Reevaluation of the Stoichiometry of Cytochrome b_{559} in Photosystem II and Thylakoid Membranes†

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ABSTRACT: The stoichiometry of cytochrome b_{559} (one *or* two copies) per reaction center of photosystem II (PSII) has been the subject of considerable debate. The molar ratio of cytochrome b_{559} has a number of significant implications on our understanding of the functional role of cytochrome b_{559}, the mechanism of electron donation in PSII, and the stoichiometry of the other redox-active, reaction center components. We have reinvestigated the stoichiometry of cytochrome b_{559} in PSII-enriched and thylakoid membranes, using differential absorbance and electron paramagnetic resonance spectroscopies. The data from both quantitation procedures strongly indicate only one copy of cytochrome b_{559} per reaction center in PSII-enriched membranes and also suggest one copy of cytochrome b_{559} per reaction center in thylakoid membranes.

The isolation and purification of membrane proteins with retention of their in vivo structures and properties is a challenging area of biochemistry. Notwithstanding, recent biochemical, biophysical, and molecular biological advances have allowed the step-by-step isolation of the PSII[1] reaction center core containing the D1, D2, and cyt b_{559} polypeptides from the multiprotein PSII complex of plant chloroplasts and a variety of cyanobacteria. Despite these developments, a remaining difficulty is the determination of the exact stoichiometry of the redox-active components. This problem is exemplified by the controversy surrounding the stoichiometry of cyt b_{559} (one *or* two copies) per reaction center in chloroplast, thylakoid membrane, PSII-enriched membrane, and/or D1/D2/cyt b_{559} preparation(s).

Long known to be an intrinsic component of the PSII complex, cyt b_{559} certainly plays an essential structural role and most likely also plays an important redox-active role in the photochemistry of PSII. The α- and β-polypeptides (one copy of each) of cyt b_{559} form part of the minimal core complex still capable of the primary charge separation, $P680^+$ $Pheo^-$ (Nanba & Satoh, 1987; Barber et al., 1987b; Telfer & Barber, 1989), and the β-subunit is required for the assembly of the PSII reaction center (Pakrasi et al., 1990). The close association of cyt b_{559} with the PSII reaction center is further emphasized by its participation in the redox chemistry of PSII (Cramer & Witmarsh, 1977; Buser et al., 1990, 1992).

Not found in the bacterial reaction center, cyt b_{559} is likely to have a function related to the unique ability of PSII to oxidize water, such as a role in the protection of PSII from the highly oxidizing environment necessary for the oxidation of water to oxygen (Thompson & Brudvig, 1988). Clearly, knowledge of the stoichiometry of cyt b_{559} per reaction center is essential in order to determine the mechanism of electron donation by cyt b_{559}, to understand its unusual redox properties, and, most importantly, to evaluate the various proposals for its structural and redox functions in PSII. However, the literature remains divided as to one or two copies of cyt b_{559} per reaction center.

The first quantitations were performed in chloroplast or thylakoid membrane preparations, which contain three redox-active cytochromes with significant spectral overlap in the red-visible region of the spectrum [reviewed by Cramer and Whitmarsh (1977)]—cyt f (α-band at 554 nm), cyt b_{559} (α-band at 560 nm), and cyt b_6 (α-band at 563 nm). In these measurements, the experimental conditions were manipulated to stably oxidize or reduce at least one cytochrome, and/or the empirical, three-wavelength equation from Heber et al. (1976) was often used to deconvolute the contribution of each cytochrome from the overall absorbance change. Most of these studies found two copies of cyt b_{559} per chloroplast or thylakoid PSII reaction center (Erixon & Butler, 1971; Vermeglio & Mathis, 1974; Whitmarsh & Cramer, 1978; Epel et al., 1972; Selak et al., 1984). The development of PSII preparations devoid of cyt f and cyt b_6 removed the spectral overlap problem but introduced conflicting reports of the stoichiometry of cyt b_{559} between different PSII preparations with some reports of one (Ford & Evans, 1983; Ghanotakis et al., 1984; Yerkes & Crofts, 1984; Yamagishi & Fork, 1987) and others of two (Murata et al., 1984; Briantais et al., 1985; Lam et al., 1983; de Paula et al., 1985). More recently, further resolution of PSII has yielded a reaction center containing the D1/D2/cyt b_{559} polypeptides. However, even at this level, some disparity as to the ratio of Chl to cyt b_{559} exists with reports of 4–5 or 8 Chl per 1 cyt b_{559} per PSII reaction center (Nanba & Satoh, 1987; Barber et al., 1987a; Gounaris et al., 1989) and of 10–12 Chl per 2 cyt b_{559} per PSII reaction center (Dekker et al., 1989).

A review of the cyt b_{559} literature suggests that one of two copies of cyt b_{559} may be lost at some purification step between the chloroplast and/or thylakoid preparation and the isolation of the D1/D2/cyt b_{559} complex. However, a more detailed study of the data and the referenced literature reveals a number of inconsistencies and ambiguities. In addition to spectral overlap, other potential problems in the earlier measurements of the stoichiometry of cyt b_{559} include the use of a range of extinction coefficients for the α-band of cyt b_{559} [from $\Delta\epsilon$

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[1] Abbreviations: BBY-type PSII, PSII prepared by the method of Berthold, Babcock, and Yocum (1981); Chl, chlorophyll; Chl_z, monomer chlorophyll involved in the photooxidation of cytochrome b_{559}; cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; kDa, kilodaltons; MES, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; RC, reaction center; tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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(559–570 nm) = 15 mM^{-1} cm^{-1} (reviewed by Cramer & Whitmarsh, 1977) to $\Delta\epsilon$ (556–539 nm) = 23.4 mM^{-1} cm^{-1} (Miyazaki et al., 1989)] and significant variances in the number of Chl per reaction center. Although the optical quantitation of cyt b_{559} requires accurate knowledge of the number of Chl per PSII reaction center, this ratio is often either assumed or determined from a measurement of C550 assuming a given dark redox state of the sample or calculated from the UV absorbance change of the Q_A^-/Q_A difference spectrum without correction for particle flattening. Also known as the sieve effect, particle flattening results in the diminution of absorbance changes of molecules in suspension due to the mutual screening of the pigments (e.g., Chl) within a given particle.

Our laboratory also investigated the stoichiometry of cyt b_{559} in PSII-enriched membranes by EPR spectroscopy in an earlier study (de Paula et al., 1985). In that work, the concentrations of cyt b_{559} and Y_D (tyrosine 160 of the D2 polypeptide) were determined independently using two different, external spin standards. Unfortunately, the significantly differing EPR conditions did not allow a direct comparison between the concentration of cyt b_{559} and Y_D, and we have since found that our earlier estimate of two cyt b_{559} per PSII reaction center was inaccurate. Also using EPR spectroscopy, Franzén et al. (1986) found a ratio of 1.2 cyt b_{559} per Y_D in an O_2-evolving PSII-enriched preparation lacking the 17- and 23-kDa extrinsic polypeptides and the light-harvesting chlorophyll protein complex.

In an effort to resolve the question of whether there is one or two cyt b_{559} per reaction center and to further characterize cyt b_{559}, we have reevaluated the stoichiometry of cyt b_{559} in PSII-enriched membranes and, to a limited extent, in thylakoid membranes by independent measurements using differential absorbance and electron paramagnetic resonance spectroscopies.

MATERIALS AND METHODS

Sample Preparation. All manipulations were performed in dim green light, on ice. Thylakoid and PSII-enriched membranes were isolated from market spinach leaves by a modified version (Beck et al., 1985) of the isolation procedure described by Berthold et al. (1981). Prior to use, thylakoid membranes were stored at a Chl concentration of 3–5 mg of Chl/mL at 77 K in a buffer containing 15 mM NaCl, 20 mM MES, 5 mM $MgCl_2$, 1 mM EDTA, 1 mg/mL BSA, and 30% (v/v) ethylene glycol at pH 6.0. Similarly, PSII-enriched membranes were stored at a Chl concentration of 4–6 mg of Chl/mL at 77 K in a buffer containing 15 mM NaCl, 20 mM MES, and 30% (v/v) ethylene glycol (denoted suspension buffer A) at pH 6.0. The Chl concentration was determined by the method of Arnon (1949) and/or Vernon (1960). It should be noted that the Chl concentrations as calculated by the method of Arnon (1949) were consistently about 10% greater than the concentrations determined by the method of Vernon (1960) in seven different Chl determinations. In our optical measurements, we have used the values from the two methods as upper and lower limits of the true Chl concentration of a given sample, whereas, in our EPR experiments, we have used only the formulation of Arnon (1949) for the determination of the Chl concentration of our EPR samples. The O_2-evolution activity was monitored by the assay described by Beck et al. (1985); rates of 300–450 and 150–200 μmol of O_2/(mg Chl·h) were measured for the individual PSII-enriched and thylakoid membrane preparations, respectively.

Optical Measurements. All absorbance data were corrected for particle flattening. The data used to determine the wavelength-dependent flattening correction factor were also corrected for light scattering. Light scattering and particle flattening measurements were performed on a Cary 1 UV–vis spectrophotometer (Varian) under the following conditions: scan rate, 150 nm/min; data interval, 0.5 nm; slit width, 2.0 nm; and scan width, 250–800 nm. In order to determine the flattening correction factor as a function of wavelength, the absorbance spectrum of the particles was measured both in suspension and in ethanol solution.

For particles in suspension, where the particle size is comparable to the wavelength of the measurement, the absorbance (E) was corrected for scattering losses according to the method of Latimer and Eubanks (1962):

$$E' = E(\gamma_1) - A[E(\gamma_2) - E(\gamma_1)] \quad (1)$$

where

$$A = \{E(\gamma_1)/[E(\gamma_2) - E(\gamma_1)]\}_{\lambda=\lambda_0} \quad (2)$$

E' is the absorbance after correction for scattering, γ_1 and γ_2 are the two angles of detection (achieved with and without diffusing plates, respectively), and A is determined at a wavelength λ_0 where no absorbance occurs. The absorbance spectrum of the same particles in solution (E_{sol}) was corrected for light scattering by the theory of Rayleigh [reviewed by Moore (1972)], which assumes isotropic particles with dimensions that are small compared to the wavelength of the light. Assuming no angular dependence to the scattering from individual Chl molecules in solution, the scattering coefficient was calculated from the value of E_{sol} at a wavelength at which no absorbance occurs (λ_0):

$$A_{sc} = [\lambda_0^4 E(\lambda_0)^4]/\lambda^4 \quad (3)$$

$$E_{sol}(\text{corrected}) = E_{sol}(\text{observed}) - A_{sc} \quad (4)$$

where A_{sc} is the scattering coefficient and λ_0 was measured at 800 nm.

Having corrected the spectra for light scattering, the flattening factor and the differential flattening factor were determined as a function of wavelength by the theory of Duysens (1956). Assuming Beer's law to be valid, Duysens (1956) derived an expression for the optical density of spherical particles in suspension in terms of the transmittance of a single particle, $T'_{p,av}$. Rewritten in terms of absorbance

$$E'(\lambda) = (p/2.303)[1 - T'_{p,av}(\lambda)] \quad (5)$$

where p is the projected area of all particles divided by the total illuminated area. Equation 5 allows the calculation of the average transmittance of a single particle as a function of wavelength from the absorbance spectrum of the suspension and the constant p. In turn, the average particle transmittance, $T'_{p,av}(\lambda)$, then allows the calculation of the flattening correction at all wavelengths (eqs 18–21, Duysens, 1956); the relationship between $T'_{p,av}$ and the flattening factor, E_{sol}/E', and the differential flattening factor for small absorbance changes, $\Delta E_{sol}/\Delta E'$ (the ratio of the first derivatives of E_{sol} and E'), is shown graphically in Figure 1. Thus, provided the constant p in eq 5 is known, the flattening correction can be determined from the absorbance spectrum of the suspension with the aid of eq 5 and Figure 1. The constant p either can be determined microscopically by counting particles and measuring their dimensions or can be approximated spectrophotometrically by measuring $E'(\lambda)$ and $E_{sol}(\lambda)$. Using the latter method, Pulles (1978) showed that the constant p could be estimated from the ratio of E_{sol}/E' at the maximum of the Chl absorbance

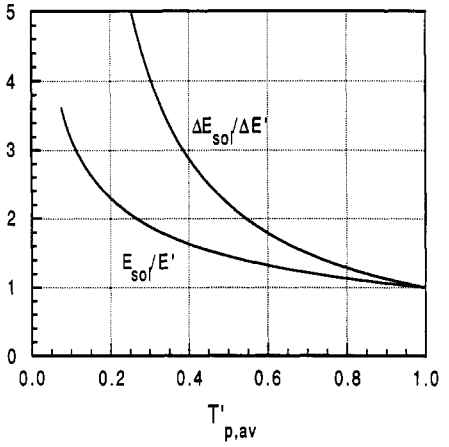

FIGURE 1: Flattening factor (E_{sol}/E') and differential flattening factor ($\Delta E_{sol}/\Delta E'$) as a function of the average transmittance of one spherical particle ($T'_{p,av}$), corrected for scattering, as calculated from the equations in Duysens (1956). See Materials and Methods for further explanation and references.

at 435 nm: the average particle transmittance, $T'_{p,av}$ (435 nm) is found from the observed ratio of E_{sol}/E'(435 nm) with the aid of Figure 1, and subsequently, the value p is obtained by eq 5 from the calculated $T'_{p,av}$ (435 nm) and the observed absorbance E' (435 nm).

Applying the above outlined methods, Pulles (1978) determined the flattening factor and differential flattening factor for spinach chloroplasts using the method of Latimer and Eubanks (1962) to correct for light scattering of particles in suspension and the theory of Duysens (1956) to correct for particle flattening [see Figures 2.3 and 2.4 in Pulles (1978)]. Assuming the extent of particle flattening to be about the same in spinach chloroplasts and thylakoid membranes, we have used these data to correct our optical measurements of thylakoid membranes. However, since a detailed study of light scattering and particle flattening in PSII-enriched membranes has not been reported, we have repeated these measurements for BBY-type PSII preparations. Shown in Figure 2a is a comparison of the absorbance spectra of BBY-type PSII-enriched membranes in suspension and in solution after correction for light scattering according to the methods of Latimer and Eubanks (1962) and Rayleigh [see Moore (1972)], respectively—the corrections are shown explicitly in Figure 2b. Repeating the measurement in Figure 2a, we find E_{sol}/E'(435 nm) = 1.16 (standard deviation = 0.05 from an average of 15 measurements), which results in p = 8.02 for BBY-type PSII-enriched membranes. With this value of p, the average transmittance of a single particle, $T'_{p,av}(\lambda)$ was calculated by eq 5 and is shown on the right-hand scale of Figure 2a. From Figures 1 and 2a, we have obtained the differential flattening factor ($\Delta E_{sol}/\Delta E'$) as a function of wavelength for BBY-type PSII-enriched membranes, shown in Figure 2c.

Differential absorbance measurements were performed at 25 °C on a flash-detection spectrophotometer, similar to that originally described by Joliot et al. (1980). Actinic flashes were provided by a xenon flash lamp (EG&G FX199, 2-μs width at half-height) filtered by a red high band-pass filter (Schott RG 5, >600 nm). Flash- and chemically-induced absorbance changes were monitored by a series of xenon flashes (EG&G, FX199U) passed through a monochromator (HL, Jobin-Yvon). Prior to the optical measurements of Q_A and cyt b_{559}, thylakoid and PSII-enriched membranes were diluted to a final Chl concentration of 20–60 μg of Chl/mL in suspension buffer A at pH 7.0; the final Chl concentration was measured for each sample.

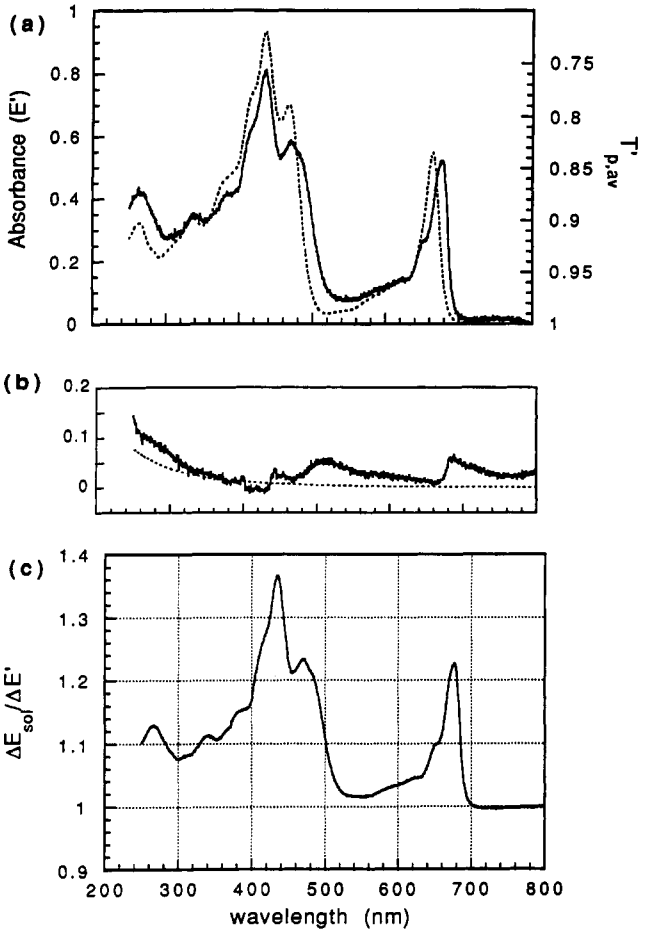

FIGURE 2: (a) Comparison of the absorbance spectra of BBY-type PSII-enriched membranes in suspension (500-fold dilution in suspension buffer A, solid line) and in solution (500-fold dilution in ethanol, broken line). The right-hand scale of $T'_{p,av}$ was calculated from Figure 1 and the observed flattening at 435 nm (see Materials and Methods). Both spectra were corrected for light scattering as described under Materials and Methods. The corrections for light scattering are shown in (b): the solid line corresponds to the value of $A[E(\gamma_2) - E(\gamma_1)]$ in eq 1 for scattering in suspension and the broken line shows the value of A_{sc} in eq 3 for scattering in solution. (c) The differential flattening factor ($\Delta E_{sol}/\Delta E'$) as a function of wavelength in BBY-type PSII-enriched membranes, calculated from eq 5, where p = 8.02 (average of 15 measurements), and Figures 1 and 2a.

For the optical quantitations of cyt b_{559}, the number of Chl per reaction center was determined from the absorbance change at 325 nm, corresponding to the reduction of the primary quinone electron acceptor, Q_A ($\Delta\epsilon$ = 13 mM^{-1} cm^{-1} for Q_A^-/Q_A; van Gorkom, 1974). Thylakoid and PSII-enriched membranes (at 20–60 mg of Chl/mL) were preincubated with 150 μM potassium ferricyanide to oxidize Q_A, 40 μM DCMU to inhibit Q_A to Q_B electron transfer, and 5 mM hydroxylamine to block Q_A^- oxidation by charge recombination. The oxidized minus photoreduced absorbance difference for Q_A was measured following a series of 15 actinic flashes and corrected for light scattering and particle flattening; a multiplicative factor 1.08 (Figure 2b) or 1.6 (Pulles, 1978) was used for the correction of particle flattening at 325 nm in PSII-enriched or thylakoid membranes, respectively.

Chemical oxidation of cyt b_{559} was achieved by incubation of thylakoid or PSII-enriched membranes (20–60 μg of Chl/mL) with 150 μM potassium ferricyanide; the chemical reduction of cyt b_{559} was achieved by pretreatment of the sample with 2 mM ascorbate and the subsequent addition of sufficient solid sodium borohydride to obtain complete

reduction. After the addition, the red-visible absorbance spectrum of cyt b_{559} (545–575 nm) was monitored over time until the oxidation or reduction had reached completion. The stoichiometry of cyt b_{559} was calculated from the optically determined number of Chl per PSII reaction center and $\Delta\epsilon$ (560–570 nm) = 17.5 mM^{-1} cm^{-1} for cyt b_{559}^{red} – cyt b_{559}^{ox} (Cramer et al., 1986).

Electron Paramagnetic Resonance Measurements. X-band EPR measurements at low temperatures were performed on a home-built EPR spectrometer (Beck et al., 1991) equipped with an Oxford ESR-900 liquid helium cryostat and interfaced to a Macintosh IIx computer. Thylakoid and PSII samples were resuspended to a final concentration of 4–6 mg of Chl/mL in suspension buffer A at pH 6.0 (MES buffer) and 8.0 (tricine buffer), respectively, since related studies have shown that Y_D is not efficiently photooxidized at acidic pH in PSII-enriched membranes (data not shown).

For the samples used in our EPR quantitations of cyt b_{559}, the ratio of Chl to reaction center was determined by the EPR spin quantitation procedure of Babcock et al. (1983), with potassium nitrosodisulfonate as a standard. This method assumes the complete oxidation of Y_D after 20 min of continuous illumination (at an intensity of 700 W/m^2) at 0 °C and a negligible contribution from $Y_Z^{\bullet}$ after an additional 1-min dark-adaption period prior to freezing at 77 K. Potassium nitrosodisulfonate [$K_2(SO_3)_2NO$] was obtained from Aldrich and dissolved, without further purification, in deaerated suspension buffer A (pH 8.0). The concentration was determined optically immediately prior to freezing at 77 K (in an EPR tube) by using an extinction coefficient of 20.8 M^{-1} cm^{-1} at 545 nm (Jones, 1963). The relative concentrations of three individual preparations of potassium nitrosodisulfonate were checked by EPR and were found to agree, within experimental error, with the optically determined concentrations.

The overlapping EPR signals of $Y_D^{\bullet}$ [g = 2.0046 and peak-to-peak width of 19 G; reviewed by Miller and Brudvig (1991)] and Chl_z^+ (g = 2.0026 and peak-to-peak width of 9–10 G) were deconvoluted by first subtracting a scaled, "pure" $Y_D^{\bullet}$ spectrum from the photoinduced EPR spectrum to determine the contribution from Chl_z^+. The resulting EPR signal of Chl_z^+ was then subtracted from the original photoinduced EPR spectrum to obtain a spectrum of only $Y_D^{\bullet}$. This deconvolution was used for all spin quantitations involving the EPR signal of photooxidized Y_D (i.e., calculations of the ratio of Chl per reaction center and quantitations of cyt b_{559}), and an example of this procedure is given in the text.

The doubly integrated area of the EPR signal of oxidized cyt b_{559} was calculated from the g_z turning point of cyt b_{559} in the first derivative spectrum, according to the method of Aasa and Vänngård (1975). Chemical oxidation of cyt b_{559} was achieved by the addition of 4–8 mM potassium hexachloroiridate (K_2IrCl_6) from a 50 mM stock solution in DMSO, followed by a 1–2-h, 0 °C dark-incubation period prior to freezing (in complete darkness) to 77 K. In our calculations, we have used the known g_z and g_y values for chemically oxidized cyt b_{559}, 3.05 and 2.18, respectively (Thompson et al., 1989), and an estimated value of 1.39 for g_x. The estimation of g_x assumes no covalency and is obtained from the relation $16 = g_x^2 + g_y^2 + g_z^2$ (Griffith, 1961).

For the quantitation of cyt b_{559} by internal spin standards, the doubly integrated areas of the EPR signals of cyt b_{559}^{ox}, $Y_D^{\bullet}$, and Chl_z^+ were all measured within the same sample. Y_D was photooxidized by a 20-min, 0 °C illumination. Chl_z was oxidized by 30 min of continuous illumination (at an intensity of 700 W/m^2) in a transparent dewar containing liquid nitrogen (77 K). The doubly integrated area of Chl_z^+ was determined after deconvolution of overlapping $Y_D^{\bullet}$ and Chl_z^+ signals. The spectra of $Y_D^{\bullet}$ and Chl_z^+ were recorded immediately after illumination, since these signals slowly decrease during dark storage at 77 K (Miller et al., 1987). For the quantitation of cyt b_{559}, Y_D, and Chl_z by an external spin standard, the doubly integrated areas of the EPR signals of cyt b_{559}^{ox}, $Y_D^{\bullet}$, and Chl_z^+ were compared to the EPR signal of potassium nitrosodisulfonate at known concentration.

To minimize the number of corrections necessary to account for differing EPR conditions, the spectrum of the g_z signal of cyt b_{559}^{ox} and all reference spectra were collected at the same microwave frequency (9.05 GHz), field modulation frequency (100 kHz), temperature (15.0 K), scan width (500 G), and number of points per Gauss (2.05). Differences in microwave power and magnetic field modulation amplitude between spectra are denoted in the respective figure legends and were accounted for in the calculations. The microwave power saturation was measured for the EPR signals of cyt b_{559}^{ox}, the internal spin standards ($Y_D^{\bullet}$ and Chl_z^+), and the external spin standard (potassium nitrosodisulfonate). At the power of our quantitative measurements (0.72 μW for the radical signals at g = 2 and 0.72–14 μW for cyt b_{559}), only the signal of Chl_z^+ was slightly saturated. In our quantitation studies, the area of the Chl_z^+ signal was corrected for 8.3% signal saturation.

RESULTS

Optical Quantitations of Cyt b_{559} in PSII-Enriched Membranes. Our optical quantitation of cyt b_{559} assumes an extinction coefficient of $\Delta\epsilon$ (560–570 nm) = 17.5 mM^{-1} cm^{-1} for cyt b_{559}^{red} – cyt b_{559}^{ox} and involves measurements of the oxidized minus reduced difference absorbance at 560 and 570 nm for cyt b_{559} and at 325 nm for the determination of the number of Chl per reaction center from the absorbance difference of Q_A^- – Q_A. Therefore, the accuracy of this measurement relies on the complete oxidation and reduction of cyt b_{559}, the absence or the deconvolution of interfering signals, and an accurate calculation of the number of Chl per reaction center (for which a correction for particle flattening and scattering is necessary; see Materials and Methods). In the redox titrations of cyt b_{559}, the potentials set by ferricyanide and dithionite are assumed to completely oxidize and reduce cyt b_{559}, respectively. However, since we often experienced significant problems with baseline drift around the isosbestic point for cyt b_{559} at 570 nm in the presence of dithionite, we used excess sodium borohydride plus ascorbate for the chemical reduction of cyt b_{559}. This dithionite-induced effect has been observed previously [Figure 3 of Thompson et al. (1989)] and may be a result of chlorophyll bleaching by dithionite.

The red-visible region of the oxidized minus reduced difference spectra of PSII-enriched membranes is shown in Figure 3. The maximum at 560 nm and the 10-nm width at half-height of the peak in the ferricyanide-oxidized minus sodium borohydride-reduced difference spectrum of Figure 3 are in good agreement with previously published spectra of cyt b_{559} in PSII-enriched membranes (Buser et al., 1990). For comparison of our data to earlier studies which use dithionite as the reductant to cyt b_{559}, we also show the ferricyanide-oxidized minus dithionite-reduced difference spectrum of PSII-enriched membranes in Figure 3. A 30% lesser absorbance change (560–570 nm) for oxidized minus reduced cyt b_{559} is observed with sodium dithionite than with sodium borohydride plus ascorbate, indicating that the latter combination of

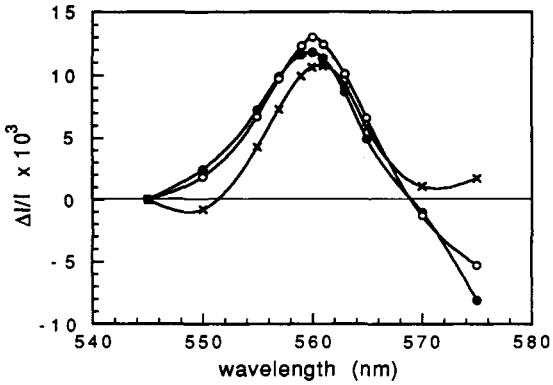

FIGURE 3: Red-visible region of the chemically oxidized minus reduced difference spectra of BBY-type PSII-enriched membranes. In each spectrum, the reference was oxidized with ferricyanide and the sample was reduced by the addition of 2 mM ascorbate (●), further addition of excess solid sodium borohydride (○), or the addition of excess solid dithionite (×). The expected absorbance change ($\Delta I/I \times 10^3$) for 1 copy of cyt b_{559} is 11.6 ± 1.2 (PSII sample 4 in Table I, [chl] = 58.6 μg/mL, and Chl/PSII = 226).

reductants is at least as effective in reducing cyt b_{559} as dithionite. Since it seems unlikely that dithionite does not fully reduce cyt b_{559}, we attribute the decrease in absorbance to interference from reduced Chl.

The same measurements as those in Figure 3 were also repeated for thylakoid membranes (data not shown). In contrast to the clean cyt b_{559} spectrum in PSII-enriched membranes, contributions from cyt f and cyt b_6 to the spectrum of cyt b_{559} were indicated by a shift in the maximum absorbance change (from 560 to 557 nm), a slight shoulder around 561 nm, and the broadened line shape (≈15-nm width at half-height) in the ferricyanide minus sodium borohydride plus ascorbate difference. Since the peaks of the contributing cytochromes are not well-resolved, we did not attempt to use these measurements in the optical quantitation of cyt b_{559} in thylakoid membranes. In fact, we were unable to obtain even an upper limit on the stoichiometry of cyt b_{559} per reaction center in thylakoid membranes, since the ratio of PSII reaction centers to b_6f complexes is variable.

The stoichiometry of cyt b_{559} per PSII reaction center was determined from the absorbance difference (560–570 nm) in the ferricyanide-oxidized minus sodium borohydride-reduced difference spectrum, the optically determined number of Chl per reaction center (see Materials and Methods), and $\Delta\epsilon$ (560–570 nm) = 17.5 mM^{-1} cm^{-1}. To account for differences in individual sample preparations, these measurements were repeated for four BBY-type PSII (one being a modified version of the BBY preparation) and two thylakoid preparations; the data are presented in Table I. After correction for particle flattening, our optical measurements of the number of Chl per reaction center are on the lower end of previously determined values in spinach thylakoid (Selak et al., 1984) and PSII-enriched membranes (Ford & Evans, 1983; Ghanotakis et al., 1984; Briantais et al., 1985). (The small discrepancy between the optical- and EPR-determined ratio of Chl per reaction center in Table I is discussed in the next section). Most important, our results from differential absorbance spectroscopy suggest one copy of cyt b_{559} per PSII reaction center.

EPR Quantitations of Cyt b_{559} in PSII-Enriched and Thylakoid Membranes. Although not often used, EPR spectroscopy is an effective and direct method for the quantitation of cyt b_{559}, since knowledge of an accurate extinction coefficient is not necessary, light scattering and

Table I: Quantitation of Cyt b_{559} by Differential Absorbance Spectroscopy

prepn[a]	sample description	cyt b_{559}/RC[b]	Chl/RC (abs)[c]	Chl/RC (EPR)[d]
1	BBY-type PSII	1.1	219–237	190
2	BBY-type PSII	1.1	206–229	230
3	BBY-type PSII	1.2–1.3	212–233	195
4	BBY-type PSII	1.1–1.3	213–240	
average		1.2 ± 0.1		
a	thylakoid		235–266	295–300
b	thylakoid		297–314	295

[a] The numbers 1–3 refer to three individual BBY-type PSII preparations; sample 4 was prepared by a modified version of the BBY-PSII preparation (Rutherford et al., 1984). The letters a and b denote two different thylakoid preparations. [b] $\Delta\epsilon$ (560–570 nm) = 17.5 mM^{-1} cm^{-1}. [c] Absorbance measurement. [d] EPR measurement.

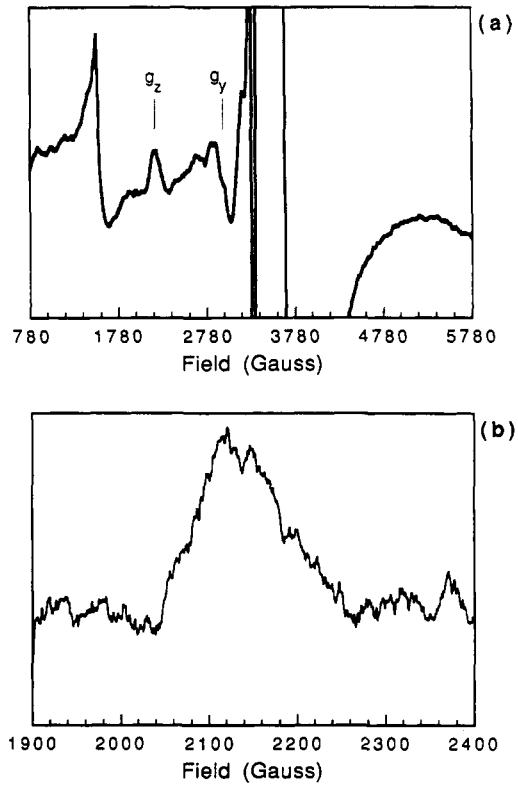

FIGURE 4: (a) EPR spectrum of dark-adapted PSII (pH 8.0) treated with 4 mM K_2IrCl_6 (sample 2 in Tables I and II). The g_z and g_y signals of cyt b_{559} are found at 3.05 and 2.18, respectively. EPR spectrometer conditions: microwave power, 11.4 μW; field modulation amplitude, 20.0 G; temperature, 7.0 K. (b) g_z EPR signal of cyt b_{559} in PSII sample 3 of Tables I and II (average of 10 scans), obtained under the EPR spectrometer conditions used for quantitations of cyt b_{559}: microwave power, 14 μW; field modulation amplitude, 20.0 G; temperature, 15.0 K (see Materials and Methods for further detail).

particle flattening are avoided, and the g_z turning point of cyt b_{559} is free of interfering signals in both PSII-enriched and thylakoid membranes. Figure 4a shows the effect of 4 mM potassium hexachloroiridate on the dark spectrum of PSII-enriched membranes: the g_z and g_y turning points of chemically oxidized cyt b_{559} are labeled and an offscale $g \approx 2$ peak is observed from the presence of excess potassium hexachloroiridate. Figure 4b shows the g_z turning point of cyt b_{559}^{ox} in PSII-enriched membranes under the EPR spectrometer conditions used for its quantitation.

In our first approach to the EPR quantitation of cyt b_{559}, we made use of two internal spin standards, Y_D and Chl_z, which are intrinsic to the sample and assumed to be present in a 1:1 stoichiometry to the reaction center. This method

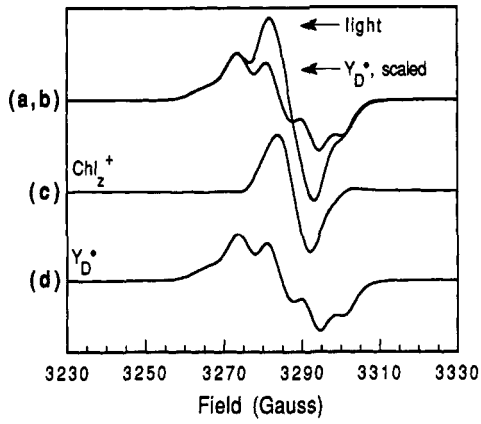

FIGURE 5: Deconvolution of the $Y_D^•$ and Chl_z^+ EPR signals observed after a 0 °C illumination period of K_2IrCl_6-treated PSII-enriched membranes. (a, light) Free radical signals after illumination. (b, $Y_D^•$, scaled) The EPR spectrum of a "pure" $Y_D^•$ signal was scaled to the outer wings of the light-induced spectrum (a, light). (c) Spectrum of photoinduced Chl_z^+ calculated from the difference of (a) minus (b). (d) Spectrum of $Y_D^•$ (dark and photooxidized fractions) determined from the difference of (a) minus (c). EPR spectrometer conditions: microwave power, 0.72 μW; field modulation amplitude, 4.0 G; temperature, 15.0 K (see Materials and Methods for further detail).

allows the direct quantitation of cyt b_{559} without any additional knowledge of the sample, such as the Chl concentration or the number of Chl per reaction center. First, we compared the signal amplitudes of cyt b_{559}^{ox} and $Y_D^•$ under conditions in which both are fully oxidized. Figure 5 shows the effect of continuous illumination at 0 °C in K_2IrCl_6-treated PSII-enriched membranes: in addition to the photooxidation of Y_D, illumination also results in the photooxidation of a fraction of Chl_z, which remains partially oxidized even after a 1-min, dark-adaption period after illumination (prior to freezing to 77 K). Spectrum d in Figure 5 shows the deconvoluted spectrum of photooxidized Y_D in PSII-enriched membranes (see legend for deconvolution procedure); the amplitude of the first-derivative EPR spectrum in (d) is assumed to correspond to one spin per reaction center. Using the method of Aasa and Vänngård (1975) to calculate the doubly integrated area of the total EPR spectrum of oxidized cyt b_{559}, the ratio of cyt b_{559} to Y_D was determined for four different BBY-type PSII and two thylakoid preparations. In agreement with our optical measurements, the results from our EPR measurements, presented in Table II (column 3), suggest one copy of cyt b_{559} in PSII-enriched membranes and also only one copy of cyt b_{559} in thylakoid membranes.

The second approach to the EPR quantitation of cyt b_{559} by internal spin standards made use of the fact that the reaction center is limited to a single turnover during low-temperature illumination. Since electron donation from Y_Z is inhibited at low temperatures, the sum of the photooxidized fractions of Y_D and Chl_z (after subtraction of the dark-oxidized fractions) should correspond to one spin per reaction center in samples with cyt b_{559} preoxidized, assuming the primary quinone electron acceptor is completely oxidized in the dark. The latter assumption is reasonable due to the pretreatment of samples with excess potassium hexachloroiridate and the relatively basic pH (8.0); separate measurements have shown that 85% of Q_A is oxidized in the dark in untreated PSII at pH 8.0 (Buser et al., 1992). Figure 6 shows the effect of continuous illumination at 77 K in K_2IrCl_6-treated PSII-enriched membranes: as expected, we observe an increase in the amplitudes of the EPR signals of $Y_D^•$ (spectra e and f) and Chl_z^+ (spectra c and d) after illumination. Assuming the increase in the signal amplitude of $Y_D^•$ and Chl_z^+ after illumination to correspond to one spin per reaction center, we find an increase of ≈35% in the signal amplitude of $Y_D^•$ after illumination, and correspondingly, the photooxidation of ≈65% of one spin Chl_z. Of significance is that we did not observe any additional photooxidation of cyt b_{559} after the 77 K illumination, indicating that the potassium hexachloroiridate treatment resulted in the complete chemical oxidation of cyt b_{559} (data not shown). In this approach, the amplitude of the EPR spectrum of oxidized cyt b_{559} was compared to the sum of the light-induced fractions of $Y_D^•$ and Chl_z^+; the data from PSII-enriched membranes are shown in Table II (column 4). Again, this experiment indicates a 1:1 stoichiometry of cyt b_{559} per reaction center. Because of complications from overlapping signals (e.g., $P700^+$) and other electron-transfer reactions, this approach was not applied for the quantitation of cyt b_{559} in thylakoid membranes.

In our final approach to the quantitation of cyt b_{559} by EPR, we used potassium nitrosodisulfonate as a spin standard to determine the concentrations of chemically oxidized cyt b_{559} and photooxidized Y_D and Chl_z in K_2IrCl_6-treated PSII-enriched and thylakoid membranes. Again, this EPR quantitation does not require knowledge of the ratio of Chl per reaction center or the Chl concentration of the sample. The results from the external EPR quantitations of cyt b_{559} are shown in Table II (columns 5–7); in agreement with our preceding data, these measurements suggest one copy of cyt b_{559} per PSII and thylakoid reaction center. In turn, the concentration of Y_D per sample was used to determine the number of Chl per reaction center, according to the method of Babcock et al. (1983). It should be noted that the EPR determination of the ratio of Chl per reaction center is consistently ≈0–15% lower than the optical quantitation of this number (see Tables I and II). Although it remains unclear which is the more accurate determination of the ratio of Chl per reaction center, we would like to briefly discuss potential problems with each measurement. In the EPR measurements, an overestimation of the amplitude of $Y_D^•$ (possibly due to some fraction of stable, oxidized Y_Z which does not recombine during the 1-min dark-adaption period after illumination prior to freezing) may lead to an underestimation of the number of Chl per reaction center. In comparison, factors which might lead to an overestimation of the Chl per reaction center from the optical measurements include the presence of photochemically inactive centers and an overestimation of the extinction coefficient for the measurement of $Q_A^- - Q_A$ at 325 nm.

DISCUSSION

Our independent measurements of the quantitation of cyt b_{559} by differential absorbance and electron paramagnetic resonance spectroscopies strongly suggest only one copy of cyt b_{559} per BBY-type PSII reaction center (Tables I and II). Although the data are not as comprehensive and the experimental variance is larger, our EPR quantitations also suggest only one copy of cyt b_{559} per thylakoid PSII reaction center (Table II). It is interesting to note that our measurements of the ratio of cyt b_{559} per reaction center are consistently slightly higher in PSII-enriched membranes (1.2 per reaction center, Tables I and II) than in thylakoid membranes (1.0 per reaction center, Table II). As mentioned previously, Franzén et al. (1986) also found a ratio of 1.2 cyt b_{559} per Y_D in O_2-evolving PSII-enriched samples (depleted of the 17- and 23-kDa extrinsic polypeptides and the light-harvesting chlorophyll protein complex) by a similar EPR approach. Both the optical and EPR quantitations of cyt b_{559} rely on completely active

Table II: Quantitation of Cyt b_{559} by EPR

prep[a]	sample description	cyt b_{559}:Y_D	cyt b_{559}:1 radical spin[b]	cyt b_{559} (μM)	Y_D (μM)	Chl_z[c] (μM)	Chl/RC
5	PSII + 4 mM K_2IrCl_6, pH 8	1.3–1.4		52–53	39		190
5	PSII + 6 mM K_2IrCl_6, pH 8	1.2		47–49	39		190
6	PSII + 4 mM K_2IrCl_6, pH 8	1.4–1.5		63–67	45		165
3	PSII + 4 mM K_2IrCl_6, pH 8	1.0–1.1		29–30	28		195
2	PSII + 8 mM K_2IrCl_6, pH 8	0.80–0.94		28–33	35		230
6	PSII + 4 mM K_2IrCl_6, pH 8		0.81–1.1	34–47	46	34	
average		1.2 ● 0.2					
a	thyl + 4 mM K_2IrCl_6, pH 6	0.79–0.91		22–26	28		300
a	thyl + 4 mM K_2IrCl_6, pH 6	0.88–0.91		25–26	29		295
b	thyl + 4 mM K_2IrCl_6, pH 6	1.2–1.3		36	29		295
average		1.0 ● 0.2					

[a] Equivalent numbers and letters in Tables I and II refer to the same preparations. [b] The doubly integrated area of the EPR signal of K_2IrCl_6-oxidized cyt b_{559} was compared to the sum of the doubly integrated areas of the EPR signals of the photooxidized fractions of $Y_D^{\bullet}$ and Chl_z^+ after a 77 K illumination period. [c] The concentration of Chl_z per reaction center was determined by comparison of the doubly integrated areas of the EPR signals of photooxidized Chl_z and the reference potassium nitrosodisulfonate. As discussed in the text, a 77 K illumination results in the photooxidation of an additional 35% of Y_D; therefore, only about 65% of 1 Chl_z is oxidized in this sample.

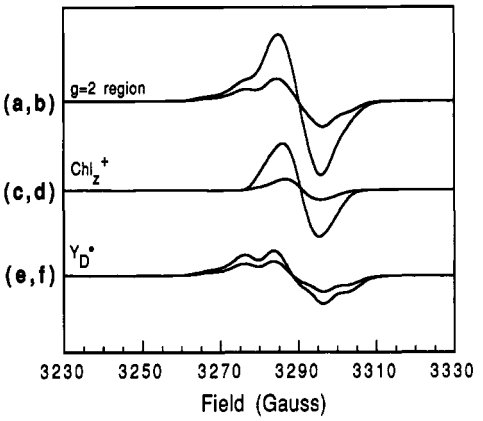

FIGURE 6: Effect of illumination at 77 K on the free radical EPR signals in K_2IrCl_6-treated PSII-enriched membranes. The spectra in (a) and (b) show the free radical signals before and after illumination, respectively. The contributions of $Y_D^{\bullet}$ and Chl_z^+ were deconvoluted by the method described in the legend to Figure 5. Spectra (c,d) and (e,f) show the contributions from Chl_z^+ and $Y_D^{\bullet}$, respectively, to the radical signals in spectra (a,b). EPR spectrometer conditions: microwave power, 0.72 μW; field modulation amplitude, 4.0 G; temperature, 15.0 K (see Materials and Methods for further detail).

samples: the optical determination depends on the formation of $P680^+ Q_A^-$ in all reaction centers and the EPR quantitation relies on the complete photooxidation of Y_D in all centers. In either case, the presence of photochemically inactive centers would lead to an overestimation of cyt b_{559}—which might explain the slightly higher stoichiometry observed for cyt b_{559} in the BBY-type PSII-enriched membranes versus the more intact thylakoid membranes.

An alternative explanation for the slightly greater than 1 cyt b_{559} per PSII reaction center (1.2:1) ratio and for the range found for this ratio, especially in thylakoid membranes, is the presence of variable amounts of cyt b_{559} associated with the PSII complex or spread heterogeneously in the membrane. There is some experimental evidence which suggests that more than one cyt b_{559} per PSII reaction center may be present in the thylakoid membrane under specific conditions such as photoinhibition (Hundal et al., 1990), during greening (Herrmann et al., 1985), or in a mutant lacking the ability to synthesize the D1 polypeptide (Nilsson et al., 1990). Although these experiments only measure apoprotein and are thus difficult to interpret in terms of assembled cyt b_{559}, the point is that all of these conditions would lead to an overestimation of the stoichiometry of cyt b_{559}. If we assume that all active reaction centers are homogeneous, then the fact that we never observe more than 1.5 cyt b_{559} per reaction center (Tables I and II) argues strongly against a 2:1 ratio of cyt b_{559} per PSII reaction center. As a result, we believe the variation in our measured stoichiometry is due to experimental error and not to heterogeneity. Clearly, a 1:1 stoichiometry of cyt b_{559} per reaction center has significant manifestations on the interpretation of its electron-transfer kinetics and redox behavior, as well as implications on the overall structure of the reaction center and the mechanisms of electron transfer.

Previously, our lab determined a stoichiometry of 2 cyt b_{559} per reaction center in BBY-type PSII-enriched membranes on the basis of earlier EPR measurements which relied on two spin standards, ferrimyoglobin azide and potassium nitrosodisulfonate, to independently determine the concentrations of fully oxidized cyt b_{559} and Y_D, respectively (de Paula et al., 1985). However, the high pH conditions (pH 8), which we have found to be necessary for the complete photooxidation of Y_D in PSII-enriched membranes, were not yet realized in the original study. In addition, the current investigation has indicated a problem with our earlier use of ferrimyoglobin azide as a spin standard for cyt b_{559} due to incomplete oxidation of the heme and a consequent underestimation of the spin standard's concentration (data not shown). Unfortunately, a direct comparison of signal amplitudes of cyt b_{559}^{ox} and $Y_D^{\bullet}$ was not possible in the earlier work due to the significantly different EPR conditions.

Throughout this paper, we have emphasized the complications and have denoted potential problems of both past studies and the present study in the quantitation of cyt b_{559} by optical and EPR spectroscopies. Some of these problems can be corrected for or altogether avoided, such as particle flattening in optical measurements or the microwave power saturation of EPR signals. Uncertainties in the extinction coefficient for cyt b_{559} in situ and side reactions of the exogenous oxidants and reductants are also a cause of inaccuracy in such quantitations. For our optical quantitation of cyt b_{559} in PSII-enriched membranes, we assumed a differential extinction coefficient of $\Delta\epsilon$ (560–570 nm) = 17.5 mM^{-1} cm^{-1} for cyt b_{559}^{red} – cyt b_{559}^{ox} in situ (determined from isolated cyt b_{559} from spinach; Cramer et al., 1986). In contrast, many earlier studies use an extinction coefficient of 15 mM^{-1} cm^{-1} (Whitmarsh & Cramer, 1978; Selak et al., 1984; Murata et al., 1984; Briantais et al., 1985; Nanba & Satoh, 1987), which leads to an overestimation of the ratio of cyt b_{559} per reaction center as compared to our calculations. Furthermore, from the measurements of particle flattening by Pulles (1978) and

ourselves, we have found significant flattening corrections necessary for the accurate determination of the number of Chl per reaction center from the UV measurement of Q_A. Taken together, our optical and EPR measurements suggest that a number of previous studies have seemingly overestimated the ratio of Chl per reaction center in PSII-enriched and thylakoid membranes. An overestimation of the number of Chl per reaction center would also lead to an underestimation of the reaction center concentration, again resulting in an overestimation of the stoichiometry of cyt b_{559} from the observed absorbance. Compounded, these two errors may lead to a 20–25% overestimation of cyt b_{559}—resulting in the calculation of 1.5 instead of 2 copies of cyt b_{559} per reaction center in some of the references cited in the introduction.

It is important to note that even the most careful measurements still depend on several inherent assumptions. In the optical measurements, we have assumed (a) the complete oxidation of cyt b_{559} by potassium ferricyanide and its complete reduction by sodium borohydride plus ascorbate, (b) a dark state with all of Q_A oxidized in the presence of ferricyanide, and (c) all reaction centers to be photochemically active. The assumptions in our EPR measurements include (a) the complete oxidation of cyt b_{559} by potassium hexachloroiridate, (b) the complete photooxidation of Y_D (without contributions from $Y_Z^{\bullet}$), and (c) one complete turnover of all reaction centers during low-temperature illumination.

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