# Pigment Quantitation and Analysis by HPLC Reverse Phase Chromatography: A Characterization of Antenna Size in Oxygen-Evolving Photosystem II Preparations from Cyanobacteria and Plants†

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ABSTRACT: Photosystem II, the photosynthetic water-oxidizing complex, can be isolated from both plants and cyanobacteria. A variety of methods have been developed for purification of this enzyme, which can be isolated in several functional and structural forms. Knowledge of the pigment content of photosystem II preparations is important for precise spectroscopic, biochemical, and functional analysis. We have determined pigment stoichiometries in oxygen-evolving photosystem II preparations from plants and cyanobacteria. We have employed a solvent system for the isocratic elution of a reverse phase HPLC column in which we have determined the extinction coefficients of the relevant pigments. Pigments were extracted from four photosystem II preparations. These preparations included spinach photosystem II membranes [Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234], spinach photosystem II reaction center complexes [Ghanotakis, D. F., & Yocum, C. F. (1986) *FEBS Lett. 197*, 244−248], spinach photosystem II complexes [MacDonald, G. M., & Barry, B. A. (1992) *Biochemistry 31*, 9848−9856], and photosystem II particles isolated from the cyanobacterium, *Synechocystis* sp. PCC 6803 [Noren, G. H., Boerner, R. J., & Barry, B. A. (1991) *Biochemistry 30*, 3943−3950]. Pigment stoichiometries were determined using two different methods of data analysis and were based on the assumption that there are two pheophytin *a* molecules per photosystem II reaction center. The pigment stoichiometries obtained were comparable for the two methods of data analysis and agreed with previous biophysical and biochemical characterizations of the preparations. The average pigment stoichiometries (chlorophyll:plastoquinone-9 per 2 pheophytin *a*) determined using the two data analysis methods were as follows: photosystem II membranes, 274:3.2; photosystem II reaction center complexes, 78:2.5; *Synechocystis* PS II particles, 55:2.4; photosystem II complexes, 121:2.0.

Photosystem II[1] is a multi-subunit membrane protein complex that carries out the photoinduced oxidation of water and reduction of plastoquinone. PS II is present in all oxygen-evolving plants, green algae, and cyanobacteria and is organized similarly, but not identically, in all these organisms [reviewed in Barry et al. (1994)]. Prosthetic groups include chlorophyll, pheophytin, a non-heme iron, manganese, plastoquinone, and carotenoids. It is widely accepted that there are 4 manganese per PS II reaction center. Three redox active tyrosines have been identified, including D and M, which have undetermined functions, and Z, which reduces P680, the primary chlorophyll donor, and then oxidizes the manganese cluster. One spin of $D^{\bullet}$ can be generated per reaction center [reviewed in Barry (1993) and Debus (1992)].

The PS II enzyme contains cytochrome $b_{559}$, which is composed of an $\alpha$ and $\beta$ subunit. These cytochrome $b_{559}$ subunits are linked by a heme to form a heterodimer (Babcock et al., 1985; Widger et al., 1985). PS II also contains the D1 and D2 subunits, which form the core of the reaction center and bind several of the prosthetic groups that are involved in electron transfer (Nanba & Satoh, 1987). Also present are the light-harvesting 43 kDa (CP43) and 47 kDa (CP47) subunits and the extrinsic 33 kDa protein, which stabilizes the manganese cluster. The 18 and 24 kDa extrinsic proteins have been identified in plants; these subunits sequester the calcium and chloride that are essential for activity [reviewed in Yocum (1991)]. These subunits have not been found in cyanobacteria. In cyanobacteria, two other extrinsic proteins have been identified (9 or 12 kDa and cytochrome $c_{550}$). It has been proposed that these cyanobacterial extrinsic proteins play an analogous role to the 18 and 24 kDa proteins in plants (Shen et al., 1992; Shen & Inoue, 1993).

A variety of procedures have been developed to isolate PS II from plants and cyanobacteria. Most of these methods use detergents to generate monodisperse preparations that are composed of PS II-detergent micelles. Chromatographic separation can then be used for purification. Depending on the choice of detergent and the quality of the chromatographic separation, there may be significant differences in the subunit and pigment composition of the final PS II particles. Several types of preparations that are capable of oxygen evolution have been developed from both plants and cyanobacteria.

In isolating the plant enzyme, purification procedures typically begin with the isolation of PS II membranes, which consist of purified grana stacks (Dunahay et al., 1984). The procedure of Berthold et al. (1981) is commonly used; this procedure uses Triton X-100 to solubilize the stromal membrane of the spinach chloroplast. As isolated in the form

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[1] Abbreviations. Chlorophyll, chl; cytochrome, cyt; pheophytin, pheo; photosystem II, PS II.

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of PS II membranes, the PS II enzyme has a large associated light-harvesting antenna. For some applications, this large antenna size is a disadvantage. Several PS II preparations have been developed that have a smaller chlorophyll antenna size and retain oxygen-evolving capacity [for example, see Enami et al. (1989), Ghanotakis et al. (1987), Ghanotakis and Yocum (1986), MacDonald and Barry (1992), Mishra and Ghanotakis (1993), and van Leeuwen et al. (1991)]. Such PS II "core" preparations are prepared by treating PS II membranes with non-ionic detergents, such as octyl glucoside or dodecyl maltoside. Chlorophyll antenna sizes range from 35 chlorophylls per reaction center (van Leeuwen et al., 1991) to 110−120 chlorophylls per reaction center (MacDonald & Barry, 1992). This variation is due to alterations in the preparation's content of chlorophyll-containing antenna subunits.

From cyanobacteria, PS II is purified by isolation of cellular membranes and solubilization of these membranes with detergent. Typically, chromatography steps are necessary in order to purify PS II-containing detergent micelles away from other contaminants.

Preparations containing D1, D2, cytochrome $b_{559}$, and one or more low molecular weight subunits are the most resolved PS II preparations. These preparations can be isolated from both plants and cyanobacteria [reviewed in Barry et al. (1994)]. These highly resolved "D1−D2 preparations" are not active in oxygen evolution but are capable of primary charge separation (Nanba & Satoh, 1987). D1−D2 preparations have been isolated and characterized by many laboratories [for example, see Akabori et al. (1988), Aured et al. (1994), Chang et al. (1994), Dekker et al. (1989), Eijckelhoff and Dekker (1995), Fotinou and Ghanotakis (1990), Ghanotakis et al. (1989), Gounaris et al. (1989, 1990), Ikeuchi et al. (1989), Kobayashi et al. (1990), Montoya et al. (1991), Omata et al. (1984), Oren-Shamir et al. (1995), Seibert et al. (1988), van Leeuwen et al. (1991), and Yruela et al. (1994)]. The pigment content and composition of these preparations have been the subject of debate. In some cases, D1−D2 preparations have been reported to contain 6 chlorophyll *a* and 2 $\beta$ carotenes per 2 pheophytin *a* molecules, while other studies have found 4 chlorophyll *a* and 1 $\beta$ carotenes per 2 pheophytin *a* molecules. Recently, Eijckelhoff and Dekker (1995) reported revised pigment stoichiometries for D1−D2 preparations. Their measurements are performed by extraction of pigments into organic solvents and by separation and purification of these pigments through the use of HPLC chromatography. The eluant's absorption at a fixed wavelength is monitored; the pigment's molar extinction coefficient at that wavelength is used to quantitate pigments. Their data support a stoichiometry of 6 chlorophylls per 2 pheophytin *a* molecules. Eijckelhoff and Dekker attribute much of the variation in the literature to error introduced by the use of solvent elution systems in which the pigment extinction coefficients are not known (Eijckelhoff & Dekker, 1995).

Knowledge of the pigment content of PS II preparations is important for accurate spectroscopic, biochemical, and functional analysis. Therefore, we have measured pigment stoichiometric ratios in four oxygen-evolving PS II preparations: spinach PS II membranes (Berthold et al., 1981); spinach PS II reaction center complexes (Ghanotakis & Yocum, 1986); PS II particles from *Synechocystis* sp. ATCC 6803 (Noren et al., 1991); and spinach PS II complexes (MacDonald & Barry, 1992). We use the pheophytin *a* content as an internal standard, by use of the assumption that there are two moles of pheophytin *a* per trap. This assumption presumes that PS II is similar to the reaction center from purple bacteria, in which two bacteriopheophytin molecules are present (Deisenhofer & Michel, 1989). For experimental support of this assumption, see Montoya et al. (1991) and Kobayashi et al. (1988). The HPLC method that we have devised is novel but is based on procedures previously described for pigment separation (Eijckelhoff & Dekker, 1995; Shioi et al., 1983). In analyzing our data, we have employed two different methods of analysis. These two methods give equivalent results. In each of these methods, chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and plastoquinone-9 extinction coefficients are employed under conditions in which their values are precisely known. The chlorophyll antenna measurements derived from these pigment stoichiometric ratios are in agreement with the results of earlier biophysical and biochemical measurements of antenna size in these preparations.

## MATERIALS AND METHODS

*Preparation and Purification of PS II Samples*. Spinach PS II membranes were prepared as described by Berthold et al. (1981) using market spinach. The specific activity of oxygen evolution of the PS II membranes was approximately 600 $\mu$mol of $O_2$ (mg of chl-h)$^{-1}$. Spinach PS II complexes were prepared as described by MacDonald and Barry (1992). The specific activity of oxygen evolution was approximately 1100 $\mu$mol of $O_2$ (mg of chl-h)$^{-1}$. Spinach PS II reaction center complexes were prepared as described by Ghanotakis and Yocum (1986), except that the dialysis time was extended to 5 h. The specific activity of oxygen evolution was approximately 1800 $\mu$mol of $O_2$ (mg of chl-h)$^{-1}$. PS II particles from *Synechocystis* sp. PCC 6803 were prepared as described in Noren et al. (1991). A glucose tolerant strain of the cyanobacterium was grown in BG-11 media, which was supplemented with 5 mM glucose and 5 $\mu$g of kanamycin/mL (Noren et al., 1991; Barry, 1995). Cultures were grown to approximately 1.3 $OD_{720nm}$ before harvesting. The final PEG precipitation was omitted to avoid possible interference with HPLC separation of the pigments. The *Synechocystis* PS II particles were concentrated using Centricon 100 microconcentrators. The specific activity of oxygen evolution was approximately 2400 $\mu$mol of $O_2$ (mg of chl-h)$^{-1}$.

*Calibration*. A Hitachi U-3000 double-beam spectrophotometer was employed for these studies. The calibration of the spectrophotometer was evaluated every few months using a set of neutral density filters for the *y* axis and a holmium oxide filter for the *x* axis. The calibration of the spectrophotometer was also evaluated more frequently using a horse heart cytochrome *c* solution of known absorbance for the *y* axis and a visible emission line of the deuterium lamp for the *x* axis.

Manipulations and dilutions of solutions were made using Rainin pipettors. The accuracy and precision of these devices were evaluated frequently using established protocols.

*Pigment Extraction*. In order to obtain reliable results, complete extraction of pigments into organic solvent is

essential. Several methods of extraction were tested to determine which method resulted in highest extraction yield. An extraction system of 80% acetone−20% methanol proved most effective (HPLC grade acetone and methanol, Mallinckrodt Chemical, Paris, KY). Pigment extractions were performed as follows.

PS II preparations with known chlorophyll concentration (spinach PS II membranes, 2.3 mg of chl/mL; PS II reaction center complexes, 1.7 mg of chl/mL; PS II particles from *Synechocystis*, 0.7 mg of chl/mL; and PS II complexes, 1.3 mg of chl/mL) were extracted by the addition of 80% acetone−20% methanol to a final volume of 1 mL. The chlorophyll sample size was less than 280 $\mu$g of chlorophyll for PS II membranes and was less than 140 $\mu$g of chlorophyll for the other samples. The mixture was vortexed with an SP Vortex Mixer (American Scientific Products, McGaw, IL) for 1 min. The sample was sonicated in a Branson 1200 water sonication bath (Branson Ultrasonic Corp., Danbury, CT). The bath was filled with ice water, and the sonication was performed in the dark for 3−4 min. This treatment was followed by a 1 min centrifugation at 13 000*g*. The supernatant was decanted and filtered through a Millipore Millex $LCR_4$ HPLC 0.5 $\mu$m filter unit (Bedford, MA). Control experiments showed that 95%−98% of the pigments were extracted by this treatment. All samples were maintained on ice or at −20 °C. In cases where the extract was not immediately used, argon was bubbled through the sample to protect against pigment oxidation. The extracted pigments were handled under dim illumination and were stored in the dark.

*HPLC Reverse Phase Chromatography*. A Zorbax C-18 column (ODS 4.6 mm × 15 cm, Mac Mod Analytical, Chadds Ford, PA) was used to separate pigments (Shioi et al., 1983). A guard column of the same material was also employed. The mobile phase was 100% HPLC grade methanol (Eijckelhoff & Dekker, 1995). These conditions resulted in the reproducible separation of chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and plastoquinone-9. The column was equilibrated with methanol for 1 h prior to sample injection. HPLC grade methanol was filtered through Millipore type GV organic 0.22 $\mu$m filters before use. The flow rate was 1.0 mL/min, and the eluant was monitored at 255 nm. A Gilson model 302 pump and a Holochrome monitor were used (Gilson Co., Madison, WI). The monitor was interfaced to a Macintosh computer (Apple Computers Inc., Cupertino, CA) through the use of an NB-A2000 data acquisition board and Labview software (National Instruments, Austin, TX). For a typical HPLC run, 3000 data points were collected with a 2 s interval between the points.

*Identification of Pigments*. Pigments were identified by UV−vis spectrophotometry and by mass spectrometry. To obtain the UV−vis spectra, an Hitachi U-3000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) was employed. Spectra were obtained at room temperature at a scan speed of 60 nm/min and a 2.0 nm slit size. For chlorophyll *a* and *b* in 100% methanol, the results were compared to spectral parameters previously described (Lichtenthaler, 1987). For comparison to spectral parameters reported for pheophytin *a*, the spectrum of pheophytin *a* was obtained in methanol acidified with one drop of 25% HCl (Lichtenthaler, 1987); this gives an approximately 100 mM final concentration of HCl. Plastoquinone-9 spectra were obtained both in the air-oxidized and reduced forms. Plastoquinone-9 was reduced by the addition of 10 mg of sodium borohydride per 0.5 mL sample. These data were compared to published spectra of plastoquinone in its two oxidation states (Barr & Crane, 1971). Filtered HPLC grade methanol was used as the solvent.

Pigment identification was also carried out through the use of mass spectrometry. Samples were concentrated with a Speed-Vac concentrator (Savant, Hicksville, NY) and were stored at −20 °C until analysis. Both plasma desorption mass spectrometry (PDMS) and electron ionization mass spectrometry (EIMS) were utilized. For PDMS, an Applied Biosystems Biopolymer Mass Analyser BIO-ION-20R (Bio-ion, Uppsala, Sweden) was used. This desorption method utilizes an energy source to cause direct formation of gaseous ions. $^{252}$Cf was used as the ionizing agent. Mass determination was made with a time of flight detector. Approximately 1.5 $\mu$L of chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and plastoquinone-9 in 80% acetone−20% methanol were used for analysis. Spectra of pigments were taken at 15 kV. For EIMS, a Kratos MS25 (Kratos Analytical, Ramsey, NJ) was tuned at 2.0 kV to optimize and focus the electron beam, and mass units were calibrated with perfluorokerosene, which gives highly reproducible and known mass unit separation. Spectra of pigments were taken at 70 eV.

*Quantitative Analysis*. Data were analyzed through the use of KaleidaGraph software (Abelbeck Software, Reading, PA). Data collected by monitoring the eluant from the HPLC separation was transferred as a Labview data file to KaleidaGraph. The area under each peak in the HPLC profile was determined by integration. This data set was manipulated in two different ways to obtain relative pigment stoichiometries. Method 1 involved the generation of a standard curve by injection of a concentration series of each purified pigment into the HPLC system. Method 2 involved direct use of the extinction coefficient at the monitoring wavelength (255 nm). To obtain relative stoichiometries, both methods assume that there are 2 mol of pheophytin *a* per PS II reaction center. Molecular weights of 892.5, 906.5, 870.6, and 748.6 g/mol were used for chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and plastoquinone, respectively. In each case, these correspond to the molecular masses of the most abundant isotopomer (see Results).

*Method 1*. Purified samples of chlorophyll *a*, chlorophyll *b*, plastoquinone-9, and pheophytin *a* were used as standards in the generation of a concentration curve for each pigment. For chlorophyll *a* and chlorophyll *b*, the concentration of each standard was determined spectrophotometrically in methanol by use of the extinction coefficient at two visible wavelengths (Lichtenthaler, 1987) (Table 1). For pheophytin *a*, the concentration of the standard was determined spectrophotometrically in acidified methanol through the use of the extinction coefficient at two visible wavelengths (Lichtenthaler, 1987) (Table 1). For plastoquinone-9, the concentration of the standard was determined spectrophotometrically in methanol by use of the difference spectrum, oxidized-minus-reduced, and the extinction coefficient previously determined in the ultraviolet (Barr & Crane, 1971). Reduction of plastoquinone-9 was carried out with sodium borohydride, as described above.

Recently, new extinction coefficients for pheophytin *a* in nonacidified methanol were reported (Eijckelhoff & Dekker, 1995). These redetermined extinction coefficients were shown to differ by approximately 10% from those previously

Table 1: Extinction Coefficients of Photosystem II Pigments [in $(g/L)^{-1}$ $cm^{-1}$]

| | Soret band[a] | $Q_y$ band[a] | 255 nm[b] |
|---|---|---|---|
| chlorophyll *a* | 77.05 | 79.24 | 24.9 ± 0.3[c] |
| chlorophyll *b* | 105.36 | 38.87 | 29.6 ± 0.4[d] |
| pheophytin *a* | 206.14 | 50.4 | 17.8 ± 1.0[e] |
| plastoquinone-9 | | | 21.4 ± 0.2[f] |

[a] Chlorophyll *a*, 665.2 nm ($Q_y$ band) and 431.8 nm (Soret band); chlorophyll *b*, 652.4 nm ($Q_y$ band) and 469.2 nm (Soret band); pheophytin *a*, 654.2 nm ($Q_y$ band) and 417.4 nm (Soret band) taken from (Lichtenthaler, 1987). [b] This work. [c] Average of 18 determinations. [d] Average of 17 determinations. [e] Average of 12 determinations. [f] Average of 10 determinations. Oxidized plastoquinone-9 based on oxidized minus reduced spectrum and the relevant extinction coefficient at 255 nm: 15 $mM^{-1}$ $cm^{-1}$ (Barr & Crane, 1971).

reported for HCl-treated pheophytin *a* (Lichtenthaler, 1987). In our study, we have used the extinction coefficients determined by Lichtenthaler, since we employed these extinction coefficients to calculate pheophytin *a* concentrations in acidified solutions. This was carried out in the following way. A stock pheophytin *a* solution was divided into two equal aliquots. One aliquot was acidified with HCl to determine the concentration of the stock solution using extinction coefficients determined by Lichtenthaler. The dilution with HCl was taken into account in this determination. The second aliquot of the stock solution was used for determination of a standard curve as described below.

After determination of the concentration of the standard, this sample was serially diluted into 80%−20% acetone−methanol. A portion of each serially injected sample was injected onto the Zorbax column using conditions identical to those employed during chromatography of the extracts from PS II. Three to five injections were performed at each concentration of each pigment. The area under each peak was determined by integration, and this area was plotted versus the known concentration to yield a standard curve (see example in Figure 1). Five or more data points were used to create each standard curve. Several standard curves were determined for chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and plastoquinone-9; the data obtained were found to be reproducible. Once standard curves were determined for each of the pigments of interest, the derived equation for the line and the molecular mass of the pigments were used to determine the concentration of pigments in each of the PS II extracts (Figure 1).

Standard curves were generated using commercially available pigments, as well as pigments isolated from large scale HPLC purifications. There was no difference when commercially available pigments were compared to pigments purified in our laboratory. Commercially available pigments included chlorophyll *a*, chlorophyll *b*, (Sigma Chemicals, St. Louis, MO), and plastoquinone-9 (Hoffman-LaRoche, Nutley, NJ).

*Method 2*. Extinction coefficients for purified chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and plastoquinone-9 were determined at 255 nm. This wavelength proved to be optimal in simultaneous detection of all four pigments. To determine the extinction coefficients at this wavelength in 100% methanol, room temperature spectra of each of the pigments were recorded from 200 to 740 nm using conditions described above. Published extinction coefficients for chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and plastoquinone-9 were used to derive the extinction coefficient at 255 nm (Table 1). Multiple determinations were performed to obtain reproducible and reliable values. For chlorophyll *a*, chlorophyll *b*, and plastoquinone-9, extinction coefficients were determined in pre-filtered HPLC grade methanol. Since the visible extinction coefficients for pheophytin *a* were determined in acidified methanol, HCl-treated HPLC grade methanol was used for determination of the 255 nm extinction coefficient of pheophytin *a*. Through a series of control experiments, the 255 nm extinction coefficient so determined was then found to be independent of acidification. The 255 nm extinction coefficient for plastoquinone-9 in the oxidized form was determined through the use of the extinction coefficient derived from the oxidized-minus-reduced spectrum of plastoquinone-9 (Barr & Crane, 1971).

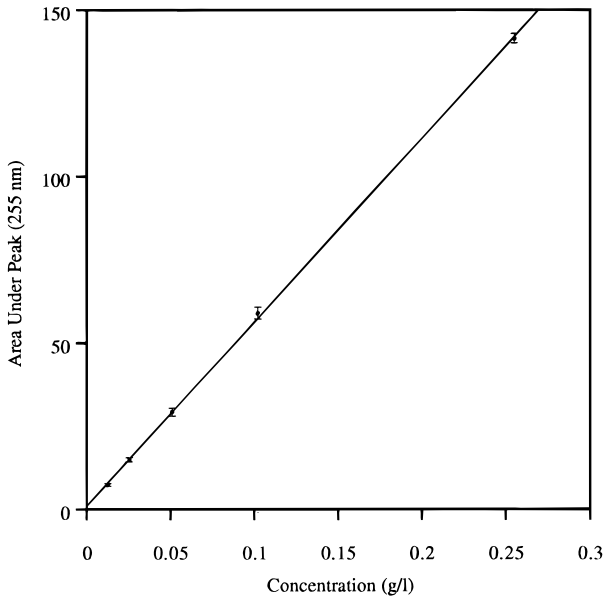


FIGURE 1: Chlorophyll *a* standard curve used for data analysis by method 1 (see Materials and Methods). Chlorophyll *a* was isolated from a large-scale HPLC separation of spinach PS II membrane pigments. Using reported extinction coefficients (Lichtenthaler, 1987), the concentration of the chlorophyll *a* stock was determined. A serial dilution of the stock chlorophyll *a* was made in acetone−methanol. Serially diluted samples were injected and analyzed by HPLC, as described in Figure 2. Areas under the chlorophyll *a* elution peak were measured using KaleidaGraph V. 3.0 software. The best fit to the data points is shown: $y = 0.998 + 553.1x$ ($R = 0.999$). The bars on each data point show the standard error of each set of measurements.

To determine relative pigment stoichiometry using method 2, the area under each relevant peak, recorded from the HPLC eluant by the UV−vis monitor, was corrected for the pigment's extinction coefficient at 255 nm and the molecular mass of the pigment.

## RESULTS

In Figure 2, we present chromatograms of pigments extracted from the four oxygen-evolving PS II preparations of interest: spinach PS II reaction center complexes (Figure 2A); *Synechocystis* PS II particles (Figure 2B); spinach PS II complexes (Figure 2C); and spinach photosystem II membranes (Figure 2D). Peaks 1−5 had elution times of 7, 12, 47, 52, and 94 min, respectively. These peaks were present in all preparations, except for peak 1, which was absent when pigments were extracted from *Synechocystis* PS II particles (Figure 2B).

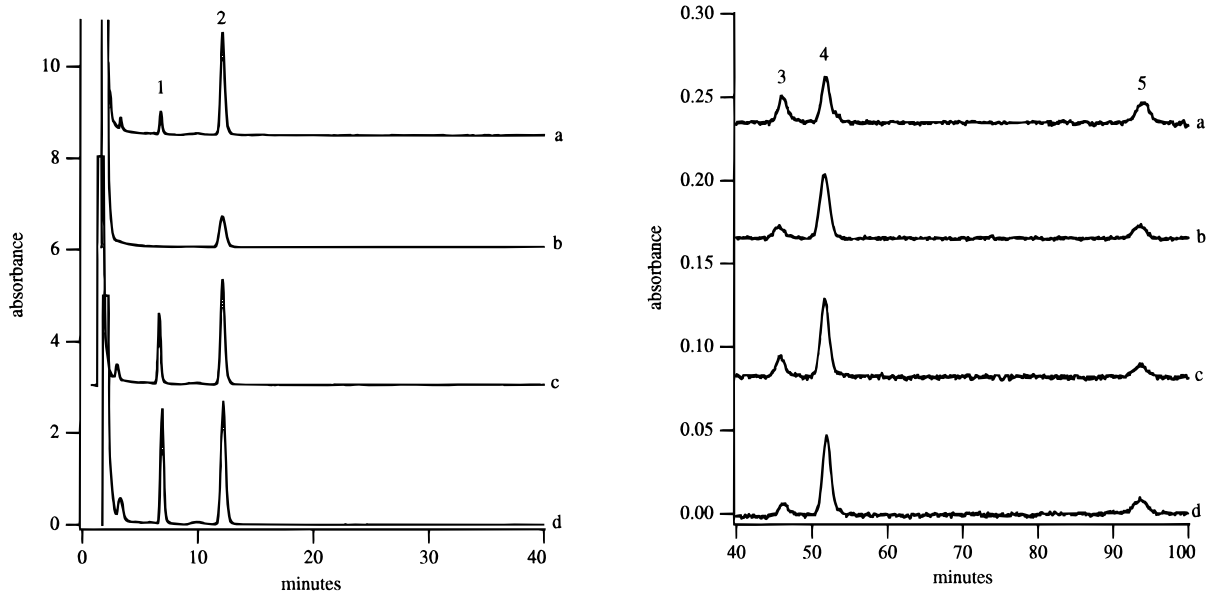


FIGURE 2: HPLC separation of acetone−methanol extracted PS II preparations. Pigments were extracted from spinach PS II reaction center complexes (a), from *Synechocystis* PS II particles (b), from spinach PS II complexes (c), and from spinach PS II membranes (d). The trace is split into two panels because of the large difference in absorbance. In the second panel, a smoothing function was applied to the traces. See Materials and Methods for conditions.

Initially, we were concerned about the reproducibility of chromatographic separations of the extracted pigments. We addressed this concern by carrying out multiple extractions of the same PS II sample. These control experiments demonstrated that the chromatographic separations gave reproducible results under our conditions. We showed that the method employed for detection of pigments in the eluant was linear with the concentrations of pigments. Injection of more concentrated samples led to a quantitative increase in the areas under the eluant peaks. We also carried out control experiments to show that chlorophyll *a* does not pheophytinize during the HPLC separation (data not shown).

The pigments from each of the peaks were collected and subjected to analysis using UV−vis spectrophotometry. In Figure 3, we present the absorption spectra of sample collected in peak 2 (Figure 3A), peak 1 (Figure 3B), and peak 3 (Figure 3C). The oxidized and reduced spectra of sample collected from peak 5 are also presented (Figure 3D). The absorption maxima of the Soret and $Q_y$ bands in Figures 3A−C are identical to the absorption maxima of chlorophyll *a*, chlorophyll *b*, and pheophytin *a*, respectively (Lichtenthaler, 1987). The oxidized and reduced spectra shown in Figure 3D are identical to published spectra of plastoquinone-9 in its two oxidation states (Barr & Crane, 1971). Peak 4 was also analyzed and was determined to be a carotenoid, probably $\beta$-carotene (data not shown). Other carotenoid peaks eluted with the solvent front (Figure 2).

The pigments from each of the peaks were collected and subjected to analysis using mass spectrometry. For a review of the applications of mass spectrometry to photosynthetic pigments, see Hunt and Michalski (1991). Calculations of the average molecular mass and the molecular mass of the most abundant isotopomer were performed with the program, RLSPEC, written by Dr. R. Lapp at the University of Nebraska. Chlorophyll *a* has an isotopically averaged mass of 893.5 g/mol; the most abundant isotopomer has a mass of 892.5 g/mol. Chlorophyll *b* has an isotopically averaged mass of 907.5 g/mol; the most abundant isotopomer has a mass of 906.5 g/mol. Pheophytin *a* has an isotopically averaged mass of 871.2 g/mol; the most abundant isotopomer has a mass of 870.6 g/mol. Plastoquinone-9 has an isotopically averaged mass of 749.2 g/mol; the most abundant isotopomer has a molecular mass of 748.6 g/mol.

Figure 4 shows the mass spectra obtained through the use of plasma desorption mass spectrometry (PDMS). Positive ions were detected. The label in each panel denotes the centroid of the broad molecular ion envelope. The shape of this envelope is influenced by the isotope pattern and by protonation of the $M^+$ ion (Hunt & Michalski, 1991).

The mass spectra shown in Figures 4A−D were obtained from peaks 2, 1, 3, and 5, respectively, and agree with the expected molecular mass of chlorophyll *a* (A), chlorophyll *b* (B), pheophytin *a* (C), and plastoquinone-9 (D). Since there was a significant amount of noise in the mass spectrum of plastoquinone-9, electron ionization mass spectrometry (EIMS) was also employed on this sample. The results of EIMS confirmed the molecular weight of the pigment in peak 5 (data not shown).

To summarize, UV−vis spectrophotometry and mass spectrometry show peak 1 to be chlorophyll *b*, peak 2 to be chlorophyll *a*, peak 3 to be pheophytin *a*, and peak 5 to be plastoquinone-9. After pigment identification, quantitative treatment of the data could be carried out.

For each of the PS II preparations, multiple extractions and multiple injections were performed, and the areas under the eluant peaks were determined. These data were then subjected to two methods of data analysis, a standard curve method (method 1; Table 2) and an extinction coefficient method (method 2; Table 3). In method 1, we used standard curves to derive the concentrations of chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and plastoquinone-9. This method does not rely on knowledge of the extinction coefficient at the monitoring wavelength and is independent of precise calibration of the UV−vis monitor. In method 2, we used extinction coefficients determined at 255 nm (Table 1) to directly determine the pigment stoichiometries from the chromatograms. This method avoids the propagation of indeterminate pipeting errors involved in serial dilution.

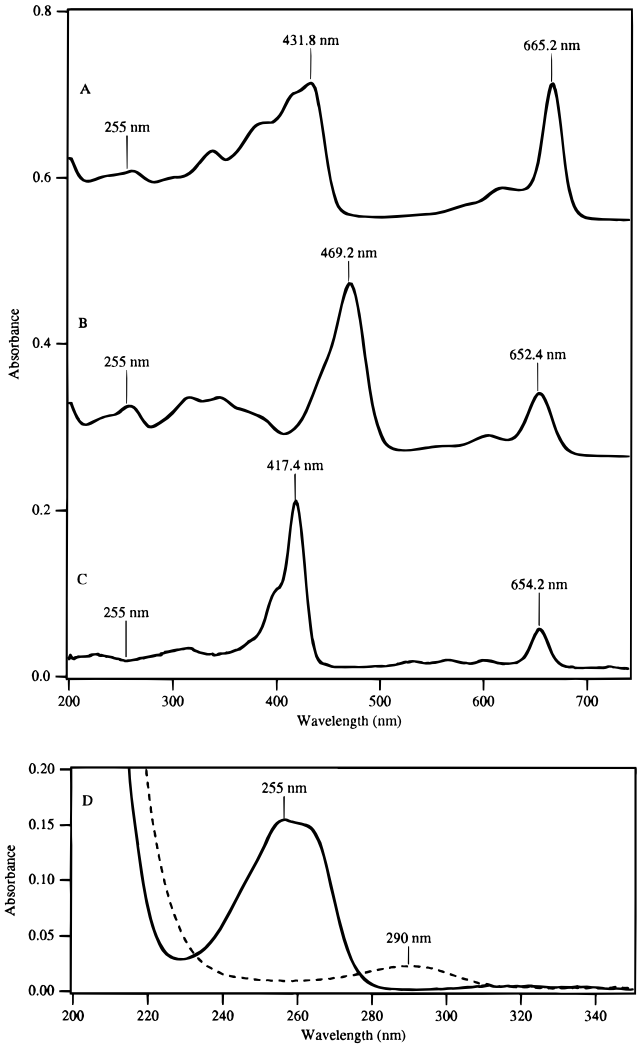


FIGURE 3: Room temperature UV−visible spectra of extracted PS II pigments. The spectra (A−D) were obtained from peaks 2, 1, 3, and 5 (Figure 2), respectively, and show chlorophyll *a* (A), chlorophyll *b* (B), pheophytin *a* (C), and plastoquinone-9 (D). For each pigment, spectra were normalized to a concentration of 1 mM. For presentation purposes, chlorophyll *a* and *b* spectra were then expanded 2-fold, and plastoquinone-9 was expanded 10-fold. See Materials and Methods for conditions.

Tables 2 and 3 show that the pigment stoichiometries derived by method 1 and by method 2 are in reasonable agreement.

The data in Tables 2 and 3 show that PS II membranes have the highest chlorophyll and plastoquinone content (278−271 chlorophylls and 3.5−3.0 plastoquinone-9 per 2 pheophytin *a*) of any of the preparations examined. This result is consistent with the relatively low specific activity for oxygen evolution that is typically found in this type of PS II preparation [600 $\mu$mol of $O_2$ (mg of chl-h)$^{-1}$]. The chlorophyll *a* to chlorophyll *b* ratio was found to be 2.1 (Tables 2 and 3), in agreement with earlier results [for example, see MacDonald and Barry (1992)].

PS II complexes are isolated by treatment of PS II membranes with dodecyl maltoside and ion exchange chromatography. Nonessential light-harvesting subunits are separated away from PS II reaction centers in this chromatography step. These PS II complexes are monodisperse, yet retain the 18 and 24 kDa extrinsic proteins (MacDonald & Barry, 1992). Compared to PS II membranes, this preparation shows a 2.3-fold decrease in antenna size and a 1.6-fold reduction in the amount of plastoquinone-9 associated with the reaction center. Note that PS II complexes still retain chlorophyll *b*, although the chlorophyll *a* to chlorophyll *b* ratio is increased to 3.0−2.8 (Tables 2 and 3), as previously described (MacDonald & Barry, 1992). The retention of chlorophyll *b* is caused by the fact that some, but not all, of the light-harvesting antenna subunits have been removed during the purification procedure (MacDonald & Barry, 1992).

PS II reaction center complexes are isolated by treatment of PS II membranes with octyl glucoside. The light-harvesting complex can then be precipitated away from the PS II reaction center. The detergent is dialyzed out of the preparation. This procedure leaves a PS II fraction that can be pelleted at low centrifugal forces (Ghanotakis & Yocum, 1986). Compared to PS II membranes, this preparation shows a 3.5-fold decrease in antenna size. There is a 1.3-fold decrease in the content of plastoquinone-9 per reaction center. This preparation has a chlorophyll *a* to chlorophyll *b* ratio of approximately 9, consistent with the removal of most of the associated light-harvesting complex from the PS II reaction center (Tables 2 and 3).

PS II particles from *Synechocystis* are isolated by treatment of cellular membranes with dodecyl maltoside. The treatment is followed by two rounds of ion exchange chromatography. This preparation has the highest specific activity for oxygen evolution of any of the preparations examined [2400 $\mu$mol of $O_2$ (mg of chl-h)$^{-1}$]. This preparation also has the smallest chlorophyll antenna size (56.1−54.9 chlorophylls per 2 pheophytin). The average quinone content is 2.4 plastoquinone-9 per 2 pheophytin. Note that there is no detectable chlorophyll *b* in the PS II preparations from *Synechocystis*, as expected for cyanobacteria (Svec, 1991).

## DISCUSSION

In this report, we describe a procedure for simple, rapid, and accurate assessment of PS II pigment stoichiometries. The extraction procedure removes 95%−98% of the pigment in one extraction step. Isocratic elution of a reverse phase column simplifies the chromatography and provides a mobile phase in which extinction coefficients can be precisely determined. As recently discussed (Eijckelhoff & Dekker, 1995), many of the previously described methods for analysis of pigments are not compatible with reported spectrophotometric extinction coefficients for chlorophylls *a* and *b*, pheophytin *a*, and plastoquinone-9. This is a result of the use of conditions for column elution (such as solvent mixtures) in which extinction coefficients have not been determined. This can lead to propagation of substantial errors in a quantitative analysis. We hope that our detailed description of extraction and chromatography conditions will help to promote a standardization of pigment analysis methods.

Our comparison of two different methods of data analysis shows good agreement between the standard curve and extinction coefficient methods. This is of importance, since most previous PS II pigment composition studies do not specify the data analysis method that has been employed. We show here that the two methods can be employed in such a way as to give equivalent results.

We can compare the results of our pigment quantitation to previous characterizations of each of these PS II preparations. These characterizations are based on four methods:

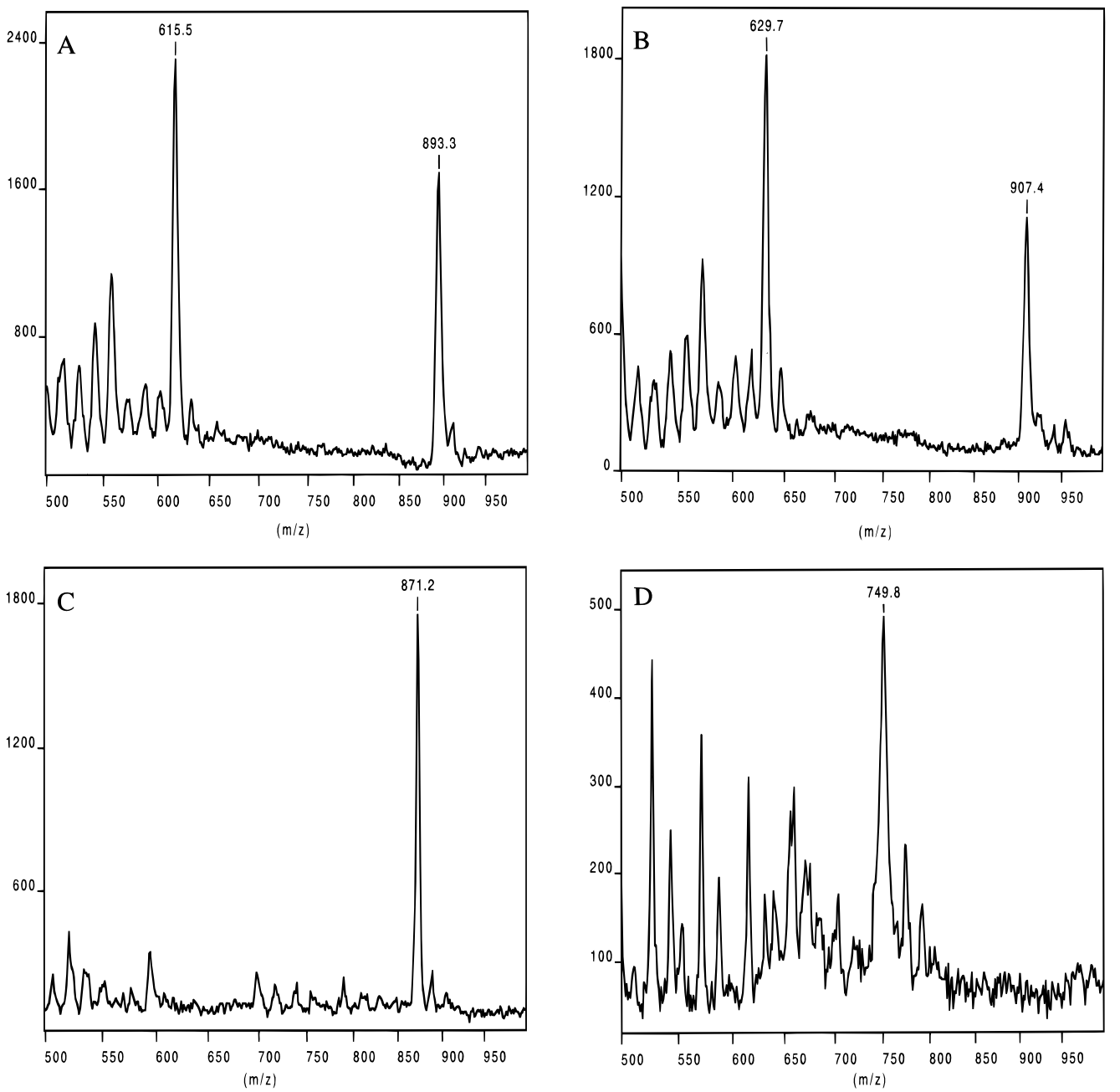


FIGURE 4: Plasma desorption mass spectra of extracted PS II pigments. The spectra (A−D) were obtained from peaks 2, 1, 3, and 5 (Figure 2), respectively, and show chlorophyll *a* (A), chlorophyll *b* (B), pheophytin *a* (C), and plastoquinone-9 (D). See Materials and Methods for conditions.

manganese quantitation and the assumption of four manganese atoms per trap; $D^{\bullet}$ spin quantitation and the assumption of one $D^{\bullet}$ spin per trap; optical quantitation of $Q_A^-$ yield through the use of the $Q_A^-$ extinction coefficient at 320 nm and the assumption of one $Q_A^-$ per trap; and previous pigment analyses [see, for example, Akabori et al. (1988), Kobayashi et al. (1988), Murata et al. (1984), Omata et al. (1984), Tang and Diner (1994), and Yamata et al. (1987)]. In some cases of pigment analysis, the hemes of cytochrome $b_{559}$ were used as an internal standard (but see below for a discussion of potential variability in this heme content). In manganese-depleted PS II preparations, $Z^{\bullet}$ can also be used to estimate antenna size, since its signal can be photoaccumulated up to one spin per reaction center.

PS II membranes from spinach have been studied extensively. On the basis of manganese quantitation, $D^{\bullet}$ and $Z^{\bullet}$ spin counts, and $Q_A^-$ optical measurements, antenna sizes in the range from 206 to 280 chlorophylls have been reported (Babcock et al., 1983; Berthold et al., 1981; Bowlby et al., 1990; Buser et al., 1992; Dekker et al., 1984; Ghanotakis et al., 1987; MacDonald & Barry, 1992). For example, Bowlby et al. (1990) found 245 ± 12 chlorophylls per 2 cytochrome $b_{559}$ hemes. The antenna size of PS II membranes would be expected to vary extensively. This is due to the fact that these preparations can exhibit significant contamination from photosystem I (Dunahay et al., 1984); this contamination will increase the apparent antenna size. Also, more extensive detergent treatments, for example, two treatments with Triton X-100 instead of one [compare Berthold et al. (1981) and Buser et al. (1992)], may reduce antenna size by removal of chlorophyll in light-harvesting subunits. Both sources of antenna size variation would also be expected to affect the specific activity of the enzyme preparation. In a study of PS II membranes with similar oxygen rates to those obtained

Table 2: Analysis of Pigment Content of Four Photosystem II Preparations Determined by Method 1 (Standard Curve Determination)[a]

| preparation | chl/2 pheo | PQ-9/2 pheo | chl *a*/*b* ratio |
|---|---|---|---|
| spinach PS II membranes[b] | 278 ± 4 (284−273) | 3.5 ± 0.2 (3.8−3.3) | 2.17 ± 0.02 (2.20−2.15) |
| spinach PS II reaction center complexes[c] | 81 ± 3 (86−77) | 2.78 ± 0.08 (2.91−2.66) | 10.2 ± 0.3 (10.6−9.7) |
| PS II particles from *Synechocystis*[d] | 56.1 ± 0.5 (56.4−55.3) | 2.24 ± 0.02 (2.26−2.22) | |
| spinach PS II complexes[e] | 126 ± 2 (129−123) | 2.2 ± 0.1 (2.3−2.0) | 3.04 ± 0.02 (3.07−3.01) |

[a] Ranges represent the results of multiple determinations performed on multiple samples. Six individual determinations were performed on spinach PS II membranes, spinach PS II reaction center complexes and PS II complexes. Four individual determinations were performed on PS II particles from *Synechocystis*. The number given after the mean is the standard deviation, *s*, for each set of measurements. [b] Berthold et al. (1981). [c] Ghanotakis et al. (1986). [d] Noren et al. (1991). [e] MacDonald and Barry (1992).

Table 3: Analysis of Pigment Content of Four Photosystem II Preparations determined by Method 2 (Extinction Coefficient Determination at 255 nm)[a]

| preparation | chl/2 pheo | PQ-9/2 pheo | chl *a*/*b* ratio |
|---|---|---|---|
| spinach PS II membranes[b] | 271 ± 5 (280−266) | 3.0 ± 0.1 (3.2−2.9) | 2.05 ± 0.02 (2.08−2.03) |
| spinach PS II reaction center complexes[c] | 74 ± 3 (79−70) | 2.20 ± 0.07 (2.31−2.10) | 8.2 ± 0.2 (8.4−7.9) |
| PS II particles from *Synechocystis*[d] | 54.9 ± 0.5 (55.3−54.1) | 2.55 ± 0.02 (2.57−2.52) | |
| spinach PS II complexes[e] | 117 ± 2 (120−114) | 1.82 ± 0.06 (1.89−1.70) | 2.80 ± 0.02 (2.82−2.77) |

[a] Ranges in parentheses represent the results of multiple determinations performed on multiple samples. Six individual determinations were performed on spinach PS II membranes, spinach PS II reaction center complexes, and PS II complexes. Four individual determinations were performed on PS II particles from *Synechocystis*. The number given after the mean is the standard deviation, *s*, for each set of measurements. [b] Berthold et al. (1981). [c] Ghanotakis et al. (1986). [d] Noren et al. (1991). [e] MacDonald and Barry (1992).

here, MacDonald and Barry (1992) found 270 chlorophylls per trap through the use of D• and manganese quantitation. This result is in reasonable agreement with our measurements of antenna size (an average of 274 chlorophylls per trap) through pigment analysis.

Other studies have estimated the antenna size of PS II reaction center complexes. Values of 68−75 chlorophylls per trap have been obtained through D• and manganese quantitation (Ghanotakis et al., 1987). Bowlby et al. (1990) found 75 ± 5 chlorophylls per 2 cytochrome $b_{559}$. These numbers are in agreement with our result of 74 ± 3 and 81 ± 3 chlorophylls per reaction center in this preparation.

PS II complexes were originally shown to contain 110 chlorophyll per 4 manganese and 100 chlorophylls per D•. The reported standard error was 7%−8% (MacDonald & Barry, 1992). A more extensive later study is summarized in Table 4 (MacDonald et al., 1994). In this study, a range of values from 89 to 110 chlorophyll per trap were found; these numbers are based on $Q_A^-$ optical quantitation, D• spin counts, and manganese quantitation. The range of antenna size values found in our pigment analysis (average of 121 chlorophylls per trap) overlaps with the range observed in these previous determinations (Tables 2−4).

Several methods of isolating oxygen-evolving PS II particles or membranes from *Synechocystis* have been described (Burnap et al., 1989; Kirilovsky et al., 1992; Nilsson et al., 1992; Noren et al., 1991; Tang & Diner, 1994). Chlorophyll antenna sizes from 37 to 74 chlorophylls per trap have been reported. The Noren et al. preparation was characterized using manganese quantitation and D• spin quantitation and found to have 50−66 chlorophylls per trap (Boerner & Barry, 1993; Boerner et al., 1992, 1993; Noren & Barry, 1992). A more extensive later study of this preparation is summarized in Table 4 (MacDonald et al., 1994). A range of values from 46 to 64 chlorophyll per trap were found; these numbers are based on $Q_A^-$ optical quantitation, D• spin counts, and manganese quantitation. The average of these values is in good agreement with the result of pigment analysis that we report here (56 chlorophylls per trap). Note that our finding of 56 chlorophylls per trap is somewhat larger than the minimum 48 chlorophylls per reaction center expected from the studies of Alfonso et al. (1994) on isolated CP47, CP43, and D1−D2 preparations. The small difference could be due to a 10%−20% loss of protein-associated pigments upon separation of CP47 and CP43 from the D1−D2 core.

We have also reported the quinone content of each of the four oxygen-evolving preparations. This is an important first step in biophysical studies of the acceptor side of PS II and in attempts to reconstitute quinones into these preparations. The values derived from PS II membranes are in agreement with the previous measurements of 3.2 plastoquinone-9 per 2 cytochrome $b_{559}$ in membranes. Our values are higher, however, than the 1.8 ± 0.1 plastoquinone-9 per 2 cytochrome $b_{559}$ obtained previously from reaction center complexes (Bowlby et al., 1990). We have found PS II membranes to have the highest quinone content of all four preparations, consistent with the fact that these membranes have not been extensively treated with detergent and are not column purified. Quinone content is reduced in reaction center complexes, probably due to the additional treatment with detergent. PS II complexes have the lowest quinone content; this low quinone content is due to the chromatographic purification in detergent-containing buffers. This treatment will remove any quinone bound to low-affinity sites on PS II. Note that our previous results have shown that PS II complexes contain only a single functional quinone, $Q_A$, in spite of the fact that our present results show that more than one quinone per reaction center are present (G. M. MacDonald, B. A. Barry, and R. J. Debus, unpublished data). Cyanobacterial PS II is also column purified and also contains no functional $Q_B$. However, its quinone content is somewhat higher than that of spinach PS II complexes. This may be due to some unexpected difference between cyanobacterial and spinach PS II or may be due to the low content of PS II and high content of quinone in membranes from cyanobacteria.

Our studies have provided information about pigment composition in several oxygen-evolving PS II preparations. Accurate knowledge of pigment composition is a prerequisite

Table 4: Characterization of Photosystem Complexes[a]

| preparation | chl/$b_{559}$ | chl/$Q_A^-$ | chl/4 Mn | chl/D$^\bullet$ |
|---|---|---|---|---|
| PS II particles from *Synechocystis* | 31 (33−29) | 46 (50−42) | 64 (66−60) | 60 (71−49) |
| PS II particles from Mn-depleted and cyt $c_{550}$ depleted *Synechocystis* | 33 (36−31) | 49 (56−41) | | 54 (59−49) |
| spinach PS II complexes | 110 (120−101) | 95 (98−91) | 110 (134−91) | 89 (93−84) |

[a] Table from MacDonald et al. (1994). The ranges represent the results of multiple determinations performed on multiple preparations. Each chl/D$^\bullet$ value is the average of two to three determinations. Each chl/4 Mn value is the average of four to seven determinations. Each chl/$Q_A^-$ value is the average of three to five determinations.

for many types of biochemical and biophysical studies. An additional reason for our study was to provide a further test of our results concerning cytochrome $b_{559}$ stoichiometry (MacDonald et al., 1994). The stoichiometry of cytochrome $b_{559}$ in PS II has been under debate. Two cytochrome $b_{559}$ hemes per PS II reaction center have been found in some studies, while other studies have found only one heme per trap [see MacDonald et al. (1994) and references therein]. Our previous work has led us to favor the hypothesis that the stoichiometry of cytochrome $b_{559}$ per reaction center is variable and depends on the method of detergent treatment (MacDonald et al., 1994). This work was based on several different types of antenna measurements on two preparations: the Noren et al. (1991) *Synechocystis* preparation and the PS II complex preparation from spinach (Table 4). Three methods were used to estimate antenna sizes in each preparation: manganese quantitation, $Q_A^-$ quantitation, and D$^\bullet$ spin quantitation. No matter which method is used to determine antenna size, the cytochrome $b_{559}$ heme content per trap differs by factors of 1.7−2.3, when the two preparations are compared (Table 4). Several hypotheses were presented to explain our results. The most favored hypothesis was that PS II complexes from plants and cyanobacteria originally contain 2 cytochrome $b_{559}$ hemes per PS II reaction center. During isolation and purification of PS II particles, one of these hemes can be lost, as occurs readily in spinach core particles (MacDonald et al., 1994). This hypothesis can explain much of the variation in cytochrome $b_{559}$ content that has been observed in the literature. The results of our pigment analysis confirm these previous antenna size measurements on the *Synechocystis* and the spinach PS II complex preparation (MacDonald et al., 1994) and support the conclusion that the cytochrome $b_{559}$ heme content is different, when the two preparations are compared. Note that our results are consistent with approximately 90% active centers in both the Noren et al. *Synechocystis* preparation and the PS II complex preparation from spinach. We had previously concluded that both preparations contain a majority of active centers from an analysis of the increase in specific activity during each purification (MacDonald & Barry, 1992; Noren et al., 1991).

Tang and Diner found 40 ± 2 chlorophylls and 1.2 ± 0.1 cytochrome $b_{559}$ per reaction center in their *Synechocystis* preparation. They have attributed the difference in antenna size between their preparation and the Noren et al. preparation as due to the presence of 50% inactive centers in the Noren et al. preparation (Tang & Diner, 1994). Our present results show that this is not a possible explanation of the observed difference between the two preparations. Our pigment analysis, which does not depend on PS II function, agrees with antenna size measurements based on electron transfer activity in each preparation (Tables 2−4).

The difference between our findings and the results of the Tang and Diner study may be due to differences in the method of analysis. For example, the Tang and Diner method extracts pigments with an aqueous acetone solution. In our hands, this procedure is not as effective as the mixture of acetone and methanol that we have employed. The apparent difference may also be due to the use of different extinction coefficients for chlorophyll *a* and pheophytin *a* in the Tang and Diner analysis. The extinction coefficients that we have employed are based on the work of Smith and Benitz (1955) who used magnesium atomic absorption to determine extinction coefficients for chlorophylls *a* and *b* in diethyl ether. Using these values as a standard, Porra et al. (1989) found an extinction coefficient of 71.43 (mM-cm)$^{-1}$ or 79.95 (g-cm/L)$^{-1}$ at 665.2 nm for chlorophyll *a* in methanol and of 38.55 (mM-cm)$^{-1}$ or 42.48 (g-cm/L)$^{-1}$ at 652.0 for chlorophyll *b* in methanol. Using the values of Smith and Benitz as a standard, Lichtenthaler (1987) independently found values for chlorophyll *a*, chlorophyll *b*, and pheophytin *a* in methanol, as described in Table 1. These extinction coefficients are in reasonable agreement with those of Porra et al. (1989).

Alternatively, some of the apparent differences in antenna size may be due to differences in the two purification procedures. For example, the Tang and Diner method involves elution from an ion exchange column under very dilute conditions. This treatment could lead to a different content of pigments, cytochrome $b_{559}$, and extrinsic subunits per reaction center. This hypothesis is supported by the observation that the Tang and Diner preparation lacks cytochrome $c_{550}$, a cyanobacterial PS II extrinsic protein found in the Noren et al. preparation (MacDonald et al., 1994).

Each of the methods used to estimate antenna size in Table 4 has its associated difficulties. The $Q_A^-$ measurement is based on an extinction coefficient; we have employed a value of 12.5 mM$^{-1}$ cm$^{-1}$ [see MacDonald et al. (1994) for procedure and references therein]. In our hands, the $Q_A^-$ measurement consistently underestimates the number of chlorophylls per reaction center by approximately 14%. Manganese quantitation biases toward large antenna sizes if the biochemical preparation contains inactive centers. D$^\bullet$ spin counts at room temperature are associated with the largest standard error. Pigment analysis is based on an assumption that there are two pheophytin per PS II reaction center. The overall good agreement between the results of pigment analysis and other antenna size measurements suggests that any of these four methods gives a reasonable estimate of the number of chlorophylls per reaction center.

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