

## Photochemistry and spectroscopy of a five-chlorophyll reaction center of photosystem II isolated by using a Cu affinity column

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**ABSTRACT** A reaction center of photosystem II was isolated from *Pisum sativum* by using immobilized metal affinity chromatography. This reaction center is photochemically active and has a room temperature  $Q_y$  chlorophyll (Chl) absorption band peaking at 677.5 nm. From HPLC analysis, the pigment stoichiometry was suggested to be 5 Chls per 1  $\beta$ -carotene per 2 pheophytins. Low-temperature absorption measurements at 77 K were consistent with the removal of one of the Chls associated with the usual form of the reaction center isolated by using ion-exchange chromatography. Transient absorption spectroscopy on the picosecond time scale indicated that the Chl removed belongs to a pool of Chl absorbing at  $\approx 670$  nm ( $C670_H$ ) that transfers energy relatively slowly to the primary donor P680 in support of our recently proposed model. The results also support the previous conclusion that radical pair formation is largely associated with a 21-ps time constant when P680 is directly excited and that the identity of  $C670_H$  is likely to be peripherally bound Chls possibly ligated to conserved His residues at positions 118 on the D1 and D2 proteins.

Photosystem II (PSII) is that part of the photosynthetic apparatus that oxidizes water to produce the hydrogen equivalents required to reduce carbon dioxide to organic substances. Dioxygen is the by-product of this light-driven process. A reaction center of PSII consisting of the D1 and D2 proteins, the  $\alpha$  and  $\beta$  subunits of cytochrome  $b_{559}$  (Cyt  $b_{559}$ ), and the product of the *psbI* gene was first isolated by Nanba and Satoh (1). Since then similar preparations have been isolated by a number of workers using different materials and slightly different procedures (2–6). Although most of the procedures used involved solubilization with Triton X-100, it has emerged that *n*-dodecyl  $\beta$ -D-maltoside (DM) was helpful for subsequent stabilization of the isolated complex (5, 7). In its most active and stable form, the isolated complex was shown to bind 6 chlorophylls (Chls) per 2  $\beta$ -carotenes per 2 pheophytins (Phs) (8, 9). Recently, it has been reported that the number of Chls bound to the reaction center complex can be reduced to 4 by extensive washing of the preparation when bound to an ion-exchange column (10, 11) and such preparations have been used for spectroscopic analyses (11–13). Curiously, the 6- and 4-Chl preparations reported showed little or no wavelength shift in their room temperature optical absorption in the long-wavelength region (13). Moreover, extensive efforts to repeat the published procedure did not yield the 4-Chl preparation (ref. 14 and C. Eijkelhoff and J. P. Dekker, personal communication), although it did remove  $\beta$ -carotene (15). The uncertainty of the pigment level in the isolated complex becomes a key issue when interpreting subpicosecond and picosecond transient absorption and emission data (16–22). For example, several groups have reported relatively slow kinetics (tens of picoseconds) in isolated PSII reaction centers

that have been assigned to energy transfer from accessory Phs and Chls to the primary donor P680 (17, 19–24). It has been further suggested that the slow energy transfer processes are associated with two additional Chls bound to the periphery of the reaction center complex (21, 24). Moreover, the presence of these slow energy transfer processes has made the determination of the time constants for primary charge separation more difficult, leading to controversy (16, 21, 24–28). It is therefore particularly interesting to compare the primary photochemistry of the 6-Chl-containing PSII reaction center with those in which 1 or 2 of the peripheral Chl molecules have been removed. In this paper, we report a procedure for isolating the reaction center of PSII by using immobilized metal affinity chromatography. HPLC analysis indicated that the complex contained 5 Chls per 2 Phs and, relative to that of the 6-Chl-containing preparation, showed a spectral shift indicative of the removal of an accessory Chl absorbing on the blue side of the main  $Q_y$  band. The role of this Chl as an accessory pigment was confirmed by time-resolved picosecond absorption spectroscopy.

### MATERIALS AND METHODS

**Preparation of PSII Reaction Centers.** Standard reaction center preparations containing  $\approx 6$  Chls and 2  $\beta$ -carotenes per 2 Phs were isolated from peas (*Pisum sativum*) as described by Chapman *et al.* (7). For our procedure, PSII-enriched membranes were prepared from peas as described (29) and washed with 2 M  $\text{CaCl}_2$ /50 mM Mes at pH 6.5. After resuspension of Chl in 50 mM Mes (pH 6.5) at 2 mg/ml, the calcium-washed PSII-enriched membranes were solubilized with Triton X-100 at a detergent/Chl molar ratio of 50:1 (corresponding to a mixture of 3 vol of PSII-enriched membranes with Chl at 2 mg/ml and 1 vol of 30% Triton X-100). The mixture was stirred at 4°C in the dark for 120 min and the nonsolubilized material was removed by a 30-min centrifugation at  $40,000 \times g$ . The supernatant was loaded on a 10 mm  $\times$  100 mm Cu affinity chromatography column at a flow rate of 4 ml/min. The loaded column was washed at a flow rate of 2 ml/min with 50 mM  $\text{Na}_2\text{HPO}_4$ /50 mM NaCl/0.2% Triton X-100/1.2 mM DM at pH 6.5 until the  $A_{435}/A_{417}$  ratio dropped to  $<1$ . At this stage, the reaction center complex was eluted from the column with 5 mM imidazole/50 mM Mes/2 mM DM at pH 6.5. When required, the eluted reaction center preparation was concentrated by using Amicon Centricon-100 tubes.

**Preparing the Cu Affinity Column.** Fast-flow chelating Sepharose (Pharmacia) was washed with distilled water and packed in a 10 mm  $\times$  100 mm column. The column was flushed thoroughly with distilled water and then with 10 ml of 0.1 M  $\text{CuSO}_4$ . The nonbound copper was removed by washing with

Abbreviations: Chl, chlorophyll; Cyt  $b_{559}$ , cytochrome  $b_{559}$ ; DM, *n*-dodecyl  $\beta$ -D-maltoside; Ph, pheophytin; PSII, photosystem II.

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100 ml of distilled water. Finally, the column was equilibrated with 50 ml of 50 mM  $\text{Na}_2\text{HPO}_4$ /50 mM NaCl/0.2% Triton X-100/1.2 mM DM at pH 6.5. After isolation of the PSII reaction centers, the column was cleaned by washing with 50 ml of 0.5 M NaOH at a flow rate of 2 ml/min, followed by 50 ml of distilled water, and equilibrated again with the phosphate buffer above.

**Spectroscopy.** All measurements were carried out in 50 mM Mes/5 mM imidazole/2 mM DM at pH 6.5. Anaerobic conditions were obtained as described (15). Steady-state absorption spectra were measured by using an SLM Aminco (Urbana, IL) model DW2000 spectrophotometer and for 77 K spectra, glycerol was added to the sample to 60% (vol/vol). Steady-state and transient light-induced optical absorption changes were measured with a Perkin-Elmer model 554 spectrophotometer fitted with side illumination (2, 7) and a nanosecond absorption system (30) but with the addition of a dye laser or an ultrafast laser system with femtosecond resolution (24, 25). In the latter cases, the experiments were conducted at 10°C and the steady-state measurements were carried out at room temperature. All studies were conducted under anaerobic conditions unless stated otherwise. Picosecond absorption difference spectra were collected at 462-fs intervals over a period of 0–70 ps by using excitation pulses centered at 665 nm or 694 nm. Data collected between 640 nm and 730 nm (spectral resolution of 0.7 nm) were globally analyzed as a sum of exponentials. Data were collected for both parallel and magic-angle configurations of the pump and probe polarizations. The same conclusions could be obtained from data collected using either configuration (see also ref. 24). However, contributions to the data from the slow energy transfer pigments can be more readily resolved by using the parallel configuration (24), and therefore, only data collected by using this configuration is shown here.

**Electrophoresis.** SDS/PAGE was performed with the buffer system of Laemmli (31) and 10–17% gradient gel containing 6 M urea. Gels were stained with Coomassie blue.

**HPLC and Pigment Analysis.** HPLC analysis was carried out by a modification of the method as described (32). Pigments were extracted into 80% (vol/vol) acetone and filtered through a 0.2- $\mu\text{m}$  (pore) membrane [poly(vinylidene difluoride), Whatman] before injection. An ODS1 reverse-phase column (Spherisorb) and isocratic elution with methanol/ethyl acetate, 68:32 (vol/vol), at a flow rate of 1 ml/min were used. All pigments were detected in the same sample by measurement at 663 nm for Chl *a* and Ph *a* and at 450 nm for  $\beta$ -carotene. The HPLC was calibrated with pure pigments whose concentrations were measured in the methanol/ethyl acetate mobile phase with extinction coefficients of 86.9  $\text{mM}^{-1}\cdot\text{cm}^{-1}$  for Chl *a*, 49.3  $\text{mM}^{-1}\cdot\text{cm}^{-1}$  for Ph *a*, and 135  $\text{mM}^{-1}\cdot\text{cm}^{-1}$  for  $\beta$ -carotene. These values were calculated for the mobile phase, based on comparative measurements in several organic solvents and published extinction coefficients (33, 34). However, throughout the preparation of the reaction center, it was convenient to estimate Chl levels by using the method of Arnon (35). The Chl/Ph ratio was calculated from HPLC data as was the Chl/ $\beta$ -carotene ratio. The Cyt  $b_{559}$  content was estimated by using an extinction coefficient of 17.5  $\text{mM}^{-1}\cdot\text{cm}^{-1}$  for the dithionite minus ferricyanide change at 559 nm.

## RESULTS

Fig. 1 shows the room temperature absorption spectrum of the PSII reaction center preparation, where it can be seen that compared to the standard preparation isolated by ion-exchange chromatography, the long-wavelength absorption maximum of our preparation is  $\approx 677.5$  nm rather than 675.5 nm (Fig. 1 *Inset*) when measured in 50 mM Mes/5 mM imidazole/2 mM DM at pH 6.5. There was also a lowering of

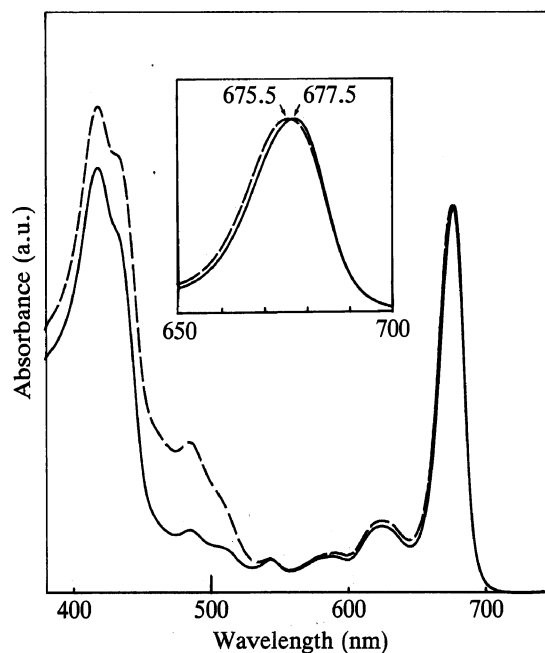


FIG. 1. Room temperature absorption spectra of our preparation (solid line) and a standard reaction center containing 6 Chls (dashed line). The two spectra were normalized at their  $Q_y$  absorption maxima, which are shown in more detail in the *Inset*. a.u., Arbitrary unit(s).

the level of  $\beta$ -carotene in our preparations, as judged from reduced absorption in the 485-nm region. SDS/PAGE revealed the presence of the D1 and D2 proteins, the  $\alpha$  and  $\beta$  subunits of Cyt  $b_{559}$ , and the product of the *psbI* gene (Fig. 2, lane 2).

The room temperature absorption spectra shown in Fig. 1 indicate that the pigment composition of our preparation is different to the normal preparation. This difference was confirmed by HPLC analysis. The quantified levels of the pigments and cofactors are given in Table 1, which shows that the preparation isolated by the use of a Cu affinity column

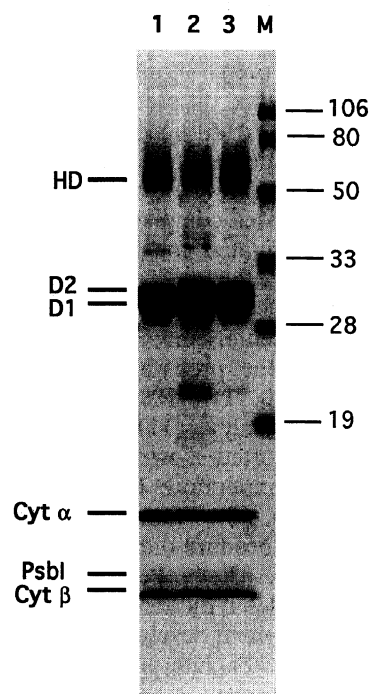


FIG. 2. SDS/PAGE profile of our PSII reaction center preparation. Lanes: 1, normal Chl preparation isolated from pea; 2, our preparation; 3, normal preparation after passing through a Cu affinity column twice; M, molecular mass markers (kDa). HD, D1/D2 heterodimer; Cyt  $\alpha$  and  $\beta$ , Cyt  $b_{559}$   $\alpha$  and  $\beta$  subunits; PsbI, product of the *psbI* gene.

Table 1. Pigment content of PSII reaction centers from *Pisum sativum* isolated by using affinity chromatography

Pigment	Content, no. per reaction center
Chl <i>a</i>	5.2 ± 0.1
Ph <i>a</i>	2.0*
β-Carotene	1.1 ± 0.1
Cyt <i>b</i> <sub>559</sub>	1.0 ± 0.1

Data of Chl *a*, Ph *a*, and β-carotene were obtained from HPLC analyses; the Cyt *b*<sub>559</sub> content was measured from reduced minus oxidized spectra.

\*Data are based on the assumption that a reaction center contains two molecules of Ph.

binds ≈5 Chls per 1 β-carotene per two Phs. We also conclude that it contains 1 Cyt *b*<sub>559</sub> per 2 Phs.

The red shift of the room temperature long-wavelength absorption spectrum compared with the normal preparation (Fig. 1 *Inset*) indicates that a short-wave absorbing Chl species has been removed during the isolation procedure. This suggestion is supported by comparison of the 77 K absorption spectra shown in Fig. 3 for the two types of preparations measured in the same medium as for room temperature spectra except for the inclusion of 60% glycerol. The two spectra have been normalized at their long-wavelength maxima. Both show a maximum at 678 nm but the shoulder at about 671 nm is less intense in the 5-Chl-compared to the 6-Chl-containing preparation, as would be expected if a Chl absorbing at ≈670 nm has been removed.

This form of the reaction center preparation was found to be photochemically active, as judged by its ability to accumulate reduced Ph (Ph<sup>-</sup>) or P680<sup>+</sup> when illuminated in the presence of dithionite (1, 2) or silicomolybdate (2), respectively. The transient yield of P680<sup>+</sup>Ph<sup>-</sup> was also investigated by monitoring nanosecond absorption changes at 820 nm after a flash excitation at 682 nm. By assuming a quantum yield per photon absorbed of 1 for the 6-Chl preparation (30), the quantum yield of radical pair formation for the 5-Chl reaction center was 1.0 ± 0.1. Despite this high photochemical capacity, we found that at 25°C the 5-Chl preparation, even under dark conditions, was rather labile (Fig. 4*A*), although 2 mM DM was present, which normally stabilizes the 6-Chl preparation. This instabil-

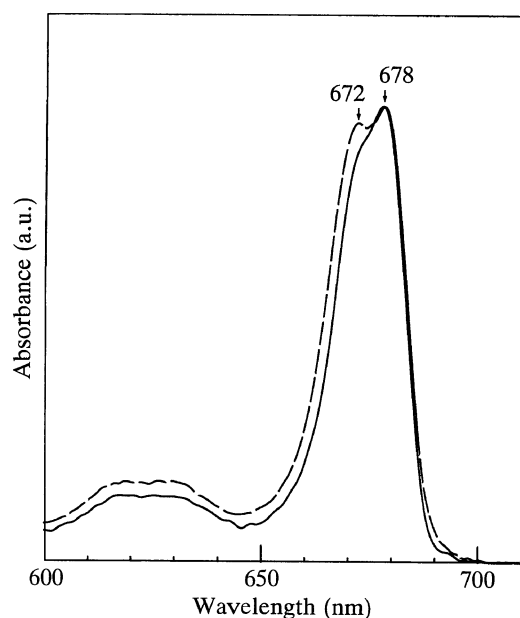


FIG. 3. Comparison of absorption spectra in the  $Q_y$  absorption region for the two types of reaction center measured at 77 K in the presence of 60% glycerol. a.u., Arbitrary unit(s).

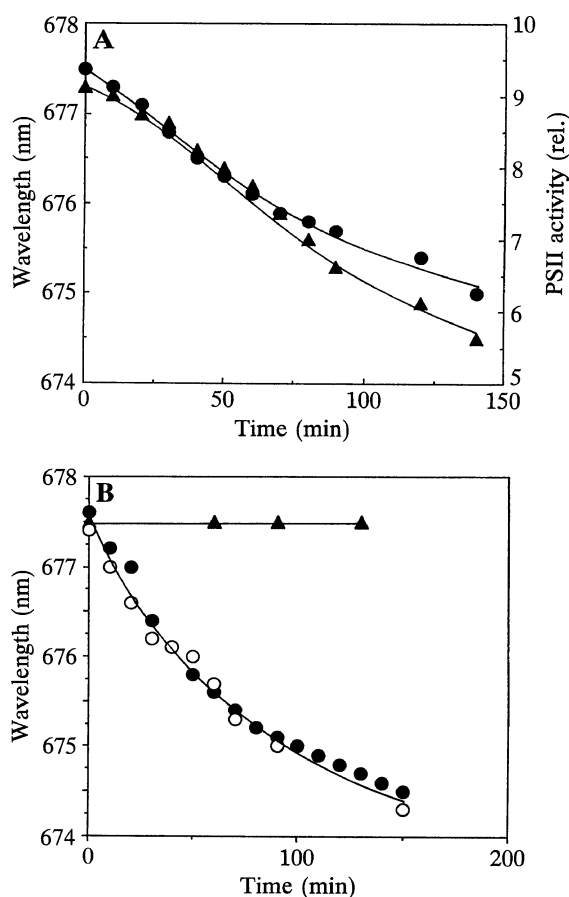


FIG. 4. (*A*) Effect of incubation in the dark at 25°C on the wavelength of the  $Q_y$  absorption maximum (circles) and the photochemical activity (triangles) of our reaction center preparation. PSII activity was measured as the ability of the reaction center to photoaccumulate P680<sup>+</sup> in the presence of silicomolybdate (2). (*B*) Effect of temperature, 10°C (triangles) and 25°C (circles), under aerobic (solid symbols) and anaerobic (open symbols) conditions, on the wavelength of the  $Q_y$  absorption maximum of our reaction center preparation.

ity was detected by a loss of photochemical activity (ability to perform light-induced electron transfer reactions) and by a concomitant shift of the  $Q_y$  maximum to the blue (see ref. 36), being persistent even under anaerobic conditions (Fig. 4). However, at 10°C, the preparation was relatively stable and as resistant to photo-induced damage as the normal type of preparation when oxygen was removed.

By using anaerobic conditions and 10°C to stabilize the preparation, picosecond transient absorption spectra were recorded. Fig. 5 shows transient absorption spectra at a time delay of 3 ps for the normal (solid line) and lower (dotted line) Chl preparations after excitation with 665-nm pulses. Transient absorption difference spectra for the two preparations are also given for a time delay of 100 ps (Fig. 5 *Inset*), assigned previously to the radical pair state P680<sup>+</sup>Ph<sup>-</sup> (16). While the two spectra at 100 ps were indistinguishable, the 3-ps transient spectrum for the 6-Chl-containing preparation is blue-shifted relative to that of the 5-Chl sample. This shift is illustrated by the observation that the difference between these two spectra has a minimum at 672 nm (Fig. 5, spectrum 6 Chl - 5 Chl). These data indicate that at this time delay [i.e., before the formation of the radical pair (24, 26)], a smaller proportion of the excitation energy remains on short-wavelength-absorbing chlorins (denoted C670<sub>II</sub>) of the 5-Chl compared with that of the 6-Chl preparation. This result is consistent with the 5-Chl preparation containing fewer slow energy transferring C670<sub>II</sub> pigments than the 6-Chl preparation.

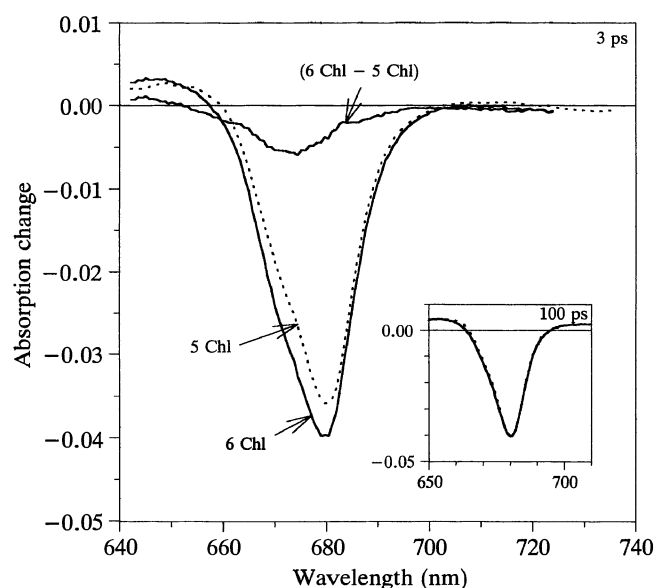


FIG. 5. Absorption difference spectra at a time delay of 3 ps after excitation of 5-Chl (dotted line) and normal (solid line) reaction center preparations at 665 nm. (Inset) Corresponding difference spectra at 100 ps. These 100-ps spectra are indistinguishable and assigned to the radical pair state  $P680^+Ph^-$ . The difference spectrum between the two 3-ps spectra is also shown (spectrum 6 Chl - 5 Chl); this difference between the data collected for the two preparations is attributed to a greater equilibration of excitation energy prior to this time delay for the 5-Chl preparation, due to a reduction in the number of slow energy transferring C670<sub>II</sub> Chls.

A kinetic analysis of the data supports this conclusion. Absorption difference spectra were collected at 150 time points on a 0- to 70-ps scale. On this time scale, the data are dominated by two components: a nondecaying component assigned to the state  $P680^+Ph^-$  (i.e., the 100-ps spectra shown in Fig. 5) and a component with a 20- to 30-ps lifetime [full

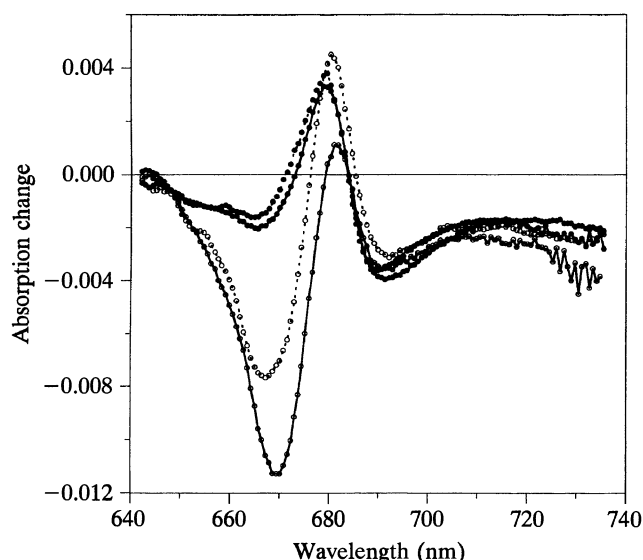


FIG. 6. Spectra of the amplitudes of 20- to 30-ps components observed after excitation of normal (solid line) or 5-Chl (dotted line) forms of the reaction center. Data were collected by using 665-nm (open symbols) or 694-nm (solid symbols) excitation (photoselective excitation of 670- and 680-nm-absorbing chlorins, respectively). The time constants of these components are  $21 \pm 1$  ps (694-nm excitation) and  $27 \pm 2$  ps (665-nm excitation) for the 6-Chl reaction centers, and  $18 \pm 3$  ps (694-nm excitation) and  $25 \pm 3$  ps (665 nm excitation) for the 5-Chl reaction centers. The negative feature at 670 nm observed by using 665-nm excitation is attributed to recovery of the bleach/stimulated emission of 670-nm-absorbing pigments.

details of the kinetic analysis and other components resolved are given in Rech *et al.* (24)]. Fig. 6 shows the kinetic spectra of the amplitudes (decay-associated spectra) of the 20- to 30-ps components for both the 5- and 6-Chl-containing preparations, using either 665- or 694-nm excitation pulses. After 665-nm excitation, the spectra are dominated by a negative feature at  $\approx 670$  nm. This feature results from recovery of the bleach/stimulated emission near 670 nm as excitation energy is transferred from C670<sub>II</sub> pigments to P680 and is subsequently trapped by radical pair formation (24). The amplitude of this feature is reduced in the 5-Chl reaction centers compared to the normal preparation by 30–50% in both the parallel (Fig. 6) and magic-angle (not shown) data. This observation is consistent with the lowering of the level of the C670<sub>II</sub> Chl associated with the slow energy transfer kinetics. In contrast, after 694-nm excitation, the data at all probe wavelengths are essentially the same for both preparations, in agreement with our previous conclusion that these excitation conditions avoid contributions to the data from slow energy transfer steps (24).

## CONCLUSION

We have isolated a reaction center of PSII estimated to contain five Chl based on HPLC analyses and published extinction coefficients. The spectral properties and the maintenance of photochemical activity indicate that this form of the PSII reaction center has lost one of its accessory Chls as compared with the normal type of preparation, which binds 6 Chls (8, 9). The reaction center of purple bacteria binds 4 bacteriochlorophyll molecules. Thus, the finding that the isolated D1–D2–Cyt  $b_{559}$  complex normally has 6 Chls associated with it indicates a difference between the two types of reaction centers. Comparison of the primary sequences of the bacterial and PSII reaction center proteins reveals that both the D1 and D2, unlike the L and M subunits, have conserved His residues in their second transmembrane segments. It has been speculated (37) that these two His residues at positions 118 could serve as ligands for the “extra” two accessory Chls. If this is the case, it is possible that by using immobilized metal affinity chromatography, we have displaced one of these peripheral Chls in the process of forming a ligand bridge between Cu and His 118 of either the D1 or D2 protein.

This conclusion is important for interpreting our transient absorption data. We have previously identified energy transfer processes occurring on two time scales in the 6-Chl preparation. Equilibration of excitation energy between  $\approx 680$ -nm-absorbing pigments (C680 pigments) and some  $\approx 670$ -nm-absorbing (C670<sub>I</sub>) pigments occurs in  $100 \pm 50$  fs (15). However, transfer of excitation energy from other 670-nm-absorbing pigments (C670<sub>II</sub>) was found to be much slower, 10–20 ps (24). These slow energy transfer processes resulted in the average lifetime for charge separation increasing from  $21 \pm 1$  ps for direct excitation of the C680 pigments to  $27 \pm 2$  ps for excitation of the C670 pigments and were also identified by differences in the spectra of these two components. It was suggested that the slow energy transfer processes were avoided by direct excitation of the C680 pigments, as back energy transfer from the C680 pigments (which include P680) to the C670<sub>II</sub> pigments was slow relative to charge separation. The data presented here for the 5-Chl reaction center provides strong confirmation of these conclusions.

We have observed that the transient absorption features at  $\approx 670$  nm attributed to slow energy transferring Chls (Figs. 5 and 6) are reduced in amplitude when Chl is removed from the reaction center. However, under conditions in which we had concluded (24) that the slow energy transfer steps were avoided (694-nm excitation), the data is the same for both the 5- and 6-Chl reaction center preparations. These results therefore provide further evidence that the 21-ps charge separation

observed after 694-nm excitation (photosensitive excitation of P680) is not limited by any slow energy transfer steps and that the C670<sub>II</sub> is likely to be peripherally bound Chls possibly ligated to D1 and D2 His-118 residues. We did observe that our isolation procedure gave rise to a preparation that was rather unstable and contained some breakdown fragments (see Fig. 2, lane 2) compared with normal 6-Chl preparations (Fig. 2, lane 1). However, when we subjected the normal type of reaction center preparation to Cu affinity chromatography (unpublished data), we observed that at least 1 Chl was removed and that in this case the resulting 5-Chl reaction centers were essentially free of breakdown products (see Fig. 2, lane 3). This latter finding indicates that by using immobilized metal affinity chromatography, it may be possible to modify the Chl level of standard preparations without incurring unwanted damage.

Throughout this paper we have assumed that the normal type of reaction center contains 6 Chls per 2 Phs as reported (8, 9), but this stoichiometry is dependent on the use of published extinction coefficients of which there are variations found in the literature (38). Therefore, although there is no question that our preparation has a lower Chl level compared to the normal preparation, the change may not be precisely 1 Chl and further work is required to confirm the exact pigment content of these preparations.

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