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Accelerated Publications

The 23 and 17 kDa Extrinsic Proteins of Photosystem II Modulate the Magnetic Properties of the S_1-State Manganese Cluster†

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ABSTRACT: An S_1-state parallel polarization "multiline" EPR signal arising from the oxygen-evolving complex has been detected in spinach (PSII) membrane and core preparations depleted of the 23 and 17 kDa extrinsic polypeptides, but retaining the 33 kDa extrinsic protein. This S_1-state multiline signal, with an effective *g* value of 12 and at least 18 hyperfine lines, has previously been detected only in PSII preparations from the cyanobacterium sp. *Synechocystis* sp. PCC6803 [Campbell, K. A., Peloquin, J. M., Pham, D. P., Debus, R. J., and Britt, R. D. (1998) *J. Am. Chem. Soc. 120*, 447−448]. It is absent in PSII spinach membrane and core preparations that either fully retain or completely lack the 33, 23, and 17 kDa extrinsic proteins. The S_1-state multiline signal detected in spinach PSII cores and membranes has the same effective *g* value and hyperfine spacing as the signal detected in *Synechocystis* PSII particles. This signal provides direct evidence for the influence of the extrinsic PSII proteins on the magnetic properties of the Mn cluster.

The oxygen-evolving complex (OEC)[1] of Photosystem II (PSII), located on the lumenal side of PSII, consists of a cluster of four Mn atoms, essential calcium and chloride cofactors, and Y_Z, tyrosine 161 of the D1 polypeptide *(1)*. Water serves as the terminal electron donor in PSII; electrons from water coordinated to the Mn cluster are transferred via the Mn cluster to the intermediate electron carrier, Y_Z (which also may be directly involved in water splitting) *(2)*, and from Y_Z to the photo-oxidized $P680^+$ Chl moiety. O_2 is produced as a byproduct of the oxidation of two molecules of water by the Mn cluster following four sequential single electron reductions of $P680^+$. This four-electron process to oxidize water to molecular oxygen is modeled by Kok's "S-state" cycle *(3)*. In this model, the OEC cycles through a series of S-states, S_0 to S_4, where the subscript refers to the

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[1] Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll; CW, continuous wave; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MMN, 15 mM NaCl, 5 mM $MgCl_2$, 50 mM MES-NaOH (pH 6.0); MSP, 33 kDa manganese stabilizing protein; OEC, oxygen-evolving complex; OGP, 1-octyl-β-D-glucopyranoside; OTG, 1-octyl-β-D-thioglucopyrano-side; PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SMN, 0.4 M sucrose, 10 mM NaCl, 50 mM MES-NaOH (pH 6.0); XANES, X-ray absorption near edge structure.

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number of oxidizing equivalents the OEC has accumulated following succeeding reductions of $P680^+$. O_2 evolution occurs during the $S_4 \rightarrow S_0$ transition. The S_1-state, in which the Mn cluster has stored one oxidizing equivalent, is the dark-stable resting state of the OEC.

While the exact structure of the tetranuclear Mn cluster is unknown, XANES, EXAFS, and EPR studies performed on PSII samples have provided much insight into possible structures *(4−8)*. XANES studies demonstrate oxidation of the Mn cluster upon the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ state transitions *(9)*. The conventional perpendicular polarization EPR spectra of PSII samples poised in the S_2- and S_0-states show signals with at least 18 hyperfine lines near $g = 2$ (termed "multiline" signals) consistent with electron spin $S = {}^1/_2$ configurations of the Mn cluster in these Kok-cycle S-states *(8, 10, 11)*. The S_2-state also presents an EPR signal near $g = 4.1$ *(12−14)*. On the basis of these EPR studies and the single electron transfer per S-state transition nature of the Kok cycle, the S_1-state of the Mn cluster is predicted to be either diamagnetic or integer-spin paramagnetic. Integer-spin transitions are typically not detected with conventional, perpendicular mode CW-EPR. However, parallel polarization CW-EPR, with the oscillating magnetic field applied in a direction parallel to the static magnetic field, allows for the observation of integer-spin transitions that would normally be forbidden or have very small transition probabilities in perpendicular mode EPR *(15−17)*. Indeed, a broad, featureless EPR signal near $g = 4.8$, detected with parallel polarization EPR, has been associated with the S_1-state and assigned to an $S = 1$ integer spin state *(18, 19)*. However, this signal is weak and difficult to detect, and it has only been reproduced by two laboratories *(19, 20)* despite significant effort by other laboratories *(21)*. Additionally, the lack of ^{55}Mn hyperfine structure limits its utility in Mn cluster structure determination.

We have recently discovered an S_1-state multiline EPR signal with parallel polarization EPR spectroscopy of PSII particles isolated from the cyanobacterium *Synechocystis* sp. PCC6803 *(4)*. This signal differs significantly from the previously detected S_1-state signal *(18, 19)* in that it has an effective *g* value of 12 instead of 4.8, and instead of being featureless, it displays at least 18 hyperfine lines with an average splitting of 32 G. No such highly resolved S_1-state multiline signal has been observed in higher plant PSII membranes or particles.

In higher plants, three extrinsic proteins (psb O, P, and Q nuclear-encoded gene products), located on the lumenal surface of PSII, have been associated with the Mn cluster *(1)*. These proteins in spinach have apparent molecular masses of 33, 23, and 17 kDa. In cyanobacteria, the 23 and 17 kDa proteins are absent and apparently substituted by a 12 kDa protein and cytochrome c_{550} *(22, 23)*. In plant PSII preparations, all three extrinsic proteins can be removed by treatment with urea and 200 mM NaCl *(24)*, whereas a high salt treatment (1 M NaCl) removes only the 23 and 17 kDa proteins *(25)*.

The 33 kDa protein appears to stabilize and protect the Mn cluster, and it is therefore referred to as the manganese-stabilizing protein (MSP). In the absence of the MSP, and without at least 100 mM Cl^-, the Mn cluster is destabilized and at least two Mn^{2+} ions are lost *(26)*. In the presence of 200 mM Cl^-, the Mn cluster remains intact, even in the absence of the MSP, although the rate of O_2 evolution is significantly decreased *(27)*. In the absence of the MSP and in the presence of 10 mM Ca^{2+}, oxygen evolution rates are approximately 30% of the rate of control samples *(28)*. The 23 and 17 kDa proteins reduce the Ca^{2+} and Cl^- requirements for O_2 evolution. In PSII membranes washed with 1 M NaCl to remove the 23 and 17 kDa proteins, normal rates of oxygen evolution only occur in the presence of additional Ca^{2+} *(29, 30)*. The 23 kDa protein appears to maintain Ca^{2+} binding *(29)*, while the 17 kDa protein appears to maintain Cl^- binding *(27)* within the OEC. In the absence of these proteins and without additional Ca^{2+}, oxygen evolution rates are decreased by approximately 50% *(31)*. Also, neither protein will bind to the OEC in the absence of the MSP, and although the 23 kDa protein will bind if the MSP is present and the 17 kDa is absent, the 17 kDa will not bind if the 23 kDa is absent, suggesting a regulatory cap structure in the order Mn cluster-33-23-17 *(1)*.

The purpose of the present study is to determine why the S_1-state multiline EPR signal has been observed in *Synechocystis* preparations and not in higher plant PSII preparations. It is possible that the signal has been observed only in *Synechocystis* for at least three reasons: (1) it is an anomaly of the specific *Synechocystis* PSII core preparation; (2) there is a change in the magnetic properties of the Mn cluster in the absence of the 23 and 17 kDa proteins; or (3) there is a difference in the inherent structure of the Mn cluster between *Synechocystis* and higher plants. To investigate these options, we have prepared both spinach PSII membrane and core samples with all three extrinsic proteins retained, with the 23 and 17 kDa proteins removed and with the 33, 23, and 17 kDa proteins removed. Additionally, spinach PSII membrane samples have been prepared depleted of the 23 and 17 kDa proteins but with the addition of 10 mM Ca^{2+}.

Parallel mode CW-EPR reveals no S_1-state multiline signal in spinach PSII samples containing all three extrinsic proteins. However, PSII samples depleted of the 23 and 17 kDa proteins, with and without additional Ca^{2+}, show an S_1 multiline signal as observed in *Synechocystis* PSII preparations. Spinach PSII samples depleted of all three extrinsic proteins show no S_1-state multiline signal.

MATERIALS AND METHODS

Synechocystis PSII Core Particles. *Synechocystis* sp. PCC6803 PSII core particles were isolated as described by Tang and Diner *(32)*. The samples contained 40 ± 2 Chl a/photoreducible Q_A 5-6 mg of Chl/mL in 50 mM MES-NaOH (pH 6.0), 20 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM $MgSO_4$, 0.03% *n*-dodecyl-*β*-D-maltoside (Anatrace, Maumee, OH) and 25% (v/v) glycerol (Gibco-BRL). The sample activity was 5100 ± 200 *μ*mol of O_2 (mg of Chl h)$^{-1}$.

Spinach PSII Membranes. PSII-enriched "BBY" membranes were prepared from fresh market spinach according to a modified method based on the preparations described by Berthold et al. *(33)* and Ford and Evans *(34)*. Spinach leaves were ground in 400 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, and 50 mM HEPES (pH 7.5) with 1.7 g/L BSA and 4.5 mM ascorbate. The pelleted chloroplasts were washed with 150 mM NaCl, 8 mM $MgCl_2$, 50 mM MES-NaOH (pH 6.0), then resuspended in 15 mM NaCl,10 mM $MgCl_2$, and 50 mM MES-NaOH (pH 6.0) and treated with 25 mg of

Triton X-100/mg of Chl at a concentration of 2 mg of Chl/mL for 30 min. The PSII membranes were subsequently washed with 400 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM EDTA, and 50 mM MES-NaOH (pH 6.0) (SMNCE buffer), and starch grains were removed via centrifugation (a "low speed cut" 2000*g* for 2 min). The supernatant was centrifuged to obtain the PSII-enriched membranes. All procedures were carried out at 4 °C under dim green light except the leaf-grinding step.

(i) Samples Depleted of the 23 and 17 kDa Proteins. PSII-enriched membranes were washed twice in a buffer containing 300 mM sucrose, 10 mM NaCl, and 25 mM MES-NaOH (pH 6.5) (buffer A) and centrifuged at 35000*g* for 10 min after each wash. The 23 and 17 kDa proteins were removed by suspending the membranes in a medium containing 1 M NaCl, 300 mM sucrose, and 25 mM MES-NaOH (pH 6.5) and incubated in room light at 4 °C for 30 min at a final concentration of approximately 2 mg of Chl/ml. Samples were then centrifuged at 35000*g* for 30 min, washed again with buffer A, and repelleted. Samples for which Ca^{2+} was reconstituted to restore O_2 evolution were prepared by washing the membranes depleted of the 23 and 17 kDa proteins with a buffer containing 300 mM sucrose, 10 mM $CaCl_2$, and 25 mM MES-NaOH (pH 6.5) instead of buffer A for the final centrifugation. Additional samples were prepared in which 200 mM Cl^- was added to the sample by using the same procedure as for the Ca^{2+} reconstitution, except the buffer contained either 100 mM $CaCl_2$ or 200 mM NaCl instead of 10 mM Ca^{2+}.

(ii) Samples Depleted of the 33, 23, and 17 kDa Proteins. PSII-enriched membranes were washed twice in buffer A and centrifuged at 35000*g* for 10 min after each wash. Removal of the 33, 23, and 17 kDa proteins was accomplished by suspending the membranes in a medium containing 2.6 M urea, 200 mM NaCl, and 25 mM MES-NaOH (pH 6.5) to a final concentration of approximately 2 mg of Chl/ml, and incubating in darkness at 4 °C for 30 min. Following incubation, samples were centrifuged at 35000*g* for 30 min, then washed with a high salt concentration buffer containing 300 mM sucrose, 200 mM NaCl, and 25mM MES-NaOH (pH 6.5) and centrifuged for 30 minutes at 35000*g*.

Spinach PSII Core Samples. PSII core samples were prepared from PSII-enriched membranes (vide supra except 2 and 5 mM $MgCl_2$ in the grinding and washing buffers, respectively, and Triton solubilization in MMN; the Triton pellets were washed with SMN only) by solubilization with OGP (from Fischer Scientific) (OGP-cores), resulting in loss of the 23 and 17 kDa proteins and retention of the 33 kDa protein, or by solubilization with OTG (Aldrich Chemical Co.) (OTG-cores), resulting in retention of the 33, 23, and 17 kDa proteins. The OGP-cores were prepared as in the Ghanatokis et al. "PSII reaction center complex" *(35)*, except both the light-harvesting complex and the PSII cores were pelleted for 30 min. The OTG-cores were prepared as described by Mishra and Ghanatokis *(36)* except solubilization was carried out at 1.4 (mg of Chl) ml^{-1} with 1.2% (w/v) OTG, with a subsequent 2.8-fold dilution with SMN. The final pellets of both preparations were washed with 0.4 M sucrose, 10 mM NaCl, 5 mM $CaCl_2$, and 50 mM MES-NaOH (pH 6.0), and resuspended in the same buffer for O_2 evolution measurements and SDS−PAGE analysis. Removal of the 33, 23, and 17 kDa proteins from the OTG- and OGP-cores was carried out as for the PSII-enriched membranes.

Gel Electrophoretic Analysis. The polypeptide composition of PSII membrane and core preparations was analyzed on a 15% polyacrylamide gel by SDS−PAGE at 80 V, using the buffer system of Laemmli *(37)*. Protein samples (6-12 μg of Chl) were incubated in 60 mM Tris (pH 7.5), 60 mM β-mercaptoethanol, 4% SDS, and 2.4 M urea at 37 °C for 1.5 h prior to loading.

Oxygen Evolution. The oxygen evolution rates of the spinach PSII samples were determined with a Clark-type oxygen electrode, with the sample suspended in the appropriate buffer with 1.6 mM PPBQ as an electron acceptor. The rates of oxygen evolution [μmol of O_2 (mg of Chl h)$^{-1}$] were 700 (OGP-cores), 400-900 (OTG-cores), 400-500 (control membranes), 160-200 (membranes depleted of the 23 and 17 kDa proteins), 200 (membranes depleted of the 23 and 17 kDa proteins but with 10 mM Ca^{2+}), 230-250 (membranes depleted of the 23 and 17 kDa with 100 mM $CaCl_2$ or 200 mM NaCl), and less than 100 (membranes depleted of the 33, 23, and 17 kDa proteins). OTG-cores depleted of the 33, 23, and 17 kDa proteins showed O_2 activity less than 25% compared with the protein intact cores.

Sample Illumination. After preparation, spinach PSII samples were loaded into quartz EPR tubes at a concentration of approximately 15 mg of Chl/mL (membranes) or 7-12 mg of Chl/mL (cores) and dark adapted at 4 °C for 1 h (membranes) or 30 min (cores) to poise them in the S_1-state prior to freezing at 77 K. All samples were advanced to the S_2-state by illumination for 5 min at 195 K (dry ice/methanol). Illumination was performed with a focused 300 W IR-filtered Radiac light source. Samples were frozen in liquid nitrogen within 2 s of illumination.

EPR Spectroscopy. EPR spectra were recorded on a Bruker ECS106 X-band CW-EPR system with a Bruker ER 4116DM dual mode cavity capable of both parallel (TE 012) and perpendicular (TE102) mode polarizations of the applied magnetic field. Cryogenic temperatures were obtained with an Oxford ESR900 liquid helium cryostat. The temperature was controlled with an Oxford ITC503 temperature and gas flow controller and measured with a carbon glass resistor calibrated from 3.6 to 300 K.

RESULTS

SDS−PAGE analysis shows the absence of a 23 and 17 kDa band in the PSII membrane samples depleted of these two extrinsic proteins and only faint bands in the OGP-core samples. PSII membrane samples depleted of all three extrinsics do not show a 23 or 17 kDa band and only a faint band of the 33 kDa protein. OTG-cores and untreated PSII membrane samples clearly show bands due to all three extrinsic proteins (Figure 1).

The S_1-state parallel mode CW-EPR spectrum from *Synechocystis* PSII particles shows a multiline signal with at least 18 resolved hyperfine lines centered about an effective *g* value of 12 (Figure 2a, "dark"). This multiline signal is significantly decreased following advancement to the S_2-state (Figure 2a, "light"). The small residual S_1 multiline in the illuminated spectrum is presumably due to incomplete advancement of all PSII centers in the sample.

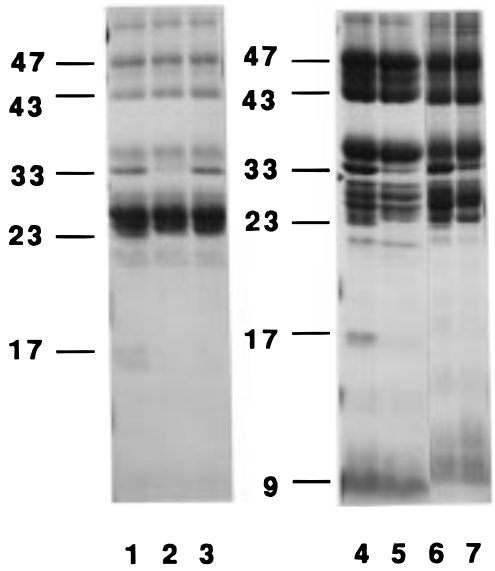

FIGURE 1: SDS−PAGE analysis of the PSII membrane and core samples used in this study. Numbers indicate apparent molecular masses (kDa). Lane 1: untreated PSII membranes. Lane 2: PSII membranes depleted of the 33, 23, and 17 kDa proteins. Lane 3: PSII membranes depleted of the 23 and 17 kDa proteins. Lane 4: untreated OTG-cores. Lane 5: OTG-cores depleted of the 33, 23, and 17 kDa proteins. Lane 6: untreated OGP-cores. Lane 7: OGP-cores depleted of the 33, 23, and 17 kDa proteins.

The number of hyperfine lines reveals that this signal arises from a multinuclear exchange-coupled paramagnetic Mn cluster as described in our previous report *(4)*. The three underlying broad background features in Figure 2a at approximately 1100, 900, and 600 G appear in most spectra with random dependence on sample concentration and illumination. These features are probably due to O_2, but further investigation is required to conclusively identify their origin. Figure 2b shows the S_2-state minus S_1-state perpendicular mode CW-EPR difference spectrum, which confirms the advancement from the S_1- to S_2-state by the appearance of the $g = 2$ multiline signal in the S_2-state.

Since the *Synechocystis* cores display an S_1-state multiline signal, it is interesting that this signal has not been observed in higher plant PSII preparations. It seemed possible that the S_1-state multiline signal is unique to the PSII *Synechocystis* core preparation. This led us to pursue looking for the signal in spinach PSII core preparations. No S_1-state multiline signal is present in the spinach PSII OTG-core preparation with all three extrinsic proteins retained (Figure 3a, "dark"). However, the OGP-core preparation, missing the 23 and 17 kDa proteins, clearly shows a strong multiline signal (Figure 3b, "dark") with hyperfine splittings identical to that found in (Figure 2a, "dark").[2] The parallel mode CW-EPR spectrum of the 23 and 17 kDa depleted PSII core sample advanced to the S_2-state shows a reduction of the S_1-state multiline signal (Figure 3b, "light"), with some residual multiline presumably due to incomplete S_2-state

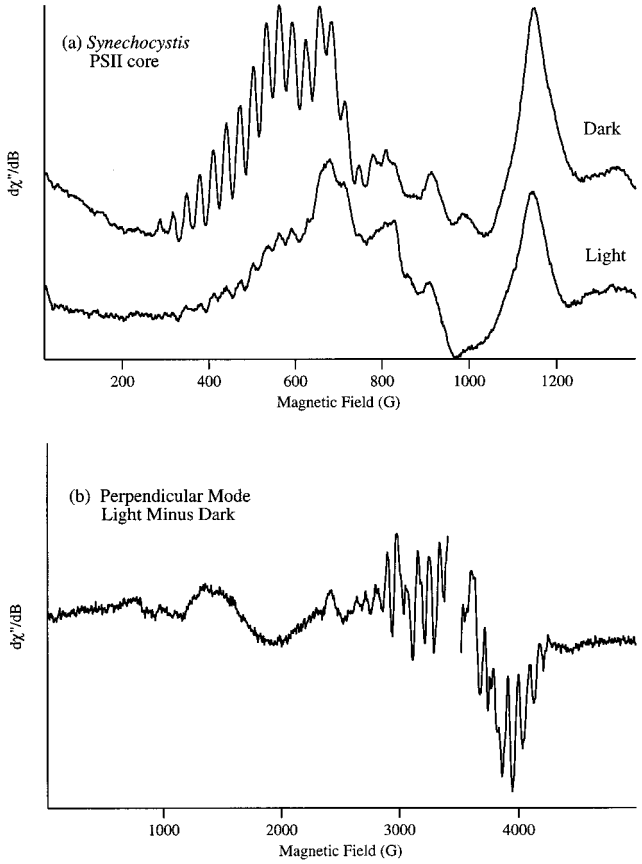

FIGURE 2: (a) "Dark" trace: the parallel polarization EPR spectrum of *Synechocystis* PSII particles. "Illuminated" trace: spectrum following 195 K illumination (5 min) to induce the $S_1 \rightarrow S_2$ transition. (b) The illuminated minus dark perpendicular polarization spectrum of the same states. The $g = 2$ multiline is partially saturated under these conditions. Experimental conditions: (a) parallel mode and (b) perpendicular mode; microwave frequency, (a) 9.41 and (b) 9.67 GHz; microwave power, (a) 80.3 and (b) 3.2 mW; temperature, 3.6 K; modulation amplitude, (a) 8 and (b) 10 G; modulation frequency, 100 kHz.

advancement as described above for *Synechocystis*. PSII OTG-cores depleted of all three extrinsic proteins do not display an S_1 multiline signal (Figure 3c, "dark"). Figure 4 shows the perpendicular mode CW-EPR light minus dark spectrum for each PSII core sample. All three samples show a perpendicular CW-EPR S_2-state multiline signal centered at $g = 2$. The immediate $g = 2$ region has been deleted from the spectra due to overlap with the large tyrosine radical (D2-Tyr-161, spinach notation) EPR signal. The $g = 4.1$ signals in Figure 3 appear normal. The difference spectrum for the sample depleted of all three extrinsics (Figure 4c) does not show a clear $g = 4.1$ signal.

Given that the spinach PSII OGP-core samples missing the 23 and 17 kDa proteins display an S_1 multiline signal, we decided to investigate whether spinach PSII "BBY" membranes depleted of the 23 and 17 kDa proteins would also display the S_1-state multiline. As expected, the PSII membrane samples containing all three extrinsic proteins show no S_1-state multiline signal (Figure 5a, "dark"). However, the S_1-state multiline signal is observable in the samples depleted of the 23 and 17 kDa proteins, without and with additional Ca^{2+}, (Figure 5b, "dark", and Figure 5c, "dark"). The addition of Ca^{2+} to the 23 and 17 kDa depleted sample does not appreciably alter the multiline structure

[2] It should be noted that evidence has been presented that cytochrome c_{550} and a 12 kDa extrinsic protein may substitute for the 23 and 17 kDa proteins in *Synechocystis* (*22*, *23*). Our results show that the magnetic properties of the S_1-state Mn cluster are identical in the *Synechocystis* core preparations to spinach PSII-enriched membranes and core preparations in which the 23 and 17 kDa proteins are removed. We are working to characterize the cyt c_{550} and 12 kDa protein content of the *Synechocystis* preparation.

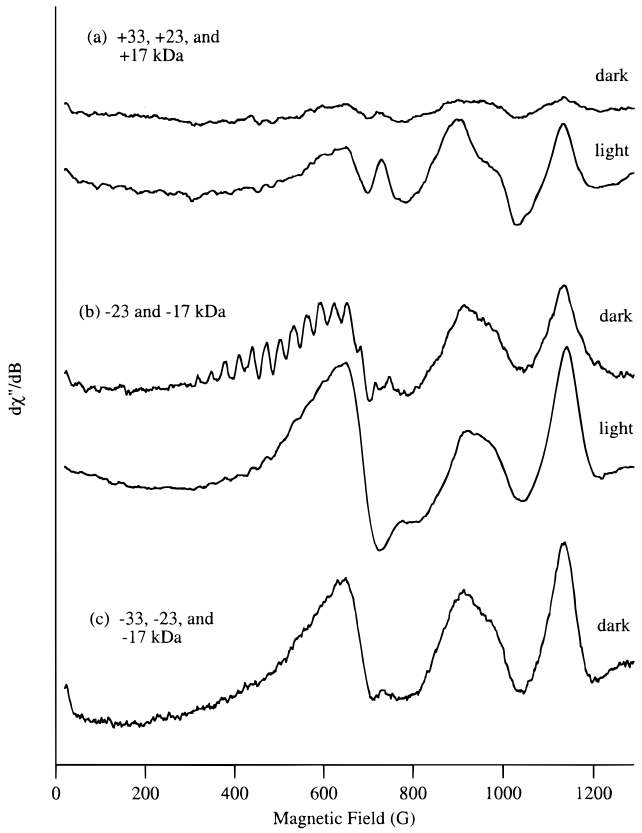

FIGURE 3: "Dark" traces: the parallel polarization EPR spectra of dark adapted PSII core particles. "Light" traces: spectra following 195 K illumination (5 min) to induce the $S_1 \rightarrow S_2$ transition. (a) Spinach PSII OTG-cores containing the 33, 23, and 17 kDa extrinsic proteins. (b) Spinach PSII OGP-cores containing the 33 kDa and lacking the 23 and 17 kDa proteins. (c) urea-treated OTG-cores lacking the 33, 23, and 17 kDa extrinsic proteins. Experimental conditions: parallel mode; microwave frequency, 9.41 GHz; microwave power, 80.3 mW; temperature, 3.6 K; modulation amplitude, 8 G; modulation frequency, 100 kHz.

(Figure 5c, "dark", compared to Figure 5b, "dark") even though O_2 evolution rates are almost fully restored upon the addition of Ca^{2+}. In addition, 23 and 17 kDa samples with an additional 200 mM Cl^- (added as either NaCl or $CaCl_2$) also exhibit the S_1-state multiline signal (data not shown).[3] The S_1-state multiline signal is absent in the PSII membrane sample depleted of all three extrinsic proteins (Figure 5d, "dark"). Each PSII membrane sample exhibited a perpendicular mode CW-EPR $g = 2$ multiline and $g = 4.1$ signal in the S_2 minus S_1-state spectrum (Figure 6).[4] It should be noted that the perpendicular mode spectra of the PSII

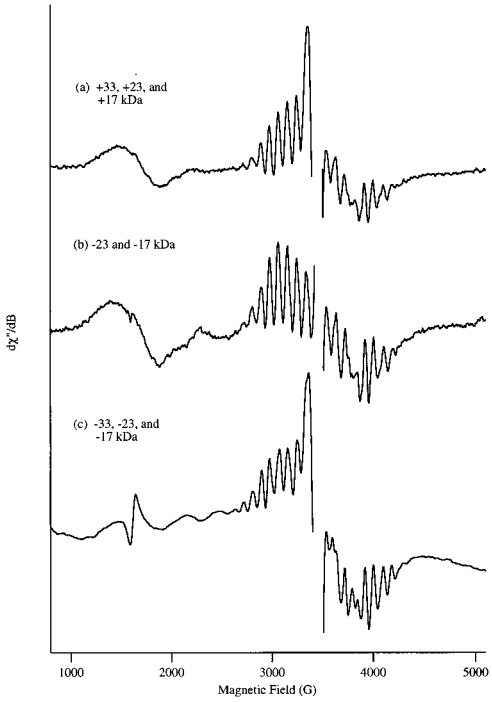

FIGURE 4: Light minus dark perpendicular polarization EPR spectra of spinach PSII core particles. (a) Spinach PSII OTG-cores containing the 33, 23, and 17 kDa extrinsic proteins. (b) Spinach PSII OGP-cores containing the 33 kDa protein and lacking the 23 and 17 kDa proteins. (c) urea-treated OTG-cores lacking the 33, 23, and 17 kDa extrinsic proteins. Experimental conditions: perpendicular mode; microwave frequency, 9.68 GHz; microwave power, 3.2 mW; temperature, 7 K; modulation amplitude, 10 G; modulation frequency, 100 kHz.

membrane samples depleted of the 23 and 17 kDa proteins, with or without 10 mM $CaCl_2$, showed no indication of free Mn^{2+} in either the S_1- or the S_2-state (data not shown). Thus, the core and membrane preparations exhibit similar behavior in the S_1-state; the samples without the 23 and 17 kDa proteins display an S_1-state multiline signal, but samples either fully retaining or completely lacking all three extrinsics do not.

DISCUSSION

The results clearly demonstrate that the S_1-state multiline signal originally observed in *Synechocystis* core particles is limited to neither *Synechocystis* nor the solubilization of PSII from the thylakoid membrane. Instead, we have established a direct correlation of the observation of the S_1-state multiline signal to the presence or absence of the extrinsic 33, 23, and 17 kDa proteins. Because we observe an S_2-state multiline signal in all cases, the effect of the extrinsic proteins on the magnetic properties of the Mn cluster is more significant in the S_1-state than the S_2-state. Perhaps the extrinsic proteins affect the zero-field splitting parameters, D and E, which play no role for the $S = {}^1/_2$, $g = 2$ multiline of the S_2-state. We have investigated the temperature dependence of the S_1-state multiline signal in *Synechocystis* over the temperature range 3.6−9 K and the signal amplitude increases with decreasing temperature following Curie-law behavior, indicating that the signal arises from an integer spin ground state or a very low-lying excited state of the Mn cluster (data not shown).

The removal of the 23 and 17 kDa proteins in higher plant

[3] These data indicate that the high chloride concentration used in the urea treatment step to remove the 33 kDa protein does not prevent the observation of the S_1-state multiline signal by damaging the Mn cluster. Additionally, the urea treatment does not damage the Mn cluster as evidenced by the observation of an S_2-state multiline signal of comparable amplitude to the untreated control sample. Therefore, the lack of an S_1-state multiline signal in urea-treated samples is due to the loss of the 33 kDa protein and not to a treatment induced disruption of the Mn cluster.

[4] Some data have indicated that in the absence of the 23 and 17 kDa proteins no S_2-state $g = 4.1$ signal is observed in spinach PSII membrane preparations [de Paula, J. C., Li, M., Miller, A.-F., Wu, B. W., and Brudvig, G. W. (1986) *Biochemistry 25*, 6487−6494]. However, we observe $g = 4.1$ signals in our PSII preparations depleted of the extrinsic proteins, in which the absence of the 23 and 17 kDa proteins was confirmed by SDS−PAGE analysis.

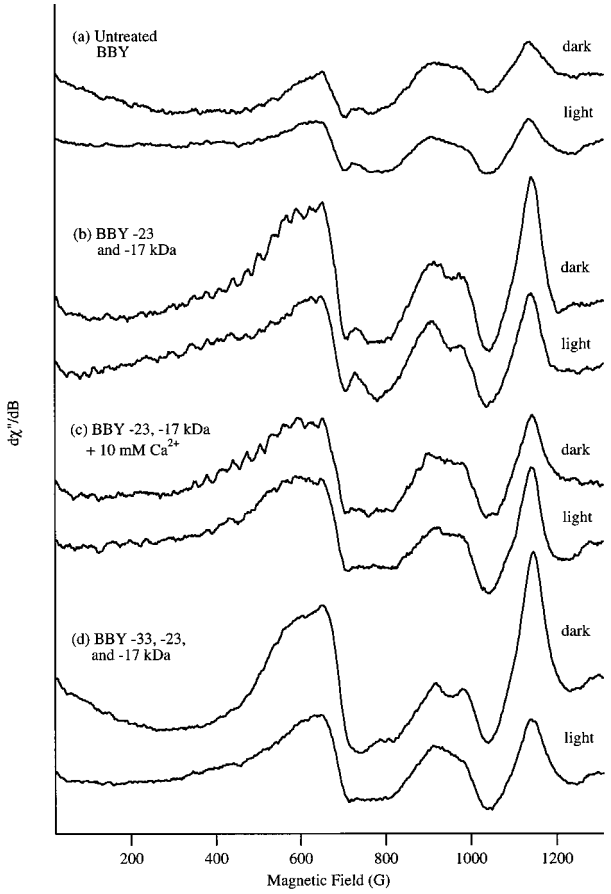

FIGURE 5: "Dark" traces: the parallel polarization EPR spectra of dark adapted PSII "BBY" membranes. "Light" traces: spectra following 195 K illumination (5 min) to induce the $S_1 \rightarrow S_2$ transition. (a) Untreated PSII membranes. (b) NaCl (1 M) washed PSII membranes containing the 33 kDa and lacking the 23 and 17 kDa proteins. (c) NaCl (1 M) washed PSII membranes lacking the 23 and 17 kDa proteins with 10 mM $CaCl_2$. (d) Urea-treated PSII membranes lacking the 33, 23, and 17 kDa proteins. Experimental conditions: parallel mode; microwave frequency, 9.41 GHz; microwave power, 80.3 mW; temperature, 3.6 K; modulation amplitude, 8 G; modulation frequency, 100 kHz.

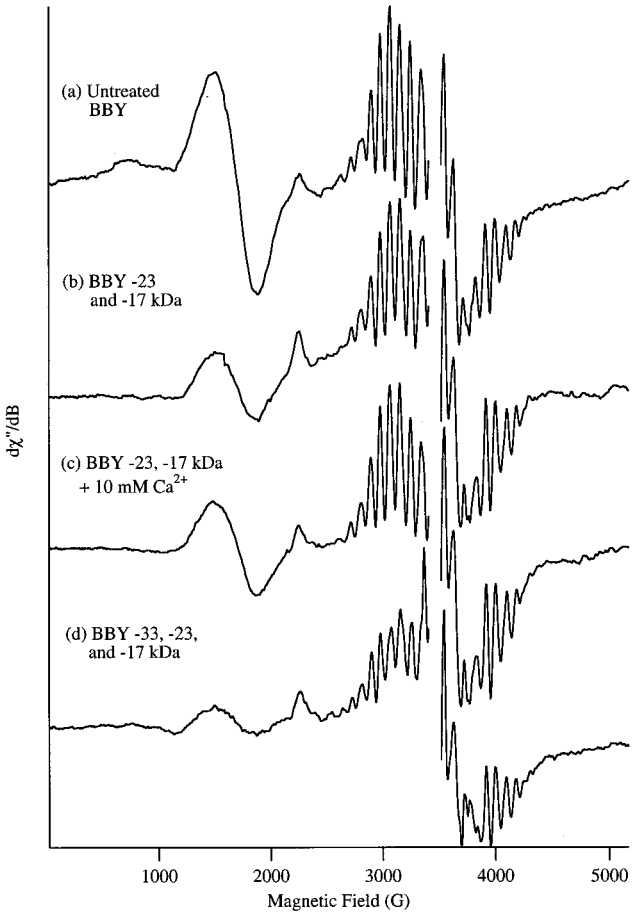

FIGURE 6: Light minus dark perpendicular polarization EPR spectra of PSII "BBY" membranes. (a) Untreated PSII membranes. (b) NaCl (1 M) washed PSII membranes lacking the 23 and 17 kDa proteins. (c) NaCl (1 M) washed BBY PSII particles containing the 33 kDa and lacking the 23 and 17 kDa proteins with 10 mM $CaCl_2$. (d) Urea-treated PSII membranes lacking the 33, 23, and 17 kDa proteins. Experimental conditions: perpendicular mode; microwave frequency, 9.68 GHz; microwave power, 3.2 mW; temperature, 7 K; modulation amplitude, 16 G; modulation frequency, 50 kHz.

PSII preparations leads to a reduction in the rate of the oxygen evolution and introduces a requirement for additional Ca^{2+} and Cl^- in order to recover full oxygen evolution activity *(27, 29, 30)*. Furthermore, the actual depletion of Ca^{2+}, but with retention of the 23 and 17 kDa proteins, prevents the S_2- to S_3-state transition. However, the addition of 10 mM $CaCl_2$ following removal of the 23 and 17 kDa proteins does not result in a change of the S_1-state multiline signal (Figure 5c, "dark"). This observation suggests that it is not simply the loss of Ca^{2+} which leads to the observation of the S_1-state multiline. Similarly, it has previously been observed that S_2-state thermoluminescence properties are abnormal in the absence of the 23 kDa protein, but not in samples only depleted of Ca^{2+} *(38)*. The possibility remains that the loss of the 23 and 17 kDa proteins prevents Ca^{2+} and Cl^- from binding properly in the S_1-state, but the removal of 23 and 17 kDa extrinsic proteins does not alter the mechanistic role of these ions in later S-states. The removal of the 23 and 17 proteins may directly alter the magnetic properties of the Mn cluster, or perhaps alter them by affecting the interaction of Ca^{2+} and/or Cl^- with the Mn cluster in the S_1-state.

The removal of all three extrinsic proteins does not induce the S_1-state multiline. However, the spectra of Figures 4c and 6d show a decrease of hyperfine resolution in the S_2-state multiline signal as evidenced by the minima of the hyperfine peaks to the low-field side of $g = 2$ not reaching the baseline. This loss of resolution could result from the production of a heterogeneous population of Mn clusters. The heterogeneity could lead to a similar loss of resolution in the S_1-state multiline.

The presence of the S_1-state multiline in native, untreated *Synechocystis* core particles suggests that the S_1-state multiline should be thought of as representing an alternative form of the Mn cluster as opposed to a perturbed form. It has been known for quite some time that the S_2-state may exist in at least two different spins states, one represented by the $g = 2$ multiline and the other by the $g = 4.1$ signal. Figure 6 shows that removal of the 23 and 17 kDa proteins does indeed lead to a decrease in the amplitude of the $g = 4.1$ signal. We have performed the S_1 to S_2 transition using 195 K illumination. It is known that illumination at temperatures lower than 195 K tends to favor formation of the $g = 4.1$ signal over the g = 2 multiline signal in the S_2-state, and that annealing a PSII sample in the S_2-state at temperatures

above 195 K leads to the recovery of the normal $g = 2$ multiline signal at the expense of the $g = 4.1$ signal. A full illumination versus temperature study will be needed to determine if there is correlation between the appearance of S_1-state multiline and the partition of the S_2-state between $g = 2$ and $g = 4.1$ forms. Dexheimer et al. performed such a study on the broad $g = 4.8$ form of the S_1-signal *(18)*. They demonstrated that upon 140 K illumination, this S_1-signal did not disappear upon advancement to the S_2-state, even though the $g = 4.1$ signal of the S_2-state was formed in full yield. It will be interesting to observe whether the same result is obtained with the $g = 12$ S_1-state multiline signal (work in progress). This relates to the vital question as to the relationship of the $g = 12$ S_1-state multiline signal to the featureless $g = 4.8$ S_1-signal, which needs further characterization. However, the existence of two spin states in the S_2-state opens the parallel possibility of two spin states existing in the S_1-state.

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REFERENCES

1. Bricker, T. M.; Ghanotakis, D. F. In *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., Eds.) 1996; pp 113−136.
2. Hoganson, C. W., and Babcock, G. T. (1997) *Science 277*, 1953−1956.
3. Kok, B., Forbush, B., and McGloin, M. (1970) *Photochem. Photobiol. 11*, 457−475.
4. Campbell, K. A., Peloquin, J. M., Pham, D. P., Debus, R. J., and Britt, R. D. (1998) *J. Am. Chem. Soc. 120*, 447−448.
5. Goodin, D. B., Yachandra, V. K., Britt, R. D., Sauer, K., and Klein, M. P. (1984) *Biochim. Biophys. Acta 767*, 209−216.
6. Kusunoki, M., Ono, T., Noguchi, T., Inoue, Y., and Oyanagi, H. (1993) *Photosynth. Res. 38*, 331−339.
7. Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
8. Dismukes, G. C., and Siderer, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 274−278.
9. Yachandra, V. K., Sauer, K., and Klein, M.P. (1996) *Chem. Rev. 96*, 2927−2950.
10. Messinger, J., Nugent, J. H. A., and Evans, M. C. W.(1997) *Biochemistry 36*, 11055−11060.
11. Årling, K., Peterson, S., and Styring, S. (1997) *Biochemistry 36*, 13148−13152.
12. Zimmerman, J.-L., and Rutherford, A. W. (1986) *Biochemistry 25*, 4609−4615.
13. Casey, J. L., and Sauer, K. (1984) *Biochim. Biophys. Acta 767*, 21−28.
14. Kim, D. H., Britt, R. D., Klein, M. P., and Sauer, K. (1992) *Biochemistry 31*, 541−547.
15. Hagen, W. R. (1982) *Biochim. Biophys. Acta 708*, 82−98.
16. Dexheimer, S. L., Gohdes, J. W., Chan, M. K., Hagen, K. S., Armstrong, W. H., and Klein, M. P. (1989) *J. Am. Chem. Soc. 111*, 8923−8925.
17. Hendrich, M. P., and Debrunner, P. G. (1989) *Biophys. J. 56*, 489−506.
18. Dexheimer, S. L., and Klein, M. P. (1992) *J. Am. Chem. Soc. 114*, 2821−2826.
19. Yamauchi, T., Mino, H., Matsukawa, T., Kawamori, A., and Ono, T. (1997) *Biochemistry 36*, 7520−7526.
20. Campbell, K. A., and Britt, R. D. Unpublished data.
21. Brudvig, G. W. (1995) In *Mechanistic Bioinorganic Chemistry* (Thorp, H. H., and Pecoraro, V. L., Eds.) pp 250−263, American Chemical Society: Washington DC.
22. Shen, J.-R.; and Inoue, Y. (1993) *Biochemistry 32*, 1825−1832.
23. Hasler, L., Ghanotakis, D., Fedtke, B., Spyridaki, A., Miller, M., Muller, S. A., Engel, A., and Tsiotis, G. (1997) *J. Struct. Biol. 119*, 273−283.
24. Miyao, M., and Murata, N. (1984) *Biochim. Biophys. Acta 765*, 253−257.
25. Kuwabara, T., and Murata, N. (1983) *Plant Cell Physiol. 24*, 741−747.
26. Mavankal, G., McCain, D. C., and Bricker, T. M. (1986) *FEBS Lett. 202*, 235−239.
27. Miyao, M., and Murata, N. (1985) *FEBS Lett. 180*, 303−308.
28. Bricker, T. M. (1992) *Biochemistry 31*, 4623−4628.
29. Miyao, M., and Murata, N. (1984b) *FEBS Lett. 168*, 118−120.
30. Yocum, C. F. (1991) *Biochim. Biophys. Acta 1059*, 1−15.
31. Miyao, M., and Murata, N. (1983) *Biochim. Biophys. Acta 725*, 87−93.
32. Tang, X.-S., and Diner, B. A. (1994) *Biochemistry 33*, 4594−4603.
33. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) *FEBS Lett. 61*, 231−234.
34. Ford, R. C., and Evans, M. C. W. (1983) *FEBS Lett. 160*, 159−164.
35. Ghanotakis, D. F., Demetriou, D. M., and Yocum, C. F. (1987) *Biochim. Biophys. Acta 891*, 15−21.
36. Mishra, R. K., and Ghanotakis, D. F. (1994) *Photosynth. Res. 42*, 37−42.
37. Laemmli, U. K. (1970) *Nature 227*, 680.
38. Ono, T., Izawa, S., and Inoue, Y. (1992) *Biochemistry 31*, 7648−7655.

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