Comparison of Cytochrome *b*-559 Content in Photosystem II Complexes from Spinach and *Synechocystis* Species PCC 6803†

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ABSTRACT: Cytochrome *b*-559 is an integral component of photosystem II complexes from both plants and cyanobacteria. However, the number of cytochrome *b*-559 associated with the photosystem II reaction center has been the subject of controversy. Some studies have concluded that there is one heme equivalent of cytochrome *b*-559 per reaction center, some studies have found two, and some studies have reported intermediate values. Most of the previous experiments have used only one method to quantitate the antenna size of the preparation. In this study, we compare the cytochrome *b*-559 content in a cyanobacterial and a plant photosystem II preparation. The plant preparation is derived from spinach, and previous work has shown that it has an antenna size of approximately 100 chlorophylls [MacDonald, G. M., & Barry, B. A. (1992) *Biochemistry 31*, 9848–9856]. The cyanobacterial preparation is from *Synechocystis* sp. PCC 6803, and previous work has shown that it has an antenna size of approximately 60 chlorophylls [Noren, G. H., Boerner, R. J., & Barry, B. A. (1991) *Biochemistry* 30, 3943–3950]. Both preparations are isolated through the use of ion-exchange chromatography, and both preparations are monodisperse in the same nonionic detergent. In our comparative study, we quantitate antenna size by three different methods. Our work shows that, depending on the method used to estimate antenna size, the oxygen-evolving spinach photosystem II preparation contains 0.82–1.0 cytochrome *b*-559 per reaction center, while the oxygen-evolving cyanobacterial preparation contains 1.5–2.1 cytochrome *b*-559 per reaction center.

Photosystem II (PSII)[1] catalyzes the light-driven oxidation of water and reduction of plastoquinone in plants, green algae, and prokaryotic cyanobacteria. In both eukaroytic and prokaryotic organisms, PSII is composed of both integral membrane proteins and extrinsic polypeptides. The integral membrane proteins, D_1 and D_2, form the heterodimer core of the reaction center and bind the prosthetic groups that are involved in electron transfer. The 47- and 43-kDa membrane proteins function as light-harvesting proteins, while the 33-kDa extrinsic protein stabilizes the tetranuclear manganese cluster, the site of water oxidation. There are two redox-active tyrosines found in photosystem II: the dark-stable radical, $D^{\bullet}$, and $Z^{\bullet}$, the electron carrier between the site of water oxidation and the primary donor, P_{680} [reviewed in Debus (1992)].

However, there are differences between cyanobacterial and plant PSII [reviewed in Barry et al. (1994)]. For example, the 33-kDa extrinsic protein is not essential for *in vivo* oxygen evolution in cyanobacteria (Bockholt et al., 1991; Burnap & Sherman, 1991; Mayes et al., 1991; Philbrick et al., 1991; Burnap et al., 1992; Vass et al., 1992) but is required for activity *in vivo* in a eukaryotic organism (Mayfield et al., 1987). Also, the plant PSII contains two extrinsic proteins with molecular masses of 18 and 24 kDa. These proteins sequester the calcium and chloride that are required for activity and protect the manganese cluster from reduction. Proteins homologous to the 18- and 24-kDa polypeptides have not been found in cyanobacteria [reviewed in Barry et al. (1994)]. However, cyanobacterial PSII preparations from *Phormidium laminosum* and *Synechococcus vulcanus* have been found to contain two other extrinsic proteins with unknown function (Stewart et al., 1985; Rolfe & Bendall, 1989; Shen et al., 1992; Shen & Inoue, 1993). One is a 9-kDa (or 12-kDa) polypeptide, and the other is cytochrome *c*-550, a low-potential cytochrome. This low-potential (−260 mV) cytochrome has a 1:1 stoichiometric relationship to the PSII reaction center and has been shown (Shen et al., 1992; Shen & Inoue, 1993) to be an integral part of the reaction center. It was proposed that this cytochrome, which is ferredoxin-reducible, could be involved in the mediation of PSI cyclic electron transfer by shuttling electrons to the quinone pool.

Both spinach and cyanobacterial PSII complexes contain cytochrome *b*-559 [reviewed in Barry et al. (1994)]. This cytochrome is made up of two subunits, α and β, and has been suggested to be a heterodimer in which the heme cross-links the two subunits (Babcock et al., 1985; Widger et al., 1985). The function of cytochrome *b*-559 is not known [reviewed in Cramer et al. (1993)]. One suggestion is that cytochrome *b*-559 is involved in cyclic electron transfer around PSII and is able to accept electrons from the reduced plastoquinone pool (Whitmarsh & Cramer, 1978) or the bound quinone, Q_B (Buser et al., 1992a). The cytochrome then reduces the primary donor directly (Heber et al., 1979; de Paula et al., 1985; Arnon & Tang, 1988; Buser et al., 1990; Canaani & Havaux, 1990) or via another chlorophyll (Thompson & Brudvig, 1988).

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[1] Abbreviations: chl, chlorophyll; cyt, cytochrome; EPR, electron paramagnetic resonance; hbw, half-bandwidth; nE, nano-Einstein; PSI, photosystem I; PSII, photosystem II; Tris, tris(hydroxymethyl)aminomethane; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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Biochemical evidence and genetic evidence suggest that cytochrome *b*-559 plays an important structural role in photosystem II. Cytochrome *b*-559 is tightly associated with D_1 and D_2, since it is found in some of the most resolved reaction center preparations from both plants and cyanobacteria (Nanba & Satoh, 1987; Gounaris et al., 1989; Tang et al., 1990). Also, deletion of the gene encoding the β subunit of cytochrome *b*-559 (Pakrasi et al., 1990, 1991) or mutations at the putative heme ligands (Pakrasi et al., 1991) lead to a failure to assemble photosystem II complexes. Truncation of the α subunit leads to a reduction in the number of assembled photosystem II centers (Tae & Cramer, 1992).

The stoichiometry of cytochrome *b*-559 per PSII reaction center has been the subject of controversy. In photosystem II preparations from plants and cyanobacteria, either one (Ford & Evans, 1983; Ghanotakis et al., 1984; Yerkes & Crofts, 1984; Barber et al., 1987; Yamagishi & Fork, 1987; Gounaris et al., 1989, 1990; Miyazaki et al., 1989; Barbato et al., 1991; Buser et al., 1992b) or two (Vermeglio & Mathis, 1974; Cramer & Whitmarsh, 1977; Lam et al., 1983; Murata et al., 1984; Selak et al., 1984; Whitford et al., 1984; Whitmarsh & Ort, 1984; Yamamoto et al., 1984; Briantais et al., 1985; de Paula et al., 1985; Dekker et al., 1989; Haag et al., 1990; Boerner et al., 1992, 1993; Noren & Barry, 1992) heme equivalents of cytochrome *b*-559 have been found per reaction center. Some groups have found intermediate values (Nanba & Satoh, 1987; Rogner et al., 1990; van Leeuwen et al., 1991). Unfortunately, it is difficult to compare these results since different PSII preparations, methods of quantitating antenna size, and extinction coefficients for the cytochrome have been used.

In this study, we compare the cytochrome *b*-559 content per reaction center in a cyanobacterial (Noren et al., 1991) and a plant (MacDonald & Barry, 1992) photosystem II preparation. The two preparations are similar in that they utilize the same detergent to solubilize the membranes; the preparations are then subjected to anion-exchange chromatography. Both preparations are monodisperse, i.e., fully solubilized in detergent. Our results demonstrate that the cytochrome *b*-559 stoichiometry is different in the two preparations. The consequences of these results are discussed.

EXPERIMENTAL PROCEDURES

Sample Preparation. Spinach PSII complexes were purified as described in MacDonald and Barry (1992), through the use of the nonionic detergent, lauryl maltoside (Anatrace, OH), and anion-exchange chromatography. This procedure results in the loss of the light-harvesting complex and yields a preparation with an antenna size of approximately 100 chlorophylls per reaction center (MacDonald & Barry, 1992). The oxygen rates of the spinach preparations were approximately 1000 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}. In spite of the reduced antenna size, the spinach preparation retains the 24- and 18-kDa extrinsic proteins (MacDonald & Barry, 1992).

Synechocystis PSII particles were prepared as in Noren et al. (1991), through the use of the nonionic detergent, lauryl maltoside, and two rounds of anion-exchange chromatography at two different pH's. The purified PSII complexes contain approximately 60 chlorophylls per reaction center (Noren et al., 1991). The oxygen rates of the cyanobacterial preparations were approximately 2000–3000 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}.

Manganese Depletion. Manganese was removed either by Tris washing (Noren et al., 1991) or by hydroxylamine treatment (MacDonald & Barry, 1992).

Room Temperature EPR Spectroscopy. Room temperature EPR was performed as in Noren et al. (1991), Boerner et al. (1992), and MacDonald and Barry (1992). The amount of the stable radical, D$^{\bullet}$, was quantitated under nonsaturating conditions by double integration of the EPR signal using the program IGOR (Wavemetrics, Lake Oswego, OR). Fremy's salt was used as a spin standard (Babcock et al., 1983; Wertz & Bolton, 1986).

Manganese Quantitation. Manganese was quantitated as in Yocum et al. (1981). PSII particles were diluted 1:1 with 1 N HCl to release total manganese, and the manganese six-line EPR spectrum was recorded as in Noren et al. (1991). $MnCl_2$ standards in the same buffer gave a linear standard curve.

Chlorophyll Quantitation. In the spinach preparation, which contains both chlorophyll *a* and chlorophyll *b* and has a chlorophyll *a*/*b* ratio of approximately 3 (MacDonald & Barry, 1992), chlorophyll was quantitated in 80% acetone by the method described in Lichtenthaler (1987). In the cyanobacterial preparation, which contains only chlorophyll *a*, chlorophyll was quantitated in methanol by the method in Lichtenthaler (1987). For all the values in Table 1, the molecular mass of chlorophyll was taken to be that of chlorophyll *a*: 894 g/mol.

Optical Quantitation of Q_A^-. The chlorophyll/Q_A ratio in PSII particles was determined from the absorption increase at 320 nm caused by a series of 15 saturating flashes given at 50-ms intervals in the presence of hydroxylamine (Rogner et al., 1990; Buser et al., 1992a). An extinction coefficient of 12.5 mM^{-1} cm^{-1} was employed (van Gorkom, 1974; Schatz & van Gorkom, 1985). To ensure the complete oxidation of Q_A, samples were pretreated in darkness with potassium ferricyanide (10 μM). To prevent the oxidation of Q_A^- by Q_B, DCMU (40 μM) was subsequently added. To eliminate absorption changes that are associated with the S-state transitions (Dekker et al., 1984; Lavergne, 1991), hydroxylamine (2 mM) was added after the addition of DCMU (Boussac et al., 1990; Lavergne, 1991). The hydroxylamine reduces Z$^{\bullet}$, thereby preventing the oxidation of Q_A^- by charge recombination. The absorption measurements were performed with a renovated CARY-14 spectrophotometer (On-Line Instrument Systems, Inc., Bogart, GA) operated in the single-beam mode (chopper motor off) with the detector housing replaced by a Hamamatsu R374 photomultiplier tube and a separate amplifier (On-Line Instrument Systems, Inc.). The amplified output was recorded with a LeCroy 9310M digital oscilloscope. The data were stored and converted to units of absorption using a personal computer with software developed in the Physics Department at University of California, San Diego. The spectrophotometer was modified locally to permit the intensity and spatial position of the monitoring lamp (Osram HLX 64640) to be adjusted remotely. Actinic flashes (approximately 2-μs width at half-maximum) were provided by an EG&G FX-193 xenon flash lamp, passed through a 2-mm-thick Corning/Kopp CS-4-96 blue filter, and directed to the sample cuvette with a Schott Flexible light-guide. The photomultiplier tube was protected by a Corion Solar Blind filter and a 320-nm interference filter (Melles-Griot FIU-006). The cuvette containing the sample was held in a thermostated jacket.

Optical Quantitation of Cytochrome b-559. Cytochrome *b*-559 content was quantitated in manganese-depleted and untreated PSII complexes from both spinach and *Synechocystis* 6803 through the use of the chemical difference spectra according to the following protocols: (A) and (B).

(A) The dithionite-reduced minus ferricyanide-oxidized spectrum used for quantitation was recorded on a Perkin-Elmer double-beam, Lambda 5 spectrophotometer. The spectrophotometer's wavelength calibration was checked using the deuterium emission line at 656.1 nm before each set of experiments. Aliquots from a stock (200 mM potassium ferricyanide) of the oxidant were added to one cuvette, and aliquots from a stock (200 mM dithionite/0.4 M glycine, pH 10.0) of the reductant were added to the second cuvette until no further change in the optical difference spectrum was detected. The difference spectrum was then recorded directly. Spectrophotometer parameters were as follows: scan rate, 60 nm/min; response time, 0.2 s; slit, 2 nm. The final concentrations of oxidant and reductant were in the range of 2–4 mM ferricyanide and 4–8 mM dithionite. The extinction coefficient used for cytochrome *b*-559 quantitation was 21.5 mM^{-1} cm^{-1} (559–577 nm) (Cramer & Whitmarsh, 1977; Cramer et al., 1986). Measurements were made in a buffer containing 25% ultrapure glycerol (BRL), 0.05% lauryl maltoside, 50 mM MES–NaOH, pH 6.0, and 7.5 mM $CaCl_2$ or in 25% ultrapure glycerol, 0.05% lauryl maltoside, 50 mM MES–NaOH, pH 6.5, and 25 mM $CaCl_2$.

(B) Absolute absorption spectra were obtained with a Uvikon 810 scanning spectrophotometer (2-nm slit) using PC-based data collection software from Research Instruments International (San Diego, CA). Room temperature chemical difference spectra were measured using a spectrophotometer constructed in-house (unless otherwise stated in the figure caption) employing an Aminco dual-wavelength monochromator with a computer (Zenith AT)-controlled stepper motor (High-Sync AC synchronous/DC stepper motor; Bodine Electric Co.). The measuring beam (1-nm bandwidth, intensity 10–20 nE m^{-2} s^{-1}) after passing through a standard cuvette was detected using an EMI 9524 photomultiplier tube coupled to a Pacific Instrument photometer (Model 124) run at the 1-μA setting. Samples were mixed before scanning using a magnetic stirring bar that was switched off 30 s prior to the scan to reduce noise. Each sample was scanned 4 times, and the results were averaged. Difference spectra were obtained by subtracting spectra of the oxidized state from those of the reduced state. To compensate for sample drift and changes in the index of refraction, the resulting spectrum was corrected (deramped) by subtraction of a linear base line connecting the spectral troughs at ca. 540 and 575 nm. The *b*-559 extinction coefficient of 17.5 mM^{-1} cm^{-1} reported previously by Cramer et al. (1986) refers to the absorbance change at 559 nm relative to the zero defined by the isosbestic point at 537 nm in the reduced-minus-oxidized spectrum. In deramped spectra, the extinction coefficient at 559 nm is 20.0 mM^{-1} cm^{-1} relative to the zero values defined at the spectral troughs. Deconvolution was accomplished using the PeakFit program (Jandel Scientific, Corte Madera, CA) assuming unrestricted Gaussian components. All measurements were made in a buffer containing 50 mM MES–NaOH, pH 6.0, 7.5 mM $CaCl_2$, 0.05% lauryl maltoside, and 25% ultrapure glycerol (BRL). The chlorophyll content of each sample was determined as described above.

RESULTS

Three methods were used to quantitate antenna size in both plant and cyanobacterial preparations: tyrosine D• spin quantitation, manganese quantitation, and Q_A^- quantitation. Figure 1 presents room temperature D• EPR spectra that were recorded on manganese-depleted PSII particles from spinach (Figure 1B) and *Synechocystis* 6803 (Figure 1A). To ensure that Z• does not contribute to the spectrum and the full D• yield is obtained, these data were recorded in the dark, 4 min following illumination, in the presence of potassium ferricyanide. The spectra of D• in oxygen-evolving preparations are similar (data not shown). Small line-shape differences are observed when Figure 1A and Figure 1B are compared, suggesting that there may be changes in the magnitude of hyperfine couplings to tyrosine protons in spinach and *Synechocystis* D•. This has been suggested previously (Hoganson & Babcock, 1992). Table 1 summarizes the result of spin quantitation under nonsaturating conditions through the use of a Fremy's salt spin standard. In contrast to an earlier report using low-temperature EPR spectroscopy (Buser & Brudvig, 1992), no difference in the yield of D• was observed at pH 6.5 and pH 8.0 in these room temperature EPR studies (data not shown). Since there is 1 D• spin per reaction center, our analysis gives an antenna size of 89 chlorophylls for the spinach preparation, 60 chlorophylls for the oxygen-evolving *Synechocystis* PSII sample, and 54 chlorophylls for the manganese-depleted (Tris washed) *Synechocystis* sample (Table 1). Quantitation of D• in 4-min dark scans taken before and after the presented scan (Figure 1) gives similar values (within 15%), indicating that, as expected, D• has long decay kinetics.

FIGURE 1: Room temperature D• EPR spectra in (A) *Synechocystis* PSII particles (0.72 mg of chl/mL) and (B) spinach PSII particles (2.6 mg of chl/mL). Potassium ferricyanide (1–3 mM) was used as an electron acceptor. Spectral conditions were as follows: microwave frequency, 9.49 GHz; microwave power, 3.5 mW; field modulation, 3.2 G; time constant, 2 s; scan time, 4 min; gain (A), 1.25×10^4; gain (B), 2×10^4. For plotting, spectra were normalized to an effective gain of 2×10^4. The *g*-value of the D• tyrosine radical is known to be 2.0046 (Miller & Brudvig, 1991).

Manganese quantitation was performed through the use of an EPR assay previously employed (Yocum et al., 1981; Noren et al., 1991; Noren & Barry, 1992). It is widely accepted that there are four manganese per reaction center [reviewed in Debus (1992)]. From evaluation of the specific activities for oxygen evolution throughout the purification (Noren et al., 1991; MacDonald & Barry, 1992), we know that the majority of centers in both preparations are active and thus contain four manganese. Table 1 summarizes the results of manganese analysis of the spinach and *Synechocystis* preparations and shows that there are 64 chlorophylls per 4 manganese or per reaction center in the oxygen-evolving cyanobacterial PSII samples. The spinach samples give 110 chlorophylls per 4

Table 1: Characterization of PSII Complexes[a]

	chlorophyll/*b*-559[b]	chlorophyll/*b*-559[c]	chlorophyll/Q_A^-	chlorophyll/4Mn	chlorophyll/D•
O_2-evolving *Synechocystis*	30 (32–29)	32 (33–32)	46 (50–42)	64 (66–60)	60 (71–49)
Mn-depleted *Synechocystis*	34 (36–32)	31 (32–31)	49 (56–41)		54 (59–49)
O_2-evolving spinach	106 (111–101)	115 (120–110)	95[d] (98–91)	110 (134–91)	89 (93–84)

[a] Unless otherwise noted, the ranges represent the results of multiple determinations performed on multiple preparations. Each chl/D• 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. [b] Determined by protocol A (Experimental Procedures) on multiple preparations. Each chl/cyt *b*-559 value is the average of three to four determinations. [c] Determined by protocol B (Experimental Procedures). Two determinations on a single preparation. [d] Five determinations on a single preparation.

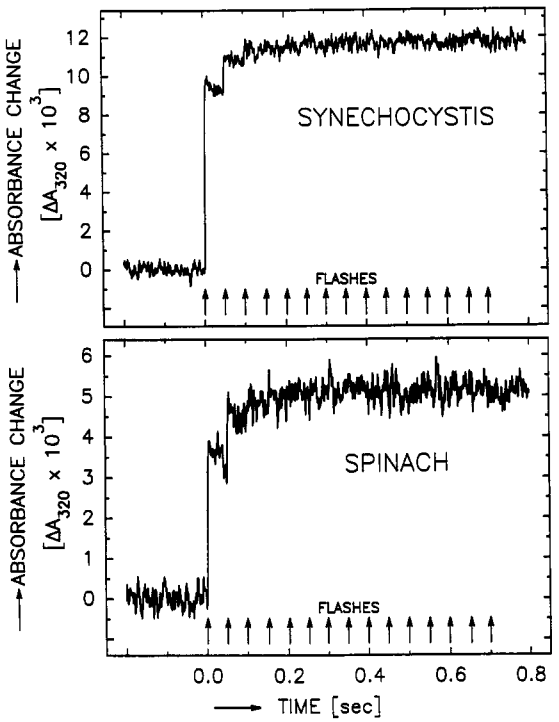

FIGURE 2: Formation of Q_A^- in PSII particles isolated from *Synechocystis* (top) and spinach (bottom) in response to a series of saturating flashes given in the presence of hydroxylamine and DCMU. Conditions: 18 μg of chl in 0.5 mL of 50 mM MES–NaOH, 7.5 mM $CaCl_2$, 0.05% lauryl maltoside, and 25% (v/v) glycerol, pH 6.0, 21.5 °C. Samples were incubated in darkness for 10 min in the presence of 10 μM potassium ferricyanide before DCMU was added to a concentration of 40 μM (the final concentration of ethanol was 1%) followed 2 min later by hydroxylamine to a concentration of 2 mM. The saturating flashes (arrows) were applied 30 s after the addition of hydroxylamine. Essentially the same data were obtained when the incubation time with hydroxylamine was shortened to 5 s or lengthened to 5 min (not shown).

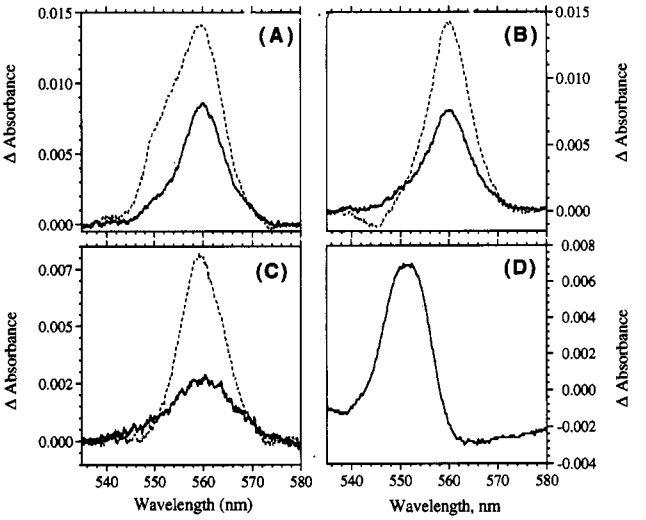

FIGURE 3: Chemical difference spectra obtained by protocol B (Experimental Procedures) of (A) oxygen-evolving *Synechocystis* PSII particles (20 μg/mL), (B) manganese-depleted (Tris washed) *Synechocystis* PSII particles (20 μg/mL), and (C) oxygen-evolving spinach PSII particles (40 μg/mL). Dithionite (~1.5 mM)-reduced-minus-ferricyanide (0.1 mM)-oxidized spectra (dashed). Menadiol (1 mM)-reduced-minus-ferricyanide (0.1 mM)-oxidized spectra (solid). (D) shows the dithionite (~1.5 mM)-reduced-minus-ferricyanide (0.1 mM)-oxidized difference spectrum of the supernatant obtained from Tris washing of oxygen-evolving *Synechocystis* PSII particles. Spectrum D was obtained using the Kontron UV–visible spectrophotometer (see Experimental Procedures).

manganese. These results are in good agreement with the D• spin quantitations described above.

Figure 2 presents the data used to quantitate Q_A^- in PSII complexes. Q_A^- was accumulated by 15 actinic flashes in the presence of hydroxylamine. The change in amplitude at 320 nm was then used to calculate the concentration of reduced quinone. The extinction coefficient derived for spinach was used for both *Synechocystis* (Figure 2, top panel) and spinach (Figure 2, bottom panel). Given that there is 1 Q_A^- per reaction center, our data give an antenna size of 95 chlorophylls for the spinach preparation, 46 chlorophylls for the oxygen-evolving *Synechocystis* preparation, and 49 chlorophylls for the manganese-depleted (Tris washed) *Synechocystis* preparation (Table 1).

For spinach, the agreement between Q_A^- and other antenna size determinations is good (Table 1). On the other hand, Q_A^- quantitation in *Synechocystis* 6803 PSII particles predicts a slightly smaller average antenna size than is found through D• and manganese quantitation, although the ranges overlap (Table 1). One possible explanation for this discrepancy might be a slightly different extinction coefficient for Q_A^- in *Synechocystis*, as compared to spinach preparations. However, this extinction coefficient has been used previously in characterization of cyanobacterial preparations (Schatz & van Gorkom, 1985; Rogner et al., 1990). A second possible explanation is a small contribution from an additional component, which gives rise to a stable light-induced absorption increase at 320 nm in the *Synechocystis* preparation. Since both preparations are monodisperse, no flattening correction is required.

Reduced-minus-oxidized difference spectra are presented in Figure 3 for oxygen-evolving *Synechocystis* and spinach PSII preparations (Figure 3A and Figure 3C, respectively) and for manganese-depleted (Tris washed) *Synechocystis* PSII preparations (Figure 3B). These data were obtained using spectrophotometric protocol B (Experimental Procedures). Both menadiol-reduced (solid curve) and dithionite-reduced (dashed curve) spectra are shown. The spectral peaks are at 559.5–560.0 nm in all cases, except possibly the dithionite-

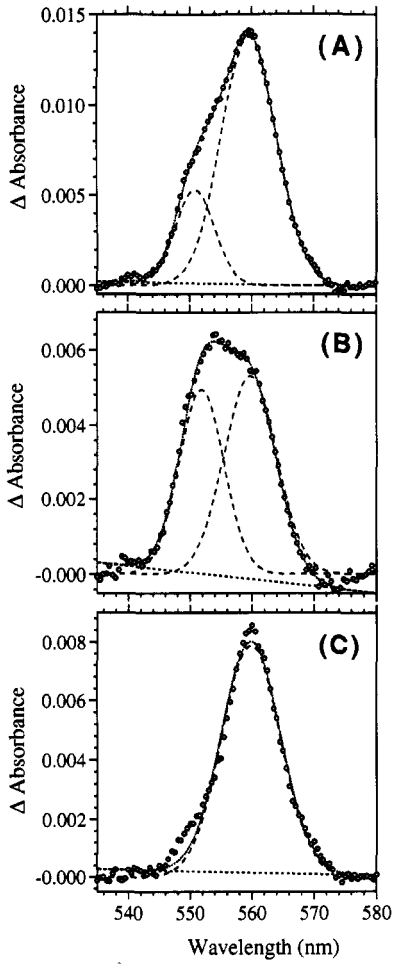

FIGURE 4: Deconvolution of the chemical difference spectra of the oxygen-evolving *Synechocystis* PSII particles. (A) Dithionite-minus-ferricyanide spectrum (repeated from Figure 3A) fit by two components with maxima at 550.8 and 559.5 nm, and hbw = 7.5 and 10.5 nm, respectively. (B) Dithionite-minus-menadiol spectrum fit by two components with 551.8- and 559.8-nm maxima and hbw = 8.6 and 10.0 nm, respectively. (C) Single component with a 559.8-nm maximum and 11.2-nm hbw of the menadiol-minus-ferricyanide spectrum (repeated from Figure 3A).

reduced spinach spectrum (Figure 3C, dashed), where the peak is shifted to a slightly shorter wavelength (approximately 559.0 nm). The half-bandwidths of all spectra are approximately 10.5 nm, except for that of the dithionite-reduced *Synechocystis* oxygen-evolving sample (Figure 3A, dashed), which shows an obvious component on the short-wavelength side and a half-bandwith of 13.7 nm. Also, the half-bandwidth of the spectrum that is obtained by menadiol-reduction of the spinach preparation (Figure 3C, solid) is 13.5–14.0 nm. However, this spectrum is of lower amplitude and is therefore more noisy than the other spectra.

To determine the origin of the more complex spectrum and larger bandwidth of the spectrum of the dithionite-reduced, oxygen-evolving *Synechocystis* preparation (Figure 3A, dashed), these data were deconvoluted as described under Experimental Procedures. Figure 4A shows that the dithionite-minus-ferricyanide spectrum can be resolved into two components with absorption maxima at 559.5 and 550.8 nm, with half-bandwidths of 10.5 and 7.5 nm, respectively, and with a 2.6 ratio in amplitude. Visual inspection of the dithionite-minus-menadiol (Figure 4B) and menadiol-minus-ferricyanide (Figure 4C) spectra shows that the ~551-nm spectral component is only present after dithionite reduction, implying that this component is low-potential. Also, the dithionite-minus-ferricyanide spectrum is approximately the sum of the other two difference spectra (compare Figure 4A with Figure 4B,C). Previously, we had attributed the broad bandwidth of cytochrome *b*-559 in oxygen-evolving *Synechocystis* preparations to cytochrome b_6f contamination (Boerner et al., 1992, 1993; Noren & Barry, 1992). However, our present results show this to be unlikely, since cyanobacterial cytochrome *f* would have a maximal absorbance at 556 nm in this difference spectrum (Ho & Krogmann, 1980) and cytochrome b_6 would have a maximal absorbance at 563–564 nm (Cramer & Whitmarsh, 1977). The spectral deconvolution (Figure 4) suggests that a low-potential component with an absorbance maximum near 550 nm is present. Since a low-potential cytochrome "*c*-550" is an extrinsic protein that has been identified in other cyanobacterial preparations (Shen et al., 1992; Shen & Inoue, 1993), the species absorbing near 551 nm in our *Synechocystis* preparation could be cytochrome "*c*-550".

Cytochrome *c*-550 is known to be released by Tris washing (Shen et al., 1992; Shen & Inoue, 1993). After Tris washing of the oxygen-evolving *Synechocystis* preparation, the "*c*-550" component of the dithionite-minus-ferricyanide difference spectrum is lost (Figure 3B, dashed). The narrow half-bandwidth and absorbance maximum of this difference spectrum are now consistent with a single component that corresponds to cytochrome *b*-559. The 551-nm component that is lost after Tris washing of the *Synechocystis* oxygen-evolving particles is recovered in the supernatant. The optical difference spectrum of the supernatant shows a broad α-band maximum at 551–552 nm (Figure 3D). It should be noted that the dithionite-minus-ferricyanide difference spectrum of a "*c*-550", partially purified from the supernatant of membranes from *Synechocystis* (D. W. Krogmann, personal communication), also showed a peak at approximately 551 nm, the uncertainty arising from a steep base line due to the presence of phycobiliproteins in the partially purified preparation (data not shown). Also, the "*c*-550" component in our oxygen-evolving particles (Figure 4) was not reducible by menadiol (Figure 4), and the "*c*-550" component in the supernatant was not reducible by 1 mM ascorbate (data not shown). These results are consistent with previous data on cytochrome *c*-550 from other cyanobacteria (Koike & Katoh, 1979) and imply that oxygen-evolving *Synechocystis* PSII preparations contain a low-potential cytochrome similar to the cytochrome *c*-550 described in the literature.

The midpoint potential of cytochrome *b*-559 can vary, with a range of +375 to +50 mV depending on the sample conditions (Cramer & Whitmarsh, 1977). The photosystem II samples employed in our studies contain approximately half "low-potential" and half "very low potential" cytochrome *b*-559, which are reducible in the *Synechocystis* preparation by menadiol and dithionite, respectively (Figure 3A,B). The ratio of menadiol-to-dithionite-reducible cytochrome also depends on sample conditions, as does the high-potential component that is reducible by hydroquinone (data not shown).

The use of dithionite as a low-potential reductant was questioned in a recent study on the stoichiometry of cytochrome *b*-559. These authors found that this reductant caused baseline drift and used borohydride as an alternative reductant (Buser et al., 1992b). In our hands, the two reductants are approximately equivalent (within 5%) as reductants of cytochrome *b*-559. However, if cytochrome b_6 is present in a preparation (e.g., thylakoids), borohydride is much less effective than dithionite in reducing it (unpublished data). This difference in reductant properties is not a factor in the

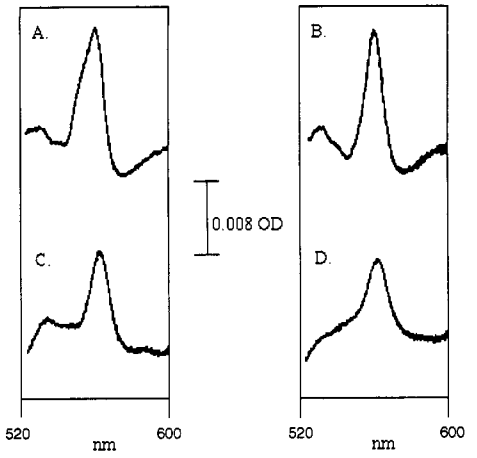

FIGURE 5: Chemical difference spectra, dithionite-reduced minus ferricyanide-oxidized, obtained by protocol A (Experimental Procedures) of (A) oxygen-evolving *Synechocystis* PSII particles (20 μg of chl/mL), (B) manganese-depleted *Synechocystis* PSII particles (21 μg of chl/mL), (C) oxygen-evolving spinach PSII particles (44 μg of chl/mL), and (D) manganese-depleted spinach PSII particles (39 μg of chl/mL).

Table 2: Stoichiometry of Cytochrome *b*-559 in Photosystem II[a]

	cyt *b*-559/ Q_A^-	cyt *b*-559/ 4Mn	cyt *b*-559/ D•
O_2-evolving *Synchocystis*	1.5	2.1	1.9
Mn-depleted *Synechocystis*	1.5		1.6
O_2-evolving spinach	0.87	1.0	0.82

[a] chl/cyt *b*-559 values that were determined by methods A and B (Table 1) were averaged to give these stoichiometries.

present experiments because cytochrome b_6 is not present to a measurable extent.

The dithionite-minus-ferricyanide spectra obtained by protocol B in Figure 3 can be used to quantitate the amount of cytochrome *b*-559 on a chlorophyll basis. For the spinach preparation, the stoichiometry is 1 cytochrome *b*-559 per 115 chlorophylls (Table 1). For *Synechocystis*, 32 chlorophylls and 31 chlorophylls per cytochrome *b*-559 were obtained for the oxygen-evolving and manganese-depleted preparations, respectively (Table 1). The estimation of the cytochrome *b*-559 content before and after deconvolution of the oxygen-evolving *Synechocystis* spectrum (Figures 3A and 4A) gives similar values (<5% difference). In other words, the "*c*-550" component has a small absorbance at 559 nm, arguing that accurate estimates of cytochrome *b*-559 content can be obtained using such data. Cytochrome *b*-559 content was also measured in these identical preparations by protocol A (Experimental Procedures). Representative difference spectra are shown in Figure 5. The agreement between the two measurements was within 5%.

Table 1 summarizes the cytochrome *b*-559 measurements on multiple preparations. On average, spinach PSII preparations were found to contain 106 chlorophylls per cytochrome *b*-559. For *Synechocystis*, 30 chlorophylls per cytochrome *b*-559 and 34 chlorophylls per cytochrome *b*-559 were found for oxygen-evolving and manganese-depleted preparations, respectively (Table 1).

On the basis of data summarized in Table 1, spinach PSII particles contain 0.82–1.0 cytochrome *b*-559 per reaction center (Table 2). Oxygen-evolving cyanobacterial PSII particles contain 1.9–2.1 cytochrome *b*-559 per reaction center when manganese and D• are used to determine antenna size and 1.5 cytochrome *b*-559 per rection center when Q_A^- is used (Table 2). For the manganese-depleted *Synechocystis* 6803 PSII particles, our results give 1.5–1.6 cytochrome *b*-559 per PSII reaction center (Table 2).

DISCUSSION

The spinach and cyanobacterial preparations used in these studies have the advantage that they contain little contaminating cytochrome b_6f and are monodisperse. In the spinach PSII complex preparation, all methods of antenna size measurement employed are in good agreement that there is one (0.82–1.0) cytochrome *b*-559 per reaction center. In the *Synechocystis* preparation, another cytochrome with an α band with maximum absorbance near 551–552 nm is present. The presence of this component does not interfere with cytochrome *b*-559 quantitation. Either in the presence or in the absence of this cytochrome, our data show that there is more than one cytochrome *b*-559 per *Synechocystis* reaction center.

For these conclusions to be in error, our antenna size measurements, which appear to be self-consistent and correct in spinach, must all seriously overestimate antenna size in *Synechocystis*, by as much as 50%. We feel that this is unlikely. For example, we are confident that our manganese quantitations are not in error by 50%, since the specific activity of oxygen evolution (on a chlorophyll basis) has been monitored during the *Synechocystis* purification protocol and has been shown to increase proportionally to loss of photosystem I (Noren et al., 1991). Also, in typical oxygen-evolving preparations under steady-state illumination, less than 20% of the centers generate Z• (Noren et al., 1991; Boerner et al., 1992). Similarly, our D• EPR measurements are correct, since no increase in the D• yield at alkaline pH has been observed and since the D• spectra at room temperature were obtained in the presence of acceptors after illumination, in order to obtain the full yield of the radical.

Our work on *Synechocystis* is in agreement with earlier studies. For example, previous characterization of a non-oxygen-evolving *Synechocystis* core preparation through the use of cytochrome *b*-559 and Q_A^- quantitation gave 1.4 cytochrome *b*-559 per Q_A^- (Rogner et al., 1990). Also, Gounaris et al. (1989) found one cytochrome *b*-559 per pheophytin in a highly resolved reaction center preparation from *Synechocystis* 6803. If we assume there are two pheophytin molecules per PSII, in analogy with the structure of the reaction center from purple bacteria (Deisenhofer & Michel, 1989), these results indicate that there are two cytochrome *b*-559 per PSII reaction center. Interestingly, in a plant reaction center preparation, Barber et al. (1987), Miyazaki et al. (1989), and Gounaris et al. (1990) assume that there are two pheophytin molecules present and find approximately one cytochrome *b*-559 per reaction center.

There are three possible explanations for our results. First, spinach and *Synechocystis* differ in the number of cytochrome *b*-559 hemes per reaction center. Second, both spinach and cyanobacterial preparations contain two cytochrome *b*-559 hemes per reaction center, but one heme is inaccessible to reductants in spinach. Third, both spinach and cyanobacterial preparations contain two cytochrome *b*-559 per reaction center, but a cytochrome or a heme is lost more easily from the spinach preparation than from *Synechocystis* particles.

While we cannot completely exclude the first explanation, we do not favor it, since many studies have found more than one cytochrome *b*-559 per reaction center in spinach preparations. Interestingly, even in studies interpreted as giving one cytochrome *b*-559 heme per reaction center, values significantly greater than one are often obtained [for example, see

Gounaris et al. (1990) and Buser et al. (1992b)]. Furthermore, in the simplest experiment that can be performed on cytochrome stoichiometry, the amount of high-potential cytochrome *b*-559, which is associated with PSII and can be oxidized by ferricyanide, can be readily seen to far exceed the amount of cytochrome *f* in thylakoid membranes (Cramer et al., 1971).

We consider the second possibility unlikely, since we have also determined cytochrome stoichiometry in the presence of additional detergent and chaotropic agents and have seen no increase in cytochrome *b*-559 heme content in the spinach preparation (data not shown).

We consider the third possibility most likely, partly because of the reasons stated just above. In addition, van Leeuwen et al. (1991) found that there was a substantial loss of cytochrome *b*-559 upon purification of spinach PSII reaction centers from spinach core particles. These results suggest that one heme or cytochrome can be lost during purification; such an explanation could account for much of the variation observed in the literature. It should be noted that the majority of centers in the spinach PSII preparation are active in oxygen evolution when there is one cytochrome *b*-559 heme per reaction center. It will be important to determine whether the cytochrome apoprotein is present in this preparation.

We conclude that one cytochrome *b*-559 per photosystem II reaction center is extremely sensitive to its environment. This has been suggested previously in a discussion of the midpoint potential of the cytochrome (Cramer & Whitmarsh, 1977). In other words, the cytochrome content of photosystem II will depend heavily on the biochemical preparation.

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