Pigment Content of D1–D2–Cytochrome b_{559} Reaction Center Preparations after Removal of CP47 Contamination: An Immunological Study[†]

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Received June 13, 1995; Revised Manuscript Received September 21, 1995[⊗]

ABSTRACT: Isolated D1–D2–cytochrome b_{559} photosystem II reaction center preparations with pigment stoichiometry higher than 4 chlorophylls per 2 pheophytins can be contaminated with CP47 proximal antenna complex. Reaction centers prepared by a modification of the Nanba–Satoh procedure and containing about 6 chlorophylls per 2 pheophytins showed immuno-cross-reactivity when probed with a monoclonal antibody raised against the CP47 polypeptide. Furthermore, they could be fractionated successfully by Superose-12 sieve chromatography into two different populations. The first few fractions off the column contained a more definitive 435 nm shoulder corresponding to increased chlorophyll content, and showed strong immuno-cross-reactivity with the CP47 antibody. The peak fractions off the column displayed a less prominent 435 nm shoulder, and did not cross-react with the antibody. Moreover, when a 6-chlorophyll preparation was mixed with Sepharose beads coupled to CP47 antibody, the eluted material corresponded to a preparation of about 4 chlorophylls per 2 pheophytins and did not show any cross-reaction with the antibody against CP47. The amount of CP47 protein in the 6-chlorophyll preparation as quantitated using Coomassie Blue staining or from gel blots was sufficient to account for most of the extra 2 chlorophylls. We conclude that D1–D2–cytochrome b_{559} preparations containing more than 4 chlorophylls per 2 pheophytins can be contaminated with small amounts of CP47–D1–D2–Cyt b_{559} complex and that native photosystem II reaction centers contain 4 *core* chlorophylls per 2 pheophytins.

Isolated D1–D2–Cyt b_{559}[1] complex [see Seibert (1993) for a review], which constitutes the reaction center (RC) of photosystem II (PSII), has been isolated from a number of different sources (Nanba & Satoh, 1987; Barber et al., 1987; Braun et al., 1990; Montoya et al., 1991). However, the pigment stoichiometry of the complex has been the source of a great deal of controversy. Although it is generally accepted that there are 2 pheophytin (Pheo) molecules per RC, different chlorophyll (Chl) per Pheo ratios have been reported. Nanba and Satoh (1987) first described a RC preparation that contained 4–5 Chl per 2 Pheo, and shortly afterward Barber et al. (1987) reported a pigment stoichiometry of 4 Chl per 2 Pheo. Seibert et al. (1988), McTavish et al. (1989), and Chapman et al. (1989) noted that the presence of Triton X-100 in these preparations resulted in unstable material that was susceptible to photodynamic damage. The introduction of milder detergents such as dodecyl maltoside (DM) into the purification protocols and elimination of O_2 provided much more stable RC material (Seibert et al., 1988; McTavish et al., 1989). However, these preparations when later quantitated were found to contain about 6 Chl per 2 Pheo (Kobayashi et al., 1990; Gounaris et al., 1990; Montoya et al., 1991). Apparently, the purification procedure affected the pigment stoichiometry of the isolated complex since small modifications, such as lengthening the ion-exchange chromatography wash step, resulted in lowering the pigment ratio from 6 Chl per 2 Pheo to 4 Chl per 2 Pheo (Montoya et al., 1991, 1993; Chang et al., 1994a).

The pigment stoichiometry found in RCs isolated from purple photosynthetic bacteria is 4 Bchl per 2 Bpheo (Straley et al., 1973; van der Rest & Gingras, 1974). Since significant sequence homology between the L and M polypeptides of the purple bacterial RC and the D1 and D2 polypeptides of the PSII RC exists in the regions thought to bind pigments (Youvan et al., 1984; Rochaix et al., 1984), the core Chl pigment content in the two types of RC could be similar. Recent reports suggest the presence of pigment–protein and/or free Chl contaminants in preparations of isolated D1–D2–Cyt b_{559} RC complex containing more than 4 Chl per 2 Pheo (Jankowiak & Small, 1993; Aured et al., 1994; Chang et al., 1994a). Resolving this issue is important since the exact chromophore stoichiometry is critical for understanding functional mechanisms in the PSII RC including excited state phenomena, energy transfer, and primary charge separation. In this paper, we demonstrate by immunological means that isolated D1–D2–Cyt b_{559} RC preparations containing more than 4 Chl per 2 Pheo can be contaminated with variable amounts of CP47, one of the proximal antenna protein complexes. Furthermore, the pigment stoichiometry can be reduced to 4 Chl per 2 Pheo by selective removal of CP47 contaminants by immunoaffinity chromatography.

MATERIALS AND METHODS

Preparation of D1–D2–Cyt b_{559} Complex and Pigment Quantitation. PSII membrane fragments were isolated from

[†] This work was supported by the DGICYT (Grant PB92-0125) (R.P.) and by the Division of Energy Biosciences, Office of Basic Energy Sciences, U.S. Department of Energy, under Contract DE-AC36-83 CH-10093 (M.S.).

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[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: Bchl, bacteriochlorophyll; Bpheo, bacteriopheophytin; Chl, chlorophyll; Cyt, cytochrome; DM, dodecyl β-D-maltoside; Pheo, pheophytin; PS, photosystem; RC, reaction center; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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spinach leaves as described by Berthold et al. (1981). D1−D2−Cyt b_{559} complex was purified according to the procedure of Nanba and Satoh (1987). However, Q-Sepharose Fast Flow (Pharmacia) was used instead of Fractogel DEAE-TSK 650S (Merck) as the ion-exchange chromatography column (2.6 × 21 cm) material, and $MgSO_4$ was substituted for NaCl as described by Yruela et al. (1994). Specifically, the Q-Sepharose column was washed with 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, plus 0.1% Triton X-100 at a flow rate of about 5 mL·min^{-1} until the 435 nm peak of the eluate was a little lower than that at 416 nm. At this point, the Triton X-100 was removed by further washing the column with the same buffer plus 0.6 mM DM in the absence of Triton until the absorption of the effluent near 280 nm was lower than 0.1. The RC material was then eluted with a 30−200 $MgSO_4$ linear gradient in the same buffer. Green fractions were pooled, desalted, and concentrated. RC preparations isolated in this manner contained about 6 Chl per 2 Pheo. The material was further purified by molecular sieve chromatography using a Superose-12 HR 10/30 column [10^3−(3 × 10^5) MW range] with a FPLC system (Pharmacia). Samples (200 μL) were loaded onto the column preequilibrated with 50 mM Tris-HCl, pH 7.2, containing 0.6 mM DM and eluted with the same buffer at a flow rate of 0.05 mL·min^{-1}. An absorption spectrum of each fraction was taken. The few first fractions (>6 Chl per 2 Pheo), which contained a pronounced shoulder at 435 nm, were pooled as a component set, and the main peak (4 Chl per 2 Pheo) was collected as another component set (Aured et al., 1994). Sometimes, complete separation was not achieved (i.e., RC preparations with 4.5−5 Chl per 2 Pheo have been observed). In those cases, a second sieve chromatography step was run under exactly the same conditions as above.

Spectrophotometric quantitation of Chl and Pheo was performed by pigment extraction in 80% acetone and subsequent acid treatment as described previously (Montoya et al., 1991). We checked the accuracy of this procedure by examining PSII RC preparations sent to us by two other laboratories. The preparations were quantitated using HPLC by the other laboratories and were 6 Chl per 2 Pheo materials. We obtained comparable results in our laboratory with these same samples using the Montoya et al. (1991) pheophytinization method.

Immunoblotting. SDS−PAGE was performed according to Laemmli (1970) with 15% acrylamide gels containing 6 M urea. D1−D2−Cyt b_{559} complex concentration was normalized using the Q_x band of Pheo (Montoya et al., 1991; Chang et al., 1994a). Immunoblotting was performed as described by Towbin et al. (1979) using a semi-dry transfer unit (TE 70 SemiPhor, Hoeffer Scientific Instruments). After transfer to a nitrocellulose membrane, the proteins were detected using a mouse monoclonal anti-CP47 antibody (a gift from Dr. Terry M. Bricker, Department of Botany, Louisiana State University, Baton Rouge). Goat anti-mouse immunoglobulin G coupled to horseradish peroxidase (Bio-Rad) was used as a secondary antibody to visualize the bands.

For Table 1, CP47 protein was purified as described previously (Alfonso et al., 1994). The amount of the purified protein was determined from the spectrum of the pigment extract in 80% acetone, using an extinction coefficient of 75 mM^{-1} cm^{-1} at 663 nm for chlorophyll (Dawson et al., 1986). We assumed a chlorophyll content of either 14 (Chang et al., 1994b) or 22 Chl per CP47 complex (Alfonso et al., 1994). Known amounts of CP47 were loaded, and a standard curve was obtained for each experiment. The gels were scanned with a Bio Image densitometer (Millipore Corp., Ann Arbor, MI) to quantitate CP47 protein content from both Coomassie Blue and blot staining. The blots in Figures 2−4 were scanned with a Hewlett Packard ScanJet IIC using Desk Scan software and the images printed using Windows Draw (Micrografx, Richardson, TX).

Immunoaffinity Purification. Mouse monoclonal anti-CP47 antibody was coupled to CNBr-activated Sepharose 4B beads (Pharmacia) according to the manufacturer's instructions. The beads with immobilized antibody were equilibrated with 50 mM Tris-HCl, pH 7.2, containing 0.2 mM DM and then incubated with 6-Chl D1−D2−Cyt b_{559} complex. The mixture was gently rotated at room temperature in the dark for 1 h to allow complete binding of CP47 to the antibody, and purified RC was recovered by filtration through a porous ceramic plate.

RESULTS

When a preparation of PSII RC complex containing 6.0 ± 0.3 Chl per 2 Pheo is further purified by sieve chromatography, the pigment stoichiometry of the resultant D1−D2−Cyt b_{559} complex can be reduced to about 4 Chl per 2 Pheo. This method of purification (Aured et al., 1994) leads to the fractionation of the initial PSII RC population (6 Chl per 2 Pheo; Figure 1A) into two components without significant pigment loss. The first component contains RC complex with more than 6 Chl per 2 Pheo (Figure 1C), and the main component (sieve chromatography peak) contains RC complex with about 4 Chl per 2 Pheo (Figure 1B). Spectra of 4- and 6-Chl preparations at room temperature look almost identical with the red band apparent at 675.5 nm (Q_y transitions of Chl and Pheo), a small band at 543 nm (Q_x transition of Pheo), β-carotene absorption in the 510−450 nm region, a band at 435 nm (Soret transition of Chl), and a band at 416 nm (the Chl vibronic band and the Soret transitions of Pheo and oxidized Cyt b_{559}). Slight differences, however, can be observed when carefully comparing the two spectra. For example, a better defined 435 nm Chl shoulder and somewhat less resolved Pheo Q_x and β-carotene bands appear in the 6-Chl preparation. The spectrum of the pooled initial fractions obtained from the sieve column (Figure 1C) was also similar, but it showed a more pronounced shoulder at 435 nm indicating higher Chl content. Other small but clear differences such as a less defined Pheo Q_x band, a smaller valley near 470 nm, and less defined β-carotene bands can also be observed. The pigment stoichiometry measured for these pooled fractions was 8.5 Chl per 2 Pheo (some of the pooled fractions showed an even higher 435 nm Chl band than that shown in Figure 1C). This result suggests a Chl-containing contaminant in the original 6 Chl per 2 Pheo preparation that elutes at a higher molecular weight than D1−D2−Cyt b_{559} RC complex. However, the sieve column did not provide complete separation of two peaks. In some cases, it was necessary to run a second sieve-chromatography column under the same conditions to obtain a clean 4 Chl per 2 Pheo preparation, as mentioned under Materials and Methods.

A faint band of apparent molecular mass of around 47 kDa can be visualized by SDS−PAGE in 6 Chl per 2 Pheo RC preparations. However, it can be seen clearly only when

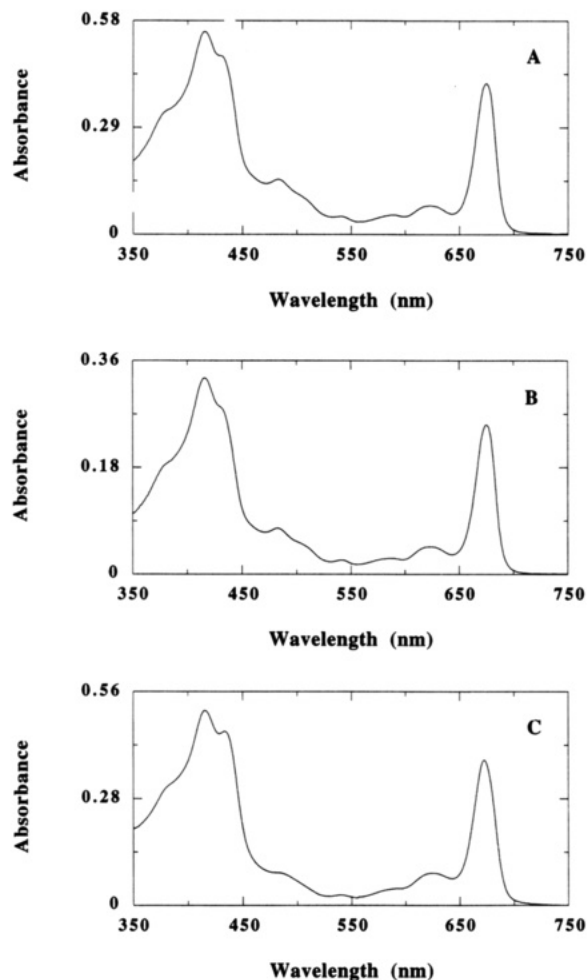

FIGURE 1: (A) Absorption spectrum of a D1−D2−Cyt b_{559} RC complex preparation containing 6 Chl per 2 Pheo prior to sieve chromatography; (B) spectrum of the pooled fractions from the main peak eluted off a Superose-12 column reflecting reaction centers containing about 4 Chl per 2 Pheo; (C) spectrum of the pooled initial fractions eluted off the Superose-12 column, containing 8.5 Chl per 2 Pheo.

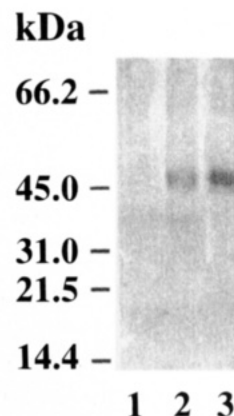

FIGURE 2: Immunoblot of different D1−D2−Cyt b_{559} RC preparations with a monoclonal antibody raised against CP47. Lanes 1−3 correspond to RC preparations with about 4, 5, or 6 Chl per 2 Pheo, respectively.

Table 1: Quantitation of the Number of Chlorophyll Molecules Associated with CP47 in a 6-Chl PSII Reaction Center Preparation

	CP47 contamination	
Chl/CP47	Coomassie[a]	blot[b]
14	1.6 ± 0.1	1.1 ± 0.2
22	2.5 ± 0.2	1.7 ± 0.3

[a] These values (means of 3 experiments) were obtained by scanning a Coomassie Blue-stained gel of a 6-Chl RC preparation to obtain the relative area under each component protein band. Protein associated with CP47 represented 4.2% of the total protein. We assumed that the ratio of CP47 to D1−D2−Cyt b_{559} is 1:1 on a complex basis in centers that bind CP47, that the molecular weight of CP47 protein is 37% in centers that bind CP47, and that all protein bands stain proportional to their molecular weights. [b] These values (means of 3 experiments) were obtained after generating a standard curve for known amounts of CP47 vs staining intensity of blots of the CP47 protein. Known amounts of 6-Chl PSII RCs were also run on the same blots, and the amount of contaminating CP 47 protein was determined by comparison with the standard curves.

the gels are heavily loaded. The molecular mass of this band coincides with the apparent molecular mass of the intrinsic proximal antenna protein CP47 (Camm & Green, 1980). To identify the contaminant conclusively, we prepared an immunoblot of PSII RC preparations with different amounts of Chl and probed it with mouse monoclonal anti-CP47 antibodies. Figure 2 clearly demonstrates the presence of CP47 antenna protein in a 6-Chl RC preparation. A lesser amount is seen in a 5-Chl preparation (corresponding to the peak fraction off the sieve chromatography column when a single chromatography step was not sufficient to give a preparation of 4 Chl per 2 Pheo), and no CP47 is detected in D1−D2−Cyt b_{559} RC complex containing about 4 Chl per 2 Pheo. While this immunoblot can identify the band conclusively as CP47, it provides no quantitation of the amount of CP47 present in the 6-Chl RC preparation. To ascertain this information, we scanned both a Coomassie Blue-stained SDS−PAGE and an immunoblot of a 6-Chl preparation. The results are shown in Table 1. The former approach detected a sufficient amount of CP47 protein (4.2% based on the relative area under the scanned CP47 band) to account for about 1.6 or 2.5 Chls depending upon whether CP47 contains 14 (Akabori et al., 1988; Kwa et al., 1992; Chang et al., 1994b) or 22 Chls (De Vitry et al., 1984; Alfonso et al., 1994), respectively. Quantitation by the Coomassie Blue-staining method probably overestimates the amount of CP47 present since CP47 stains more heavily than D1 and D2, even though we have corrected for the higher molecular weight of CP47. However, this staining difference appears to be reduced in more purified PSII preparations such as D1−D2−Cyt b_{559}−CP47−CP43 core complex (Ghanotakis & Yocum, 1986; Ghanotakis et al., 1987, 1989; Picorel and Seibert, unpublished results). To avoid this uncertainty, we also quantitated the amount of contaminating CP47 in transblots of 6-Chl RC preparations by comparison to a standard blot curve generated from known amounts of isolated CP47. The contaminating CP47 protein detected by this approach was sufficient to account for 1.1 or 1.7 Chls depending on whether CP47 contains 14 or 22 Chls, respectively.

In order to verify if the contaminant eluted in the initial fractions of the sieve chromatography (Figure 1C) was CP47, we ran an immunoblot comparing the RC preparation loaded onto the column (Figure 1A; 6 Chl per 2 Pheo) and the pooled initial fractions coming off the column (Figure 1C; 8.5 Chl per 2 Pheo). Figure 3 shows that the initial fractions contain a much higher amount of CP47 protein. The fact that the contaminant elutes slightly ahead of the 4-Chl RC complex indicates that, under the conditions used to produce PSII RCs that contain 6 Chl per 2 Pheo, some intrinsic CP47

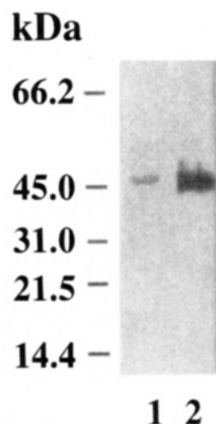

FIGURE 3: Immunoblot of a D1−D2−Cyt b_{559} preparation containing 6 Chl per 2 Pheo (lane 1) compared to the pooled initial fractions obtained by sieve chromatography with a pigment ratio of 8.5 Chl per 2 Pheo (lane 2).

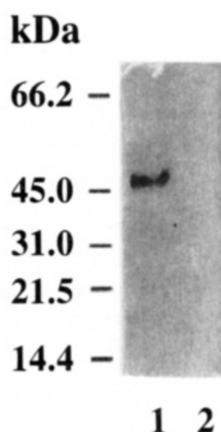

FIGURE 4: Immunoblots of D1−D2−Cyt b_{559} complex containing 6 Chl per 2 Pheo (lane 1) and the same D1−D2−Cyt b_{559} complex containing about 4 Chl per 2 Pheo after immunoaffinity purification (lane 2).

antenna protein is not completely removed from D1−D2−Cyt b_{559} and thus elutes as CP47−D1−D2−Cyt b_{559} complex.

These results suggest that preparations of 6-Chl D1−D2−Cyt b_{559} complex used in many laboratories may contain CP47 antenna protein. This contaminant artificially modifies the pigment stoichiometry of a PSII RC preparation by raising the ratio of Chl/Pheo to around 6 Chl per 2 Pheo. In order to further demonstrate CP47 contamination and its contribution to the Chl content of 6-Chl RC preparations, we eliminated the contamination selectively by incubating a 6-Chl preparation with Sepharose 4B beads coupled to monoclonal anti-CP47 antibody. The complete removal of CP47 from RC material not bound to the couple antibody was verified by immunoblotting (Figure 4). The purified D1−D2−Cyt b_{559} complex thus obtained had a pigment stoichiometry of 4.1 ± 0.2 Chl per 2 Pheo. An experiment performed the same way, except in the absence of antibody, resulted in a control RC preparation with 6 Chl per 2 Pheo.

DISCUSSION

The pigment stoichiometry of the PSII RC has been a subject of much discussion in the literature. Recently, D1−D2−Cyt b_{559} preparations containing either 6 Chl per 2 Pheo or 4 Chl per 2 Pheo have been isolated from the same species, and their spectroscopic and photochemical characteristics have been compared (Montoya et al., 1993, 1994; Chang et al., 1994a). The absorption spectra of the two preparations at room temperature are quite similar, although significant differences were observed at helium temperature (Chang et al., 1994a). Hole-burning studies of 6-Chl RC preparations (Chang et al., 1994a) concluded that the material contains contaminant CP47, non-native Chl, and a Chl band absorbing at 684 nm. Aured et al. (1994) have also suggested the presence of an antenna pigment−protein complex contaminant in 6 Chl per 2 Pheo RC preparations. However, neither of these studies confirmed the conclusion by immuno-cross-reactivity as is reported in the current work.

The possibility of Chl-containing contaminant proteins in 6-Chl PSII RC preparations has not been considered seriously until recently, probably because gel electrophoretograms stained with Coomassie Blue did not show any prominent peptide contamination. Nevertheless, some published RC preparations containing more than 4 Chl per 2 Pheo show a very faint, sometimes smeared band which could correspond to CP47 (Akabori et al., 1988; Fotinou & Ghanotakis, 1990; van Leeuwen et al., 1991; Moskalenko et al., 1992). If one assumes a value of 14 Chl *a* molecules per CP47 molecule (Akabori et al., 1988; Kwa et al., 1992; Chang et al., 1994b) and 4 Chl per 2 Pheo for the RC, the expected CP47 contamination in our 6-Chl RC preparation should be about 14% on a complex basis. This corresponds to CP47 contamination of 5.2% on a protein basis, since the molecular weight of CP47 is about 37% of that of the CP47−D1−D2−Cyt b_{559} RC complex. If a value of about 22 Chl *a* per CP47 molecule is accepted (de Vitry et al., 1984; Alfonso et al., 1994), the CP47 contamination would be about 3.3% on a protein basis. These amounts of CP47 contamination are close to the 4.2% value estimated above from the SDS−PAGE densitometer scan of a 6-Chl RC preparation mentioned under Results. This level of contamination is not easily detected by gel electrophoresis stained with Coomassie Blue unless the gels are heavily loaded, since the CP47 band tends to smear and thus can be lost in the background. Because the CP47 Chl contamination estimates above and in Table 1 based on Coomassie Blue staining of gels are probably overestimated, we also used calibrated gel blots to refine our quantitation. The resultant value of 1.1−1.7 contaminating CP47 Chls in a 6-Chl RC preparation in Table 1 demonstrates that CP47 Chl contamination can be substantial. Furthermore, this range of contamination is consistent with that estimated by low-temperature spectroscopic techniques (Chang et al., 1994a) and can explain most of the "extra" Chl found in an isolated 6-Chl PSII RC preparation.

Finally, the Chl content of our 6-Chl PSII RC preparation can be reduced by removal of contaminating CP47 protein by immunoaffinity purification to yield a minimal RC complex containing about 4 Chl per 2 Pheo (Figure 4). PSII RC complexes with such stoichiometry are photochemically active in charge separation (Montoya et al., 1994; Chang et al., 1994a). We conclude that native PSII RC complex from higher plants contains 4 *core* Chl per 2 Pheo analogous to the pigment stoichiometry of its ancestral RC from purple bacteria. This does not preclude the possibility of additional linker-type Chls binding to RC proteins and not associated with CP47 (Seibert, 1993; Chang et al., 1994a) under RC isolation conditions different from those used in this study.

We also believe that the use of homogeneous 4 Chl per 2 Pheo preparations of D1−D2−Cyt b_{559} complex could help resolve many of the contradictory high-resolution spectroscopic results found in the literature. Moreover, the use of homogeneous preparations could be essential to groups attempting to crystallize this important membrane chromoprotein.

ACKNOWLEDGMENT

We thank María V. Ramiro for her technical assistance, Prof. Terry M. Bricker for the gift of CP47 antibodies, and Dr. Lew Brown for scanning and printing the blots for us. R.P. is grateful for the hospitality at NREL while in the U.S.A.

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BI9513315