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Chlorophyll and cytochrome *b*-559 content of the photochemical reaction center of photosystem II

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Photosystem II reaction centers comprised of the D1, D2 and cytochrome *b*-559 polypeptides were isolated from well-defined oxygen-evolving reaction center preparations using either a combination of $LiClO_4$ and dodecylmaltoside, or Triton X-100 alone. Yields of chlorophyll and cytochrome *b*-559 for both preparative methods were compared, and pigment contents were compared based on 2.0 *b*-559 per reaction center, a standard derived from photosystem II components found in the starting material. Results obtained with dodecylmaltoside suggest that the reaction center of photosystem II binds 10–12 Chl *a* along with 2 Cyt *b*-559 molecules. Reaction centers prepared with Triton X-100 bind about 8 Chl *a* per 2 Cyt *b*-559 molecules, a finding which indicates that Triton X-100 extracts pigments from the reaction center polypeptides. Our results contradict the widely held notion that the pigment binding properties of the photosystem II reaction center are similar to those of the bacterial reaction center.

Photosystem II; Cytochrome *b*-599; Reaction center; Chlorophyll; Pheophytin

1. INTRODUCTION

Knowledge of the structure of photosynthetic reaction centers is based in large part on the crystallization and determination of the reaction center structures of the purple bacteria *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* [1,2], where 4 bChl *a* and 2 bPheo *a* pigments along with 1 carotenoid, 1 non-heme iron and 2 quinones comprise the components of the reaction center. In the absence of detailed structural models for the reaction center of PS II, analogies to the bacterial reaction center have been proposed, based on amino acid sequence similarities between the so-called D1 and D2 proteins of PS II and the L and M subunits of the reaction center of purple bacteria [3]. Experimental support for similarities between bacterial and higher plant reaction centers has been provided by Nanba and Satoh [4], who isolated a complex consisting of D1, D2, and Cyt *b*-559, which retained the ability to form a spin-polarized triplet [5]. This material contains 4–5 Chl *a*, 2 Pheo *a* and 1–2 Cyt *b*-559 molecules along with carotenoid and non-heme iron; this pigment composition is similar to that of the bacterial reaction center.

The procedure developed by Nanba and Satoh employs Triton X-100 as the detergent for separation of the PS II reaction center proteins from the other intrinsic proteins; later reports have shown that the reaction centers are unstable, a situation which can be ameliorated to some extent by exchanging Triton X-100 for dodecylmaltoside [6]. An alternate method for the isolation of the D1–D2–*b*-559 reaction center complex [7] avoids Triton X-100 by the use of a combination of dodecylmaltoside and the chaotropic agent $LiClO_4$. Most of the properties of this complex (absorption spectrum, light-induced formation of a spin-polarized triplet) are similar to those obtained

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Abbreviations: Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-hydroxymethylpropane-1,3-diol; Chl, chlorophyll; Cyt, cytochrome; DM, β-dodecyl-D-maltoside; Pheo, pheophytin; PS, photosystem; TX, Triton X-100

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with Triton X-100; the most obvious difference is improved stability [7].

In order to verify if Triton X-100 and dodecylmaltoside preparations are similar in other respects, we have compared the 2 isolation methods in more detail, especially with regard to the yields and pigment compositions of the complexes. A CP47–D1–D2–*b*-559 complex was used as starting material, allowing for estimates of the yields of chlorophyll and Cyt *b*-559 in both cases. Our results reveal that Triton X-100 extracts pigments from the reaction center of PS II, and that the D1 and D2 polypeptides contain condiderably more chlorophylls than earlier reported. Insofar as pigment content and organization are concerned, the reaction center of purple bacteria cannot be regarded as a general model for the reaction center of PS II.

2. MATERIALS AND METHODS

Subchloroplast PS II membranes and oxygen-evolving PS II reaction center complexes were prepared from spinach [8]. The procedure for the purification of the CP47–D1–D2–*b*-559 complexes is a modification of the method introduced by Ghanotakis et al. [7]. Oxygen-evolving core complexes were Tris-washed, solubilized at 0.5 mg/ml Chl with 0.5% dodecylmaltoside in buffer A (20 mM Bis-Tris, 20 mM NaCl, 10 mM $MgCl_2$, 1.5% taurine, pH 6.5), passed through an S-Sepharose cation-exchange column at 22°C to remove proteins with apparent molecular masses of 22 and 10 kDa, and subsequently loaded on a Q-Sepharose anion-exchange column. The column was washed with buffer A containing 0.03% dodecylmaltoside/30 mM $MgSO_4$ in order to remove chlorophyll-binding proteins with apparent molecular masses of 43 and 28 kDa, after which the CP47–D1–D2–*b*-559 complex was eluted with 100 mM $MgSO_4$. The eluate was diluted 4-fold in buffer A containing 0.03% dodecylmaltoside and recycled through the anion-exchange column as described above to yield final preparations (about 1.5 mM Chl) which were supplemented with 400 mM sucrose and stored at −60°C.

Dodecylmaltoside/$LiClO_4$ D1–D2–*b*-559 complexes (DM-complexes), were isolated by adjusting CP47–D1–D2–*b*-559 complexes to 2 M $LiClO_4$ and 0.5% dodecylmaltoside at a final chlorophyll concentration of 1 mM, followed by incubation for 15 min at 4°C and subsequent dialysis against buffer A for 2 h. The dialyzed material was loaded onto the Q-Sepharose anion-exchange column at 22°C, the 47 kDa chlorophyll-binding protein was removed by washing with buffer A containing 0.03% dodecylmaltoside and 28 mM $MgSO_4$, and the pure D1–D2–*b*-559 complex was eluted with 100 mM $MgSO_4$; subsequent washing of the column with 400 mM $MgSO_4$ did not reveal additional proteins or pigments.

Triton X-100 D1–D2–*b*-559 complexes (TX-complexes), were prepared by diluting CP47–D1–D2–*b*-559 material 5-fold with a buffer containing 50 mM Tris-HCl (pH 7.2) and 4% Triton X-100. After incubation for 15 min at 4°C, the material was applied to the Q-Sepharose anion-exchange column at 4°C. The column was washed extensively with a buffer containing 50 mM Tris-HCl (pH 7.2), 30 mM NaCl and 0.2% Triton X-100, after which the D1–D2–*b*-559 complex was eluted with 190 mM NaCl. Residual material eluted from the column with 400 mM NaCl was also analyzed.

Optical spectroscopy was performed immediately after the preparation of the complexes using an Aminco DW-2 spectrophotometer. The amount of Cyt *b*-559 was quantitated by recording the dithionite-reduced minus ferricyanide-oxidized difference in absorbance at 559 nm [9] as described in section 3. Chlorophyll was quantitated using 80% acetone by the method of Arnon [10], or, in case of Chl *b*-free preparations, by determining the amplitude of the absorption band at 675 nm; an extinction coefficient of 74 000 $M^{-1} \cdot cm^{-1}$ was used since results obtained with this value correlated closely with the results obtained with 80% acetone. The absorbance at 675 nm (or at 663 nm with 80% acetone) includes the contribution of pheophytin *a* with an extinction coefficient of about 40 000 $M^{-1} \cdot cm^{-1}$. Therefore, we define the magnitude of the absorbance at 675 nm as pigment content (Chl *a* + Pheo *a*) in this communication; it should be noted that the use of this extinction coefficient (74 000 $M^{-1} \cdot cm^{-1}$) may result in a somewhat underestimated value in case of relatively high Pheo contents. SDS-polyacrylamide gel electrophoresis was carried out as described earlier [11].

3. RESULTS

In order to make comparisons between preparative methods using Triton X-100 or dodecylmaltoside, it was necessary to start with well-defined material. Oxygen-evolving preparations described in [8,11] contain D^+ (1 per 75 Chl) and about 4 Mn atoms per 65 Chl [12]. This preparation, which evolves oxygen in the light with rates of 1200–1700 μmol O_2/mg Chl per h, does not contain significant amounts of inactive centers, based on a comparison of the specific activity with that found in salt-washed PS II membranes supplemented with $CaCl_2$ [12]. We have used this preparation as starting material, and have also determined that it contains 2 Cyt *b*-559 per 63 Chl, in reasonable agreement with D^+ and manganese content. Quantitation of *b*-559 depends on the differential extinction coefficient for the reduced-minus-oxidized forms of the cytochrome at 559 nm; for the quantitations used here, a value of 17 500 $M^{-1} \cdot cm^{-1}$ was used, as determined from the results of Cramer et al. [9] for the purified protein, and which yielded 2 *b*-559 molecules per 250 Chl in PS II membranes, in agreement with results from other methods of determining reaction center

contents [13–15]. Spin-counts of the EPR signal from oxidized *b*-559 also indicate the presence of 2 Cyt per center [16] in PS II membranes, and for thylakoids, the presence of 2 Cyt *b*-559 per photosynthetic unit has also been determined [17].

Using oxygen-evolving preparations, we first isolated material containing CP47, D1, D2, and Cyt *b*-559 as described in section 2. The final yields from this procedure were 41% for pigment (defined here as the combination of Chl *a* and Pheo *a*) and 83% for Cyt *b*-559, indicating that the product retains both cytochromes; SDS-PAGE analysis (fig.1, lane 2) shows no contamination by other Chl-binding proteins. The CP47–D1–D2–*b*-559 preparation was then used for the isolation of

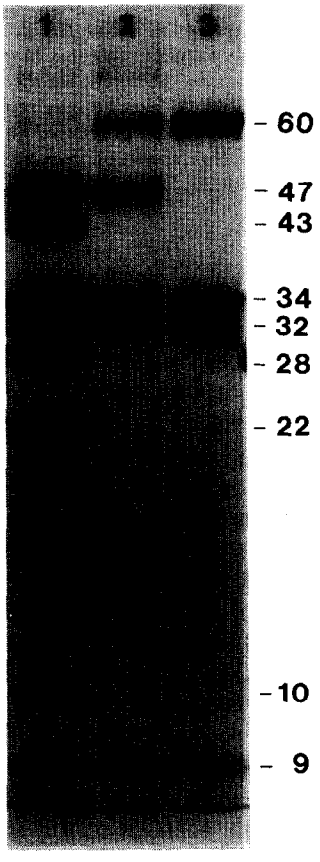

Fig.1. SDS-PAGE of PS II fractions. Gels contained 13.5% acrylamide and 4 M urea; proteins were stained with Coomassie brilliant blue. Fractions shown are the oxygen-evolving reaction center complex (1), the CP47–D1–D2–*b*-559 complex (2), and the dodecylmaltoside/$LiClO_4$-prepared D1–D2–*b*-559 complex (3). The weakly staining band migrating just below the 32 kDa polypeptide may arise from phosphorylation of D1 (see [20]).

D1–D2–*b*-559 by alternative procedures. Table 1 documents the results of pigment and *b*-559 quantitations on the DM- and TX-complexes prepared as described in section 2. As shown in table 1, the dodecylmaltoside method produces the reaction center material in high yield (83%) based on the recovery of Cyt *b*-559. The purity of the DM-complex was examined by SDS-PAGE (fig.1, lane 3); no detectable amount of CP47 is observed, and therefore the pigments found in this preparation must be associated with the D1 and D2 polypeptides. Spectrophotometric analyses of pigments and Cyt *b*-559 yields 13 pigments (10–12 Chl and 2–3 Pheo) per 2 *b*-559 molecules (table 1) for DM-complexes. The absorbance spectrum of this material (fig.2, solid line) is similar to that reported earlier [7], with a red absorbance maximum for the chlorophylls at 674–675 nm; similar spectra have also been reported for material prepared with Triton X-100 and subsequently suspended in dodecylmaltoside [6].

Inspection of table 1 also shows that the yield of the TX-complexes of D1–D2–*b*-559 is lower; approximately one-third of the cytochrome is removed during column washing to produce final yields of 61% (*b*-559) and 16% (pigment) of the contents of the starting material (the CP47–D1–D2–*b*-559 complex). Using the same approach as for the DM-complexes, we find that TX-complexes contain 10 pigments/2 *b*-559, and we therefore estimate that the TX-complexes contain 2–3 Pheo *a*, about 8 Chl and 2 *b*-559. The absorbance spectrum (with a red absorbance maximum of Chl at 674 nm (fig.2, dashed line)) is similar to those reported earlier [4,6]. Thus, the number of Chl per center is considerably lower in case of the TX-complexes, and we conclude that Chl (and perhaps Pheo) has been extracted from the D1–D2–*b*-559 proteins during purification. This extraction is most probably due to the action of Triton X-100, which has already been shown to destabilize the final product [6].

During preparation of the TX-complexes, we repeatedly observed a fraction eluting from the column at high salt concentration. This fraction was enriched in Cyt *b*-559 (i.e., it contained about 5 pigments/2 *b*-559), contained about 8% *b*-559 in the original CP47–D1–D2–*b*-559 complex, and showed a Chl absorbance maximum at 672 nm (fig.2, dotted line). This material probably represents an artifactual aggregation product, since

Table 1

Purification of D1-D2-*b*-559 with dodecylmaltoside or Triton X-100: comparison of pigment and Cyt *b*-559 yields

Fraction	Yields (%)		Pigments/2 *b*-559
	Pigments[a]	Cytochrome[b]	
CP47-D1-D2-*b*-559	100	100	32
DM-complex	34	83	13
TX-complex	16	61	10

[a] Chl *a* + Pheo *a* from absorbance at 674–675 nm

[b] From reduced-minus-oxidized difference spectrum as described in section 3

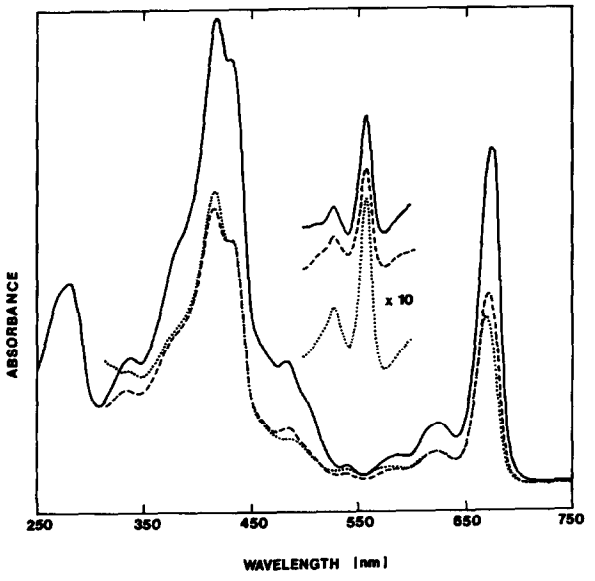

Fig.2. Room temperature absorbance spectra of the dodecylmaltoside/$LiClO_4$-prepared D1-D2-*b*-559 complex (solid line), the Triton X-100-prepared D1-D2-*b*-559 complex (dashed line), and the fraction eluted with 0.2% Triton X-100 and 400 mM NaCl (dotted line).

it is not observed when dodecylmaltoside and $LiClO_4$ are used for the purification of the complex.

4. DISCUSSION

The results presented here show that the use of dodecylmaltoside and $LiClO_4$ for preparation of D1-D2-*b*-559 reaction centers of PS II contrasts markedly with the results obtained with Triton X-100; there is no specific loss of Cyt *b*-559, and the final product is stable and contains more pigment. The exact pigment distribution between Chl *a* and Pheo *a* has not yet been determined; the most probable numbers are 10–12 Chl and 2–3 Pheo.

Our data and other reports (e.g. [6]) show that the use of Triton X-100 for the isolation of the reaction center of PS II produces side-effects which alter stability, pigment binding, and final yields. We estimate that approx. 2–4 pigments are extracted from the complex during exposure to Triton, so the pigment composition of TX-complexes does not represent the in vivo situation in the reaction center.

Although the method employed here for the isolation of TX-complexes differs from other methods [4,6,18] in the choice of starting material, the final products are comparable. Nanba and Satoh [4] estimated a ratio of 5 Chl, 2 Pheo and 1.3 *b*-559, and Barber [18] reported 4 Chl and 1 *b*-559 in a preparation from peas, not unlike the ratio of our estimated content of 8 Chl and 2 *b*-559. The latter group [19] has also reported a preparation from the cyanobacterium *Synechocystis* with a much higher (8:1) Chl/*b*-559 ratio and a very different absorption spectrum. Without yields such as we present here, it is difficult to compare those results with our data from spinach.

The pioneering work of Deisenhofer et al. [1] on the structure of the reaction center of the purple bacterium *Rps. viridis* has generated a number of speculations on the use of this structure as a model for the structure of PS II. Our results indicate that analogies (and speculations) should be restricted to properties related to the reducing sides of the two photoreactions; PS II is distinguished by the unique structures necessary to provide manganese binding, to sustain the high-potential redox reactions leading to water oxidation, and, as we show

here, by a substantially larger pigment content. At first sight, it might seem unrealistic to locate more than twice as many pigments on the reaction center proteins of PS II. On the other hand, it could be a general characteristic of higher plant systems since the PS I reaction center proteins each contain about 30 Chl on 11 transmembrane helices, and the chlorophyll-binding proteins CP47 and CP43 contain up to 20 Chl on 6 transmembrane helices. Thus, the location of 6–7 (Chl + Pheo) molecules on each of the D1 and D2 proteins (each having 5 transmembrane helices) should perhaps not be viewed as unusual.

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