to have its peak absorbance at about 672 nm at room temperature (32).

In order to verify the above deduction, we decided to generate Car<sup>+</sup> through photoinitiated electron transfer in acetonitrile (AcN) and evaluated the molar absorption coefficient of Car<sup>+</sup> by recording the doublet-minus-singlet (DmS) spectrum of Car (Figure 5). The spectrum of Car<sup>+</sup> in AcN, which peaks around 930 nm with  $\epsilon_{930}^{\text{Car}^{++}} = 2.2 \times$

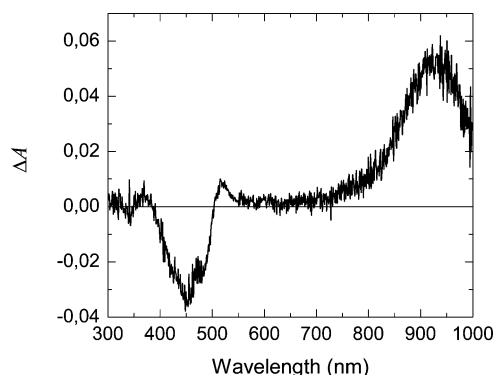


FIGURE 5: Doublet-minus-singlet spectrum of  $\beta$ -carotene in acetonitrile. The molar absorption coefficient of the ground state  $\beta$ -carotene is  $140 \text{ mM}^{-1} \text{ cm}^{-1}$  at 450 nm in acetonitrile.

$10^5 \text{ M}^{-1} \text{ cm}^{-1}$ , was fitted to a noise-free curve (a sum of Gaussians) that will be denoted here as  $\Sigma(\lambda)$ . Since the band attributed to  $\text{Car}_{\text{D1}}^{+\bullet}$  is slightly blue-shifted with respect to  $\Sigma(\lambda)$ , it was modeled as  $M(\lambda) \equiv \Sigma(\lambda + \lambda_0)$ , and  $\lambda_0$  was found by fitting  $M(\lambda)$  to  $\Delta@d$  (the average of  $\Delta@5$ ,  $\Delta@10$ ,  $\Delta@20$ , and  $\Delta@30$ ) in the spectral range where  $\text{Car}_{\text{D1}}^{+\bullet}$  is the dominant absorber (880–1000 nm). The difference  $M(\lambda) - \Sigma(\lambda)$  is barely above the noise level (with a peak value around 0.001), but its overall shape and location (data not shown) are consistent with the known spectrum of  $\text{Chlz}_{\text{D1}}^{+\bullet}$  (8). Adopting the value of  $\epsilon_{815}^{\text{ZD1}^{+\bullet}} = 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  for the peak molar absorption coefficient of  $\text{Chlz}_{\text{D1}}^{+\bullet}$  (8), and assuming that  $\epsilon_{910}^{\text{CarD1}^{+\bullet}} = 2 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ , the value found in AcN, we conclude tentatively that the molar ratio  $\rho \equiv [\text{Chlz}_{\text{D1}}^{+\bullet}]/[\text{Car}_{\text{D1}}^{+\bullet}]$  is approximately 1.7.

To consolidate the above estimate through an analysis of the bleaching signals around 680 nm in Figure 4, we assumed first that the absorption coefficients of  $\text{P}_{680}^{+\bullet}$  and  $\text{Chlz}_{\text{D1}}^{+\bullet}$  are negligible in this spectral region and, second, that the absorption spectrum of  $\text{Chlz}_{\text{D1}}$ , which is believed to absorb maximally at 672 nm (32), can be approximated by red-shifting the spectrum of Chla in an organic solvent. Each member of the set  $\Delta@d$  was modeled, in the 550–700 nm region, as a superposition of  $\Delta A(\lambda; 5 \mu\text{s})$  and  $Z_1(\lambda) \equiv C(\lambda - s)$ , where  $C(\lambda)$  is the absorption spectrum of Chla in 95% acetone and  $s$  (i.e., the magnitude of the wavelength shift) is chosen so as to bring the peak absorbance of  $Z_1(\lambda)$  at 672 nm. The correspondence between each experimental spectrum and its simulation, illustrated by the plots in Figure 6, is sufficiently close to justify placing some confidence in our spectral decomposition. Further support for the decomposition can be derived by comparing  $|\Delta_{672}^{\text{ZD1}}|$ , the amplitude of the bleaching of  $\text{Chlz}_{\text{D1}}$ , with  $\Delta_{910}^{\text{CarD1}^{+\bullet}}$ , the absorbance of  $\text{Car}_{\text{D1}}^{+\bullet}$ . If we take  $\epsilon_{672}^{\text{ZD1}} = 8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  for the peak molar absorption coefficient of  $\text{Chlz}_{\text{D1}}$ , we can calculate  $\rho$  by using the relation  $\rho = |(\Delta_{672}^{\text{ZD1}} \epsilon_{910}^{\text{CarD1}^{+\bullet}})/(\Delta_{910}^{\text{CarD1}^{+\bullet}} \epsilon_{672}^{\text{ZD1}})|$ , and it comes out to be  $2.1 \pm 0.3$ , in fair agreement with the crude value inferred earlier. Our two estimates indicate that about a third of the delocalized positive charge between  $\text{Chlz}_{\text{D1}}$  and  $\text{Car}_{\text{D1}}$  resides on the latter molecule. Our analysis of bleaching signal around 680 nm shows that the growth of  $\text{Chlz}_{\text{D1}}^{+\bullet}$  and the incomplete recovery of  $\text{P}_{680}$  parallel the growth of  $\text{Car}_{\text{D1}}^{+\bullet}$  which suggests that, at the longest delay, all three cationic species are almost in equilibrium. Equilibrium between Chlz and  $\text{P}_{680}$ , but in D2, has also been

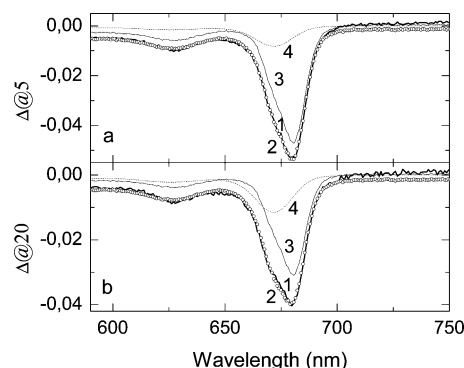


FIGURE 6: Absorption difference spectra (experimental and simulated) of the five-chlorophyll reaction center of spinach photosystem II in the presence of 0.5 mM silicomolybdate under anaerobic conditions: (a) 5 ms delay,  $\Delta@5$  and (b) 20 ms delay,  $\Delta@20$ . Experimental transient absorption difference spectrum (curve 1), linear combination (curve 2) of the transient absorption spectrum at 5  $\mu\text{s}$ ,  $\Delta A(\lambda; 5 \mu\text{s})$  (curve 3), and the absorption spectrum of  $\text{Chlz}_{\text{D1}}$  (curve 4, obtained by shifting the absorption spectrum of chlorophyll *a* in 95% acetone). For more details, see text.

observed at long delays in RC-6 when using SiMo as electron acceptor (33); the difference absorption spectrum displays a clear shoulder at 672 nm and resembles  $\Delta@30$ .

Our main conclusions may be summed up as follows: the missing pigments in RC-5 are  $\text{Chlz}_{\text{D2}}$  and  $\text{Car}_{\text{D2}}$ ; secondary electron transfer in D1, which comes to the fore only when  $\text{Chlz}_{\text{D2}}$  and  $\text{Car}_{\text{D2}}$  are both absent, has been observed for the first time; the absorption spectrum of  $\text{Car}_{\text{D1}}^{+\bullet}$  is blue-shifted with respect to that of  $\text{Car}_{\text{D2}}^{+\bullet}$ , and the positive hole is shared among  $\text{P}_{680}$ ,  $\text{Car}_{\text{D1}}$ , and  $\text{Chlz}_{\text{D1}}$ . Our study has revealed the possibility of an alternative electron transport for  $\text{P}_{680}^{+\bullet}$ , when the side electron transport in D2 is functionally impaired; this secondary electron-donation pathway in D1 is expected to occur with low yield in active PSII complexes and, therefore, not likely to play a major role in vivo.

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