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Biochimica et Biophysica Acta 1321 (1997) 21–30

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Topology of pigments in the isolated Photosystem II reaction center studied by selective extraction

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Received 10 March 1997; accepted 9 April 1997

Abstract

Pigments in the purified spinach Photosystem II reaction center (D1-D2-Cyt *b*-559) complex were extracted with diethyl ether containing varied amounts of water. The purified reaction center originally contained approximately six molecules of chlorophyll *a*, two β-carotene and two pheophytin *a* per one photochemically active pheophytin *a*. The treatment with 30–50% water-saturated ether extracted one β-carotene, as well as one chlorophyll *a* that absorbs at 677 nm, remaining 62% of the photochemical activity to reduce pheophytin *a*. With 60–80% water-saturated ether, almost all the β-carotenes were extracted, remaining the 49% activity without additional loss of chlorophyll. The absorption, fluorescence excitation and linear dichroism spectra demonstrated two spectral forms of β-carotene. The short-wavelength form of β-carotene with absorption peaks at 429, 458 and 489 nm was selectively extracted with ether at low water content, whereas the long-wavelength form with peaks at 443, 473 and 507 nm was extractable only at the higher water content. The extraction enhanced the photobleaching of chlorophylls. The results suggest that chlorophyll *a* forms with peaks at 667 and 675 nm are located close to the short-wavelength form of β-carotene that can transfer excitation energy to the photoactive pheophytin *a* on the D1 protein. © 1997 Elsevier Science B.V.

Keywords: Photosystem II; Reaction center; Carotenoid; Chlorophyll

Abbreviations: Car_{489} and Car_{507}, short and long-wavelength forms of β-carotene with longest-wavelength absorption peaks at 489 and 507 nm, respectively; CD, circular dichroism; Chl, chlorophyll; CP47, chlorophyll protein of 47 kDa; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; LD, linear dichroism; HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma; LHC, light harvesting complex; P-680, primary electron donor of Photosystem II; ^{T}P-680, P-680 triplet; P-680^{+}, P-680 cation; PS II, Photosystem II; Pheo a_{inact} and Pheo a_{act}, photochemically active and inactive forms of pheophytin *a*, respectively

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PII S0005-2728(97)00037-6

1. Introduction

The primary charge separation in Photosystem (PS) II of oxygenic photosynthesis takes place in the reaction center pigment-protein complex that consists of the D1 and D2 proteins [1,2]. The D1 and D2 proteins show some homologies in their amino acid sequences to each other and also to the L and M subunits of the purple bacterial reaction center, respectively, whose structure has been determined by X-ray crystallography [3–5]. The homology, together with the similarities in the organization of cofactors, suggests that these two photosystems have evolved from a common ancestor [6]. However, they differ from each other in the extent of oxidizing power generated and the numbers of pigment contained. The purple bacterial reaction center is known to have four molecules of bacteriochlorophyll, one carotenoid and two bacteriopheophytin [7]. On the other hand, typical preparations of PS II reaction centers, isolated to date, contain six molecules of chlorophyll *a* and two β-carotene per two molecules of pheophytin *a* [8,9].

There is some controversy as to the number of pigment molecules associated with the PS II reaction center [9–14], as well as to the assignment of the absorption bands of chlorophyll *a* and pheophytin *a* [15–21]. The localization and the function of the extra two molecules of chlorophyll *a* and one β-carotene, are also unclear at present. It is generally assumed that the two carotenoids in the PS II reaction center exhibit different spectral [22,23] and photochemical properties [24]. Recently, we reported that two molecules of β-carotene in the reaction center have been shown to be in the 15-*cis* configuration [25].

In the present study, we selectively extracted pigments from isolated spinach PS II reaction centers with ether containing different amounts of water. The extraction produced photochemically active PS II reaction centers remaining either one or no β-carotene molecule, with the concomitant loss of one chlorophyll *a*. We will discuss the localization and the function of individual β-carotene in the reaction center complex based on optical analysis.

2. Materials and methods

2.1. Preparation of PS II reaction centers

PS II reaction centers were isolated from spinach leaves as previously described [26], with replacement of the original detergent (Triton X-100) by 0.15% sucrose-monocaprate.

2.2. Extraction with ether

The extraction of pigment from the PS II reaction centers with diethyl ether was essentially performed as previously described in the extraction of the PS I reaction center [27,28]. The PS II reaction centers were washed twice with distilled water, carefully lyophilized and stored at −80°C under nitrogen atmosphere. The lyophilized powder was extracted at 4°C with ether that contained varied concentrations of water. The water content of ether was adjusted by mixing appropriate amounts of ether dehydrated by the addition of anhydrous Na_2SO_4 with the water-saturated ether that contained approximately 1.0% (w/w) water on ice. The extracted pigments were removed by centrifugation at $37\,000 \times g$ for 5 min. This procedure was repeated once more. The resultant ether-extracted PS II reaction centers were dried and solubilized by a 30-min incubation on ice with a medium containing 50 mM HEPES-NaOH buffer (pH 7.5) and 0.3% sucrose-monocaprate.

2.3. Quantitation of pigments and the other components

Analysis of pigments in the PS II reaction centers by HPLC was performed as described elsewhere [8] using a normal phase column: Senshupak Silica-1251N (4.6 mm i.d. × 250 mm; Shimadzu, Kyoto) at or below 4°C with hexane, isopropanol, methanol (100:0.8:0.4, v/v), as an eluent at a flow rate of 1 ml/min. Extinction coefficients of 135 000 (at 453 nm in 100% hexane), 81 300 (at 661.6 nm in 100% acetone) and 46 000 (at 665.9 nm in 100% acetone) $M^{-1} \cdot cm^{-1}$ were used for β-carotene, chlorophyll *a*

and pheophytin *a*, respectively [29]. Cytochrome *b*-559 was spectroscopically determined using a reduced-minus-oxidized difference millimolar absorption coefficient at 559 nm of 15 cm^{-1} [30]. The total metal content of PS II reaction centers was estimated by inductively coupled plasma (ICP) atomic emission spectroscopy with a model SPS2100A spectrometer (SEIKO Instruments, Tokyo).

2.4. Spectroscopic analyses

Absorption spectra were recorded with Carry 5G (Varian, Mulgrave) and Miltonroy 3000 Array spectrometers (Spectronic, Rochester). Linear dichroism (LD) spectra were recorded at 283 K by the gel-squeezing method with a J-200B spectropolarimeter (JASCO, Tokyo). The concentrations of acrylamide and bisacrylamide were 12% and 0.24%, respectively, and the gel was placed in a custom-made cuvette holder [31]. Circular dichroism (CD) spectra were also recorded at 283 K with the model J-200B spectropolarimeter. Fluorescence spectra at 77 K were measured with a Hitachi 850 spectrofluorometer.

2.5. Measurement of photochemical activity and flash spectroscopy

The photochemical accumulation of the reduced photoactive pheophytin (Pheo a^-_{act})was monitored by the absorption change induced by continuous irradiation with saturating light from a xenon lamp (500 W) at 277 K with a Hitachi-557 spectrophotometer equipped with a cross-illumination system. The reaction mixture contained methyl viologen (2 μM) and sodium-dithionite (2 $mg \cdot ml^{-1}$).

Time-resolved nanosecond difference absorption spectra were recorded as described elsewhere at 77 K [32]. Samples mixed with glycerol (to give a final concentration of 60%) in a 1 cm × 1 cm × 4 cm cuvette were excited by a 532-nm flash, with a 10-ns half-peak width, from a frequency-doubled Nd-YAG laser at almost saturating intensity (8 $mJ \cdot cm^{-2}$). The difference absorption spectra were obtained with a spectrograph that was equipped with a combination of a microchannel plate and a multichannel photodiode array with a wavelength accuracy of 1 nm. The microchannel plate was activated for 5 ns at the desired time after or before the laser peak. The chlorophyll *a* fluorescence excited by a laser flash was also detected and subtracted in the calculation of the absorption change.

2.6. Photobleaching

Irreversible absorption change induced by a continuous actinic illumination was recorded by a Miltonroy 3000 spectrometer at 290 K. Red actinic light was obtained from a slide projector with a 650-W tungsten lamp through R-66 (Toshiba, Tokyo) and HA-50 (far-red and UV cutoff; HOYA, Tokyo) filters at 110 μmol photons $\cdot m^{-2} \cdot s^{-1}$.

3. Results

3.1. Ether-treated PS II reaction centers

When the lyophilized PS II reaction centers were treated with ether, the amounts of pigments extracted increased in a step-wise manner as the increase in the water content of ether. The extracted reaction centers showed different absorption spectra as shown in Fig. 1a–c. The absorption due to β-carotene, in a 430–530 nm range, dramatically decreased with the increase in the water content in ether, and almost all the β-carotene molecules were extracted by treatment with ether at 60% water saturation. The treatment also decreased the Q_y peak of chlorophyll *a* by 18%. This was accompanied by a 2–3 nm blue shift of the main absorption peak. However, neither the peak wavelengths of carotenoid, pheophytin and P-680^+ pheophytin anion (Pheo a^-_{act}) radical, nor the CD spectra at the UV region were altered by the treatment as described below. The apparent blue shift of the Q_y band seemed to be due to the removal of a chlorophyll *a* form that absorbed around 677 nm, as shown by the difference spectrum (Fig. 1d,e). The extent of pigment extraction did not depend on the duration of extraction, and the repeated extractions at the same water content resulted only in a small additional release of pigments.

Using millimolar absorption coefficients for chlorophyll *a* of 81.3 at 661.6 nm in acetone [29] and β-carotene of 135 at 453 nm in hexane [33], respectively, the number of β-carotene molecules remaining in the reaction centers was determined to be

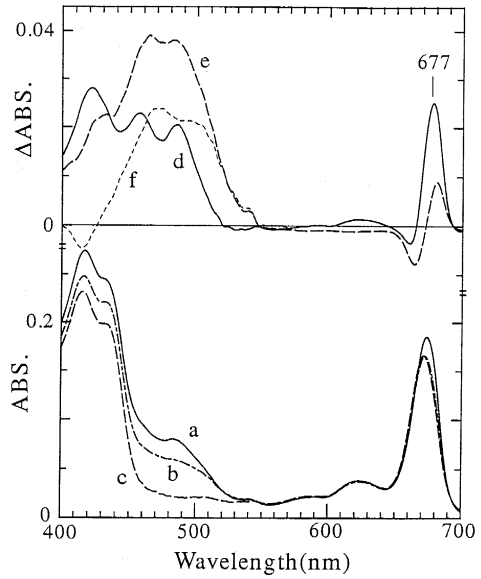

Fig. 1. Absorption spectra of the ether-extracted PS II reaction centers at 290 K. Lower panel: absorption spectra of PS II reaction centers extracted with (a) 0%; (b) 30%; and (c) 60% water-saturated ether. The upper panel shows the difference absorption spectra d = a − b and e = b − c. Broken line f represents the spectrum of long-wavelength form of β-carotene between 400 and 570 nm, calculated as e − d × 0.8. A negative change at 415 nm in a trace f represents the uncompensated chlorophyll bleaching.

almost zero, one and two per reaction center (per two molecules of pheophytin *a*), for the preparations treated with 60, 30 and 0% water-saturated ether, respectively. Further quantitation of pigments was performed by HPLC, as shown in Fig. 2. The starting material contained 6.23 ± 0.26 molecules of chlorophyll *a* and 2.18 ± 0.24 molecules of β-carotene per two molecules of pheophytin *a*. The free chlorophyll *a*, i.e., nonfunctional in terms of energy transfer, was estimated to be less than 2% of the total chlorophyll in this type of preparation [23]. The extraction with dehydrated ether (0% water-saturation) did not change the pigment composition. However, upon extraction with 20–50% water-saturated ether, the amount of β-carotene decreased to 1.08 ± 0.15 molecules per molecules of two pheophytin *a*. Further increase in the water content of the ether, to more than 60% saturation, resulted in complete loss of β-carotene from the reaction center. The ICP atomic emission spectroscopy gave a 4.87 ± 0.09:1 ratio of Mg (chlorophyll *a*) to Fe (cytochrome *b*-559) in the preparations that had been treated with 30–70% water-saturated ether (no non-heme iron remains in the isolated reaction center).

The number of cytochrome *b*-559 in the PS II complex has been a subject of debate and reported to be between 1 and 2 per two pheophytin [9]. We previously estimated the cytochrome *b*-559 content to be one per reaction center based on optical [34] and ICP analysis in the present preparation (data not shown). The stoichiometry among cytochrome *b*-559 and other constituent subunits was also analyzed based on the amino-acid composition of the isolated PS II reaction center and the deduced primary sequence of each subunit. This analysis indicated that cytochrome *b*-559 and other constituents are present in equimolar ratios in the complex (data not shown). Therefore, the 4.9:1 ratio of chlorophyll *a* to cytochrome *b*-559 (that was contained at 1 molecule per reaction center), was consistent with the estimations by HPLC and optical absorption analysis. The PS II reaction center treated by ether with different water content lost neither pheophytin *a* nor cytochrome *b*-559.

The change in secondary structure of the PS II

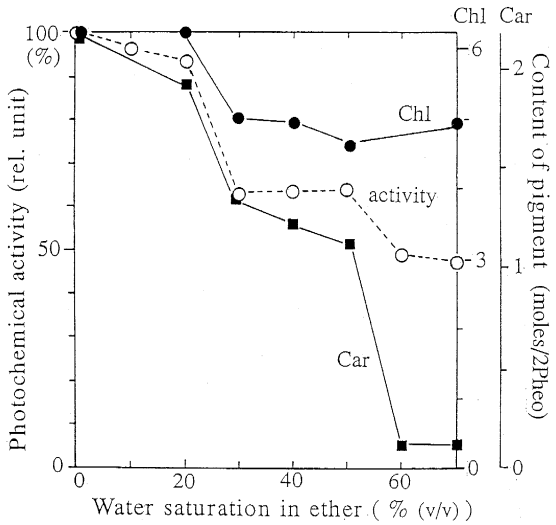

Fig. 2. Pigment contents and the activity of photochemical reduction of pheophytin (accumulation of Pheo a^-_{act}) in PS II reaction centers treated with ether with different water content. Closed circles and squares, numbers of chlorophyll and carotenoid, respectively, expressed per two pheophytin *a*. molecules. Open circles, Pheo a^-_{act} photo-accumulation activity measured at 290 K.

reaction center was checked by the CD spectra in the UV region. The 208 nm (n–π^* transition) and 222 nm (n–π^* transition) negative peaks in the CD spectra at 283 K of the ether-treated PS II reaction centers showed almost no differences after the ether treatments (data not shown). Thus, the extraction of β-carotene molecules does not seem to have significantly altered the tertiary structure of the PS II reaction center as also expected from the little changes in the peak positions of carotenoid, pheophytin, and the time-resolved transient spectra.

SDS-PAGE pattern of the ether-treated reaction centers revealed the polypeptide bands of the D1 and D2, the α and β of cytochrome *b*-559 and the product of the *psbI* gene, with no appreciable changes in molecular mass or evidence of degradation products (data not shown).

3.2. Electron transfer activities

The photo-accumulation of the anionic radical of photoactive pheophytin (Pheo a_{act}^-) was measured under steady-state illumination at 277 K (Fig. 2). Treatments with dry ether affected neither kinetics nor the extent of accumulated Pheo a_{act}^-. However, the amount of Pheo a_{act}^- decreased to 62 and 49% of the control after the treatment with ether at 30 and 60% water saturation, respectively. It is noted that the PS II reaction centers with practically no β-carotene retained substantial photochemical activity.

In order to examine the efficiency of primary charge separation, the PS II reaction center was excited at 77 K by a 532 nm laser flash, and the transient differences in absorption in the Q_y region were measured (Fig. 3). A transient change with a negative peak at 680 nm was observed at 10 ns. It can be ascribed to the flash-induced formation of P-680$^+$ and Pheo a_{act}^- [35,36]. Pheo a_{act}^- gave an absorption decrease also at 543 nm as reported [35,36] (data not shown). At 1 μs, a peak was detected at 681.5 nm which can be ascribed to the formation of P-680 triplet (^{T}P-680) produced in the charge recombination reaction between P-680$^+$ and Pheo a_{act}^-. The P-680 content, which was estimated from the extent of ^{T}P-680 induced upon the excitation by a flash of saturating intensity, decreased to 53% of the control, in both the one-carotene and zero-carotene preparations. The shape of the time-resolved absorption spectra and the kinetics did not change, although the number of chlorophyll *a* molecules decreased from six to five and that of β-carotene from two to one or zero in these preparations.

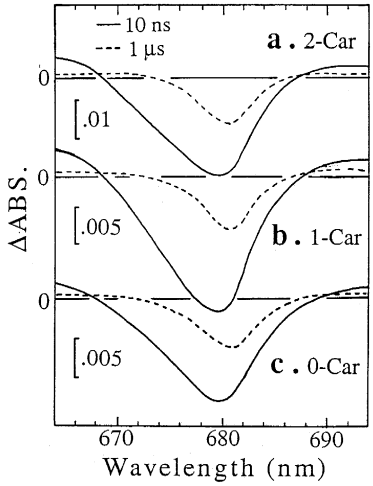

Fig. 3. Time-resolved difference absorption (ΔABS) spectra of the PS II reaction center at 77 K. a: two-carotene reaction centers; b: one-carotene reaction centers; c: zero-carotene reaction centers. Vertical bars and numbers in the figure represent units of absorbance change. See Section 2 for details of the measurements. Solid and broken lines represent ΔABS at 10 ns and 1 μs, respectively.

The 686 nm fluorescence of the PS II reaction center decayed in two phases with 3.5 and 50 ns decay times, respectively, at 77 K. The slow phase corresponded to the time for the charge recombination between P-680$^+$ and Pheo a_{act}^- [37,38]. The ether-treated PS II reaction centers yielded the same time constants as those of the untreated controls (data not shown).

3.3. Two spectral forms of β-carotene

Some reports suggested the presence of two spectral forms of β-carotene, i.e., long- and short-wavelength forms in vivo in the PS II reaction center based on the studies of fluorescence excitation spectra [22,23] or photochemical analysis [24]. The curve d in Fig. 1 shows the difference absorption spectrum between the spectra of one- and two-β-carotene preparations. This spectrum clearly reveals that the short-wavelength form of β-carotene which absorbs at 458 and 485 nm was selectively extracted with

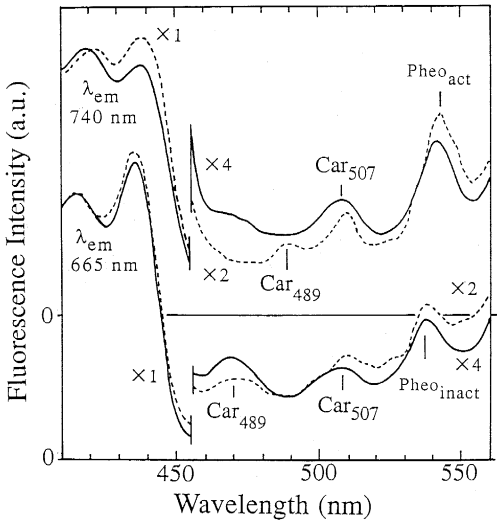

Fig. 4. Fluorescence excitation spectra of one- and two-β-carotene containing PS II reaction centers at 77 K. Upper and lower panels represent excitation spectra for 740 and 665 nm, respectively. Solid and broken lines represent the spectra of two- and one-carotene reaction centers, respectively. Intensity was normalized at the Soret peak in each case, and the intensity between 455 and 560 nm was multiplied as shown in the figure. Peaks of the short- and long-wavelength forms of β-carotene (Car-489 and Car-507, respectively) and those of pheophytins on the active and inactive branches of pigments (Pheo a_{act} and Pheo a_{inact}) were also indicated by vertical bars.

ether at low water content. The peaks shift to the shorter wavelength on cooling as shown by the linear dichroism spectrum in Fig. 5 so that this β-carotene was designated as Car_{489} after its longest peak position at 77 K. The peak wavelengths correspond to those previously estimated [22,23]. By contrast, the difference spectrum in the 400–500 nm region of one-carotene minus zero-carotene reaction centers reveals a longer-wavelength form of β-carotene that has peaks at 473 and 507 nm (designated as Car_{507}) in addition to the peaks of Car_{489} (curve e, but see also curve f in Fig. 1). The result indicates that Car_{507} can be extracted only with ether containing a higher amount of water.

In order to confirm the selective extractions of two spectral forms of β-carotene, we recorded fluorescence excitation spectra at 77 K and the linear dichroism (LD) spectra at 283 K. In the fluorescence excitation spectra (Fig. 4), a peak at 489 nm, which corresponds to the longest peak of Car_{489}, disappeared in the preparation treated with ether at low levels of water content. The LD spectrum (Fig. 5) for the two-carotene preparation showed two types of β-carotenes with positive peaks absorbing at 445, 475 and 507 nm and with negative peaks at 460 and 489 nm, respectively. They correspond to Car_{507} and Car_{489} previously postulated, respectively. In the one-carotene reaction center, only positive peaks of Car_{507} at 443, 472 and 507 nm were observed (Fig. 5). These data confirm that Car_{489} and Car_{507} are selectively extracted by the ether treatments with low- and high-water contents, respectively. The Car_{489} and Car_{507} can be estimated to be oriented parallel and perpendicular to the membrane surface from their negative/positive LD values as previously estimated [22].

The untreated PS II reaction centers gave a fluorescence emission maximum at 683 nm at 77 K as previously reported [22,23,39] upon excitation either at 435 nm (the chlorophyll peak) or at 415 nm (the pheophytin peak). The fluorescence excitation spectra did, however, differ depending on the monitoring wavelength (Fig. 4, solid curves). Moreover, they gave extents much lower than the extents in the absorption spectra in the carotenoid region, indicating that β- carotenes have low efficiency in energy transfer to P-680 and that their major roles are protection

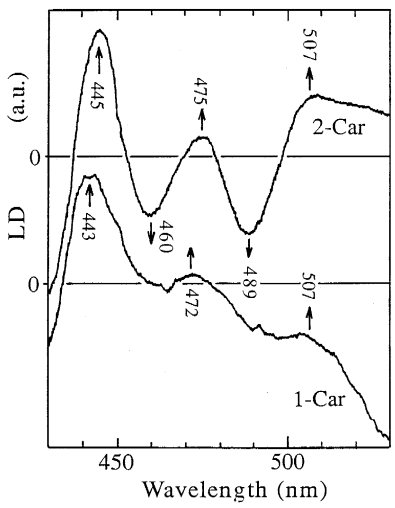

Fig. 5. Linear dichroism spectra of the PS II reaction center containing two- (upper panel) and one-β-carotene molecules (lower panel) at 290 K. Down and up arrows show the peak positions of short- (Car-489) and long-wavelength (Car-507) forms of β-carotene molecules, respectively.

from photodamage through the quenching of singlet oxygen [40]. When fluorescence was monitored at 740 nm (in the vibrational bands of chlorophyll/pheophytin fluorescence) or at 685 nm (in their Q_y peaks, data not shown), excitation peaks were observed at 489, 507 and 542 nm (Fig. 4 upper panel, broken lines). The 542 nm peak corresponds to the Q_x band of Pheo a_{act}. By contrast, the excitation spectra of fluorescence at shorter 665 nm (Fig. 4 lower panel) showed pronounced peaks at 538 nm corresponding to the photoinactive, short-wavelength form of pheophytin *a* (Pheo a_{inact}) and at 470 and 507 nm Car_{507} peaks, but showed no 489 nm peak of Car_{489}. Therefore, it is confirmed that Car_{489} more significantly contributes to the fluorescence at 740 nm where the longer-wavelength form of pheophytin *a* (Pheo a_{act}), which has a 542 nm Q_x peak and is known to be the primary electron acceptor [35,36], has higher contribution as previously reported [23]. On the other hand, Car_{507} seems to transfer energy to Pheo a_{inact} that has a Q_x peak at 538 nm and gives fluorescence in the shorter 665 nm region.

In the one-carotene preparation, the contribution of Car_{507} was also observed at 470 and 507 nm in the excitation spectrum of fluorescence either at 665 or at 740 nm (solid lines in Fig. 4). However, no peaks around 489 nm of Car_{489} were detected in both spectra. The fluorescence emission spectra suggest that Car_{489} can be selectively extracted by ether with a low water content, whereas Car_{507}, which is more resistant to the extraction, seems to preferentially transfer energy to Pheo a_{inact}. When monitored at 665 nm, the relative height of the pheophytin a peak in the Soret region at around 415 nm was lower than that in the spectrum monitored at 740 nm. This result suggests that the energy transfer from Pheo a_{inact}, which gives relatively strong fluorescence in the 665 nm region, to chlorophyll *a* or to Pheo a_{act} might be rather inefficient in this type of reaction center. One interpretation of the results is that the easily extractable Car_{489} is located in the vicinity of Pheo a_{act} that seems to be situated on the active branch on the D1 protein of the PS II reaction center (see Fig. 7).

3.4. Photobleaching of chlorophylls

We measured photobleaching of pigments in the ether-treated PS II reaction centers under aerobic

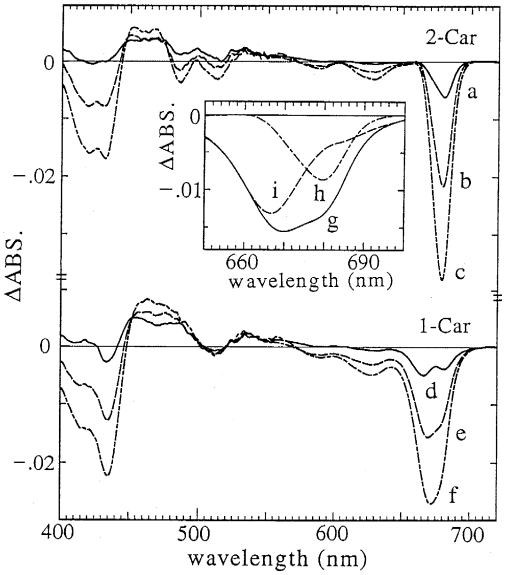

Fig. 6. Irreversible photobleachings of PS II reaction centers that contained two (upper) and one molecules of β-carotene (lower) at 290 K. Difference spectra for irreversible absorption changes in samples illuminated for 1 (a,d), 3 (b,e) and 5 min (c,f). Inset: g, same as d; h, b×0.4. i, g, h. Other experimental conditions were similar to those in Fig. 1.

conditions with no electron donors and acceptors. In the untreated two-carotene reaction center (curves a–c in Fig. 6), rapid bleaches were observed at 680 nm in the Q_y region of chlorophyll/pheophytin and at 489 and 507 nm of the carotenoid region. The latter two peaks may be ascribed to the oxidation of Car_{489} and Car_{507}, respectively, as suggested by Telfer et al. [24]. In the one-carotene reaction center (curves d–f and inset in Fig. 6), photobleaching of a new bleach at 667 nm was detected in addition to the bleach at 680 nm and that at 507 nm of Car_{507}. After prolonged illumination, an additional bleaching with a peak at 675 nm also developed as seen by the filling of the trough between the 667 and 680 nm bands in the difference spectrum at 5 min (deconvoluted data were not shown). The zero-carotene reaction center yielded the same results, although the 507 nm peak was not detected and the extent of bleachings were a little greater (data not shown). The absorption increase around 460 nm was probably caused by the generation of breakdown products of chlorophyll *a* and/or carotenoid. These results confirm the selective extraction of carotenoids.

In the absence of oxygen, the extent of photo-

bleachings in the two- and one-carotene preparations decreased to be 1/2.9 or 1/4.7 of those under aerobic conditions, respectively. The bleaching of chlorophyll *a* at 667 nm, therefore, was only observed in the presence of oxygen. According to Telfer et al. [41], singlet oxygen, generated by ^{T}P-680 under strong illumination, is preferentially quenched by β-carotene. Thus, it seems likely that the 667 nm chlorophyll *a* band is located near Car_{489}.

4. Discussion

In the PS II reaction center, the number of chlorophyll molecules in the complex has been a subject of controversy over the past decade, and their localizations are not yet clear [9–14]. The purified PS II reaction center preparation used in the present study has six chlorophyll *a* and two carotenoid molecules. The sequence homology of the constituent D1 and D2 proteins, as well as the structural and functional organization of the reactants on the acceptor side, predicts that the organization of pigments in this reaction center should be basically similar to that in the purple bacterial reaction center [9]. The reaction centers of the purple bacteria contain four molecules of bacteriochlorophyll and two bacteriopheophytin, which are arranged with pseudo-C_2-symmetry [3–5]. The reaction center also contains one molecule of carotenoid residing close to the 'accessory' bacteriochlorophyll on the inactive branch on the M subunit [3]. It appears that the PS II reaction center contained two more chlorophylls and one more carotene molecule in addition to the pigment set required for the purple bacterial-type reaction center.

In the present study, pigments were selectively extracted from the purified PS II reaction center with ether that contained different amounts of water. A possible assignment of the six molecules of chlorophyll *a* per two pheophytin is as follows: two correspond to the primary donor (P-680); the other two correspond to the accessory chlorophylls, as in the purple bacterial counterpart; and the remaining two seem to function as antenna. Some authors have claimed that contaminating CP47 is the origin of one or two molecules of chlorophyll *a*, so that the number of chlorophyll *a* molecules in the PS II reaction center is actually four, as it is in purple bacteria [12,13,42]. However, we did not detect contaminating proteins in our preparations, even on overloaded gels and western blots (Wolfgang Schröder, personal communication). Spectroscopic analysis also demonstrated the presence of six molecules of chlorophyll *a* in this preparation [23].

The ether treatment selectively extracted one molecule of chlorophyll *a* that absorbed at 677 nm, as well as one or two molecules of β-carotene. The extracted chlorophyll *a* might have contained a small amount of P-680 because we detected a 38% decrease in photoactivity. However, the spectral shape of the P-680^{+}pheo a^{-}_{act} biradical state and its decay kinetics did not change, suggesting that the released chlorophyll might not have been directly associated with the primary photochemistry. The extraction of PS I particles even with the 0%-water-saturated diethyl ether depleted almost all the carotenoids as well as phylloquinone and majority of antenna chlorophylls [27]. This indicates the different environments of carotenoids in the PS I and PS II reaction centers. Whereas, the ether-extraction in PS I neither extracted the reaction center or electron acceptor chlorophylls nor affected the activity of the primary charge separation. [27]. These results suggest that the pigments in the central core portions in both types of reaction centers are resistant to extraction with ether.

It has been reported that two spectroscopically different β-carotene molecules exist in the PS II reaction center [22,23]. One is the short-wavelength form that absorbs at 429, 458 and 489 nm denoted as Car_{489} in this work, and the other is the long-wavelength form Car_{507} that absorbs at 443, 473 and 507 nm. Some authors detected only one molecule of β-carotene in the reaction center and still indicated the presence of heterogeneous spectral forms of β-carotene [10,12]. PS II reaction centers prepared with Triton contained one molecule of β-carotene per reaction center and still showed two spectral forms of β-carotene when inspected by LD and fluorescence excitation spectra (data not shown). In the present study, Car_{489} and Car_{507} were selectively extracted by treatments with 30–50% and more than 60% water-saturated ether, respectively, whereas it appears that they can be extracted non-selectively by the detergent-treatment [10–12].

Mimuro et al. [23] suggested that Car_{507} transfers energy to the pheophytin (Pheo a_{inact}) on the inactive

branch of pigments in the reaction center. The fluorescence excitation spectra in Fig. 4B indicated that Pheo a_{inact} is energetically coupled to Car_{507}. On the basis of the structural analogy between PS II and the reaction center of purple bacteria, the inactive branch of pigments can be estimated to be located on the D2 protein. Therefore, Car_{507} may be also located on the D2 protein because the energy transfer from β-carotene to other pigments occurs only in a short distance [23]. This estimation is further supported by the result that the direction of the dipole moment of Car_{507}, estimated by LD spectroscopy, is consistent with that of the carotenoid on the M subunit, which is a counterpart to the D2 protein, in the reaction center of purple bacteria. On the other hand, Car_{489} might be located on the D1 protein in a position symmetrical to that of Car_{507}, in view of the predicted molecular topology of the prosthetic groups in the PS II reaction center. This β-carotene has no counterpart in the reaction center of purple bacteria.

The selective removal of β-carotene from the reaction center is expected to accelerate the photo-bleaching of chlorophyll *a* because β-carotene is known to quench the singlet oxygen that is generated at the reaction center [41,43,44]. After the removal of Car_{489} by the ether treatment, photobleaching of the chlorophyll *a* that absorbed at 667 nm (Chl_{667}) was induced under aerobic conditions (Fig. 6). Chl_{667} may, therefore, be in close proximity to Car_{489}. As the progression of light treatment, the bleaching at 675 nm (Chl_{675}) also became marked in the PS II reaction center with zero or one β-carotene molecule, but not in the two β-carotene reaction centers; Chl_{675} may, thus, also be located in close proximity to Car_{489} and might be located on the D1 protein together with photoactive Pheo a_{act}. Chl_{667} and Chl_{675} are also present in the deconvoluted Q_y spectra of the PS II reaction center [19]. The spectral hole burning study at 1.2 K [38] and the sub-picosecond kinetics study at 77 K [39] also indicated that the excited states of chlorophylls with peaks at 669, 675 and 676–679 nm decay with life times of 12 ps, 200 ps and 475 fs, respectively. We can estimate the assembly of pigments in the PS II reaction center by summarizing the results in the present study; Car_{489}, Pheo a_{act} and Chl_{667} reside on the D1 protein, whereas Car_{507} and Pheo a_{inact} reside on the D2 protein (see Fig. 7). Chl_{675} might also be located on the D1 protein. The precise assignment of their localization a waits the improvement of structural analysis.

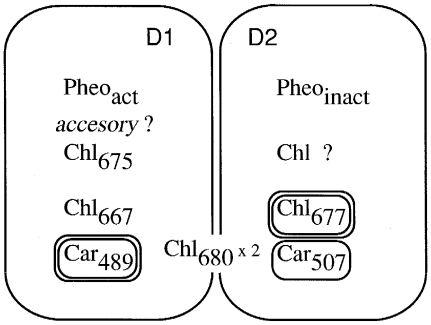

Fig. 7. Proposed arrangement of pigments in D1 and D2 polypeptides. Car-489 and Chl-677 were extracted by 30% water-saturated ether and Car-507 by 60% water-saturated ether.

The spectral hole burning study at 1.2 K [45] and sub-picosecond kinetics study at 77 K [46] indicated that the excited states of chlorophylls with peaks at 669, 675 and 676–9 nm decay with life times of 12 ps, 200 ps and 475 fs, respectively. If the life time of the excited state of Chl_{667} is around 12 ps, Chl_{667} is estimated to function as an antenna located outside the electron transferring pigment pathway in D1 protein rather than as one of the accessory chlorophylls adjacent to P-680. On the other hand, Chl_{677}, which was easily extracted with ether without serious loss of activity, can also be assumed to be outside the core portion of the reaction center. It is, thus, an attractive idea to assume that Chl_{667} is located in the D1 protein outside the core moiety of the reaction center, in a position symmetrical to that of Chl_{667} which might be on the D2 protein. Based on the above estimations, we summarized the results in this study in Fig. 7.

Acknowledgements

The authors thank Dr. A. Murakami (NIBB, Japan) for performing the ICP spectroscopy and the Instrument Center of NIBB, for use of the spectrophotometer and spectrofluorometer. T.T. is a recipient of a pre-doctoral fellowship from JSPS. The work was supported by Grants-in-Aid for Scientific Research in Priority Areas (no. 04273101 to K.S. and no. 08218261, 08228225, 08247216, 08249244 to S.I.), for General Research (no. 0604003 to K.S., no.

08836013 to M.M. and no. 08836001 to M.K.) from the Japanese Ministry of Education, Science, Culture and Sports and by a grant from the Human Frontier Science Program to K.S.

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