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An Alternative Method for Calcium Depletion of the Oxygen Evolving Complex of Photosystem II As Revealed by the Dark-Stable Multiline EPR Signal[†]

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ABSTRACT: The dark-stable multiline EPR signal of photosystem II (PSII) is associated with a slow-decaying S_2 state that is due to Ca^{2+} loss from the oxygen evolving complex. Formation of the signal was observed in intact PSII in the presence of 100−250 mM NaCl at pH 5.5. Both moderately high NaCl concentration and decreased pH were required for its appearance in intact PSII. It was estimated that only a portion of oxygen evolving complexes was responsible for the signal (about 20% in 250 mM NaCl), based on the loss of the normal S_2-state multiline signal. The formation of the dark-stable multiline signal in intact PSII at pH 5.5 could be reversed by addition of 15 mM Ca^{2+} in the presence of moderately high NaCl, confirming that it was the absence of Ca^{2+} that led to its appearance. Formation of the dark-stable multiline signal in NaCl-washed PSII, which lacks the PsbP (23 kDa) and PsbQ (17 kDa) subunits, was observed in about 80% of the sample in the presence of 150 mM NaCl at pH 5.5, but some signal was also observed under normal buffer conditions. In both intact and NaCl-washed PSII, the $S_2Y_Z\cdot$ signal, which is also characteristic of Ca^{2+} depletion, appeared upon subsequent illumination. Formation of the dark-stable multiline signal took place in the absence of Ca^{2+} chelator or polycarboxylic acids, indicating that the signal did not require their direct binding as has been proposed previously. The conditions used here were milder than those used to produce the signal in previous studies and included a preillumination protocol to maximize the dark-stable S_2 state. Based on these conditions, it is suggested that Ca^{2+} release occurred through protonation of key residues that coordinate Ca^{2+} at low pH, followed by displacement of Ca^{2+} with Na^+ by mass action at the moderately high NaCl concentration.

Photosystem II (PSII)[1] is the light absorbing protein complex that oxidizes water to produce molecular oxygen in higher plants and cyanobacteria (*1*−*3*). Water serves as the electron donor to the electron transfer chain of PSII, which ultimately supports the photosynthetic process. The catalytic center of oxygen production is a Mn_4Ca cluster, which cycles through five oxidation states designated S_0 through S_4. Each step in oxidation of the Mn_4Ca cluster is coordinated with light absorption by the chlorophylls of the PSII reaction center, which provides the energy boost for electron transfer and the formation of oxygen. A closely interacting tyrosine residue known as Y_Z transfers electrons from the Mn_4Ca cluster to the reaction center, while transferring a proton to a base acceptor.

The Ca^{2+} requirement for oxygen evolution (*4*, *5*) was well-known even before its presence at the Mn_4Ca cluster was shown by X-ray diffraction studies (*6*−*8*). The direct interaction of Ca^{2+} with the Mn cluster was indicated in electron paramagnetic resonance studies of Ca^{2+}-depleted PSII, which revealed two characteristic signals. The first one is a multiline signal from an abnormally stable S_2 state, which is produced by treatment of PSII lacking the PsbP and PsbQ subunits (also known as the 23 and 17 kDa subunits) with EDTA or EGTA or by treatment of intact PSII with citrate (*9*−*11*). The signal apparently arises from an $S = {}^1/_2$ spin state and shows 25 or more lines of about 55 G spacing, which is closer than the spacing of the normal S_2-state multiline signal. The signal persists for hours at 0−20 °C in the dark after illumination, hence its designation as the "dark-stable multiline signal". The second signal is a metalloradical signal that is due to the coupling between the S_2 state of the Ca^{2+}-depleted Mn cluster and the Y_Z radical (*12*, *13*). This "inhibited" $S_2Y_Z\cdot$ signal appears as a result of incomplete transition to the S_3 state after illumination at 250 K or above and is observed in samples that show the dark-stable multiline signal (*9*−*11*, *14*, *15*). However, several other treatments that do not produce the dark-stable multiline signal result in the appearance of an inhibited $S_2Y_Z\cdot$ signal. These include incubation with fluoride (*16*−*18*), acetate (*19*, *20*), or ammonia (*15*, *21*). The dark-stable multiline signal, therefore, appears to require special conditions that include the presence of a Ca^{2+} chelator or complexing agent.

Because of the apparent requirement for EDTA/EGTA or citrate, it has been suggested that the dark-stable multiline signal is produced by the direct associate of a polycarboxylic acid chelator with the Ca^{2+}-depleted manganese cluster (*22*). EDTA and EGTA were also proposed to bind directly to the Mn cluster in Ca^{2+}-depleted PSII based on results of an FTIR study in which carboxylate stretching bands for the S_2/S_1 transition were observed only to change after EDTA was added to Chelex 100-treated PSII (*23*). Conversely, it has been suggested that the apparent requirement for a chelator to observe the dark-stable multiline

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[1]Abbreviations: Chl, chlorophyll; EPR, electron paramagnetic resonance; OEC, oxygen evolving complex; PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II.

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signal is explained by the presence of residual Ca^{2+} in medium that lacks a soluble chelator, coupled with the rapid high-affinity binding of the residual Ca^{2+} in the absence of the PsbP subunit (*4*). In this paper, we show that the dark-stable multiline signal can be observed in intact PSII at pH 5.5 in the presence of moderately high NaCl concentrations. In addition, the signal can be observed in NaCl-washed PSII, where its intensity is promoted by conditions of pH 5.5 and moderately high NaCl concentrations. In each case, the signal was produced without the use of Ca^{2+} chelator or other polycarboxylic acids, which have previously complicated the interpretation of its origins. The new method used here to produce the dark-stable multiline signal includes milder conditions than those used previously, leading to a more straightforward interpretation of how Ca^{2+} is removed. These results have implications for understanding the origin of the dark-stable multiline signal and the conditions that lead to the Ca^{2+}-depleted state it is associated with.

EXPERIMENTAL PROCEDURES

Chemicals. Most chemicals were obtained from Fisher Scientific (Fair Lawn, NJ). Itaconic acid was obtained from Sigma Chemical (St. Louis, MO). Phenyl-*p*-benzoquinone was obtained from Aldrich Chemical (Milwaukee, WI) and was purified by recrystallization.

Preparation of PSII Samples. PSII-enriched thylakoid membranes (intact PSII) were prepared from fresh market spinach by extraction with Triton X-100 as described by Yocum and colleagues (*24*) and modified by others (*25*, *26*). No EDTA or other chelator was used during the preparation. Intact PSII was stored in liquid nitrogen in buffer containing 20 mM MES−NaOH, pH 6.3, 0.4 M sucrose, and 15 mM NaCl. The use of the term "intact PSII" refers to the preparation before sample treatment, since some loss of the extrinsic PsbP (23 kDa) and PsbQ (17 kDa) subunits took place as a result of elevated NaCl concentrations.

NaCl-washed PSII, for removal of the PsbP (23 kDa) and PsbQ (17 kDa) subunits, was prepared essentially as described previously (*27*). Intact PSII was incubated in buffer containing 1.5 M NaCl in 20 mM MES−NaOH, pH 6.3, and 0.40 M sucrose for 30 min on ice in the dark. After centrifugation for 10 min at 17400*g* in a Beckman Avanti J-25 high-speed centrifuge, the pellet was washed by centrifugation twice in the same buffer containing 15 mM NaCl for EPR experiments and three times in buffer without NaCl for O_2 evolution experiments.

O_2 evolution assays were measured at 25 °C using a Clark-type O_2 electrode (Yellow Springs Instruments, model 5331) using phenyl-*p*-benzoquinone (PPBQ) as electron acceptor as described previously (*28*), except that the light intensity was decreased to below saturation to avoid negative effects of excessive light. A Dolan-Jenner Fiberlite 180 illuminator was used at a setting that resulted in 70−75% the O_2 evolution activity observed under saturating light. Assays were carried out in 20 mM MES−NaOH, at pH 5.5 or 6.3, 0.4 M sucrose, and the indicated amounts of NaCl and/or $CaCl_2$. The O_2 evolution rates under control conditions (including saturating light) were 500−600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} for intact PSII and 250−350 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} for NaCl-washed PSII. Values represent the average of three measurement; errors were taken to be either 5% or the standard deviation of the average, whichever was larger.

PSII Sample Treatment. To remove Ca^{2+} from PSII by the Ca^{2+} chelator method, NaCl-washed PSII was treated with EDTA by suspending in 20 mM MES−NaOH, pH 6.3, 0.40 M sucrose, and 2 mM EDTA and incubating on ice for 30 min in the dark. After centrifuging as above, the pellet was washed by centrifugation in 20 mM MES−NaOH, pH 6.3, 0.40 M sucrose, 25 mM NaCl, and 0.1 mM EDTA. The PSII pellet was then resuspended in a small amount of the same buffer.

Preparation of PSII at pH 5.5/moderate NaCl concentration was carried out by suspending intact PSII or NaCl-washed PSII in buffer containing 20 mM MES−NaOH or itaconic acid−NaOH, pH 5.5, 0.40 M sucrose, and the indicated concentration of NaCl (75−250 mM NaCl). After incubation on ice for about 15 min in the dark, the PSII was pelleted by centrifugation at 24000−26000*g* in a Beckman Avanti J-25 high-speed centrifuge. The PSII pellet was then resuspended in a small amount of the same buffer. Control samples at pH 6.3 and/or 15−25 mM NaCl were prepared simultaneously. At no point were the samples exposed to EDTA or other chelator.

For the samples shown in Figure 3, intact PSII was first prepared in 20 mM MES−NaOH, pH 5.5, and 0.40 M sucrose, with 25 mM NaCl (trace b) or 250 mM NaCl (traces a, c, and d) as above. After centrifuging, samples were resuspended in 20 mM MES−NaOH, pH 5.5, and 0.40 M sucrose with 250 mM NaCl (trace a), 25 mM NaCl (traces b and c), or 220 mM NaCl plus 15 mM $CaCl_2$ (trace d) and centrifuged again. Each PSII pellet was resuspended in a small amount of the same buffer.

SDS−PAGE. SDS−polyacrylamide gel electrophoresis was carried out essentially as described by Kashino and co-workers (*29*), with the exception of the sample preparation. Gels contained 13% acrylamide/bisacrylamide and 3 M urea. PSII samples were prepared by diluting 1:1 or more with denaturing buffer (0.1 M Tris/3% SDS/20% glycerol/2% β-mercaptoethanol/3 M urea/0.01% bromophenol blue) and incubating at 38 °C for 1 h. Each lane was loaded with 5 μg of chlorophyll sample. Electrophoresis was carried out using an EC120 minigel system (E-C Apparatus Corp.). Analysis of protein band intensities was carried out with Gel-Pro Analyzer (Media Cybernetics), using the band at ~28 kDa to normalize bands for comparison between lanes.

EPR Spectroscopy. For EPR sample preparation, PSII samples were resuspended to 8−11 mg of Chl mL^{-1} and transferred to 4 mm outer diameter clear fused quartz EPR tubes (Wilmad Glass, Buena, NJ). Except where otherwise noted, samples contained 2 mM PPBQ, which was added from a 50 mM stock solution in dimethyl sulfoxide either before or after the preillumination/dark-adaptation protocol described below. Samples were dark adapted on ice for 1−3 h before freezing in liquid nitrogen for storage.

The following illumination protocol was used for production of the EPR signals observed in this study. Just before EPR spectroscopy was carried out, samples were thawed quickly in room temperature water for a few seconds and then illuminated for 5−30 s at room temperature. Samples were then dark adapted on ice for about 1 h before refreezing, after which the spectrum of the dark-adapted state was taken. Next, samples were illuminated at 195 K in a dry ice/ethanol bath for 4 min, which induced the normal S_2 state in uninhibited centers. Finally, samples were brought to 273 K by transferring first to a dry ice/ethanol bath and then to an ice water bath and illuminated for 30 s at 273 K in the ice water bath, which induced the $S_2Y_Z^{\cdot}$ state in inhibited centers. In each case illumination was carried out with a 300 W halogen lamp using a projector to direct the light beam to a patch of approximately 2 cm^2, after passing through 6 cm of 5 mM $CuSO_4$ solution in a cylindrical glass container.

X-band EPR spectroscopy was carried out using a Bruker Instruments EMX 10/12 EPR spectrometer equipped with an

ER4116DM dual mode cavity (9.65 GHz). Temperature was controlled with an Oxford Instruments ESR 900 liquid He cryostat. Signals were observed at 10.0 K using a microwave power of 20 mW, modulation frequency of 100 kHz, and modulation amplitude of 17 G.

Signal heights of the two multiline signals were found by averaging the peak-to-trough heights of several peaks that did not overlap with interfering background signals. For the normal multiline signal, these included three peaks downfield from the center (at about 2960, 3050, and 3140 G). For the dark-stable multiline signal, these included three peaks downfield (at about 3000, 3055, and 3120 G) and two peaks upfield from the center (3760 and 3830 G). For the data in Figure 5, an estimate of the centers leading to the dark-stable and normal multiline signals within each sample was based on the relative signal heights, assuming that the two signals accounted for 100% of the S_2-state population of each sample. This was done using the simple equation, $c_aH_a + c_bH_b = 100$, where H_a and H_b are signal heights and c_a and c_b are coefficients introduced to account for the different transition probabilities of the signals. Because no sample contained either signal exclusively, c_a and c_b were estimated by combining the height data from two of the samples, chosen to be pH 5.5/150 mM NaCl and pH 6.3/15 mM NaCl.

RESULTS

Appearance of the Dark-Stable Multiline Signal in Intact PSII. Intact PSII samples were prepared at pH 5.5 in the presence of 100−250 mM NaCl and dark adapted after a brief preillumination at room temperature. These samples showed a dark-stable multiline signal (Figure 1). The signal was observed to form in buffer prepared with MES (Figure 1, traces b and c), a morpholino sulfonic acid with a pK_a of 6.1. The working pH of 5.5 was chosen because it is the lowest pH at which MES can be used reliably; other morpholino sulfonic acid buffers with lower pK_as are not generally available. The signal was also observed from PSII in buffer prepared with itaconic acid (Figure 1, traces d and e), a dicarboxylic acid with pK_as of 3.85 and 5.45, which can be easily adjusted to pH 5.5. Comparison of the signals observed in MES and itaconic acid shows that the type of buffer, in particular the presence or absence of carboxylate groups, had no observable effect on the characteristics of the signal. Two spectra for each buffer system are included to show reproducibility. The presence of PPBQ also did not appear to affect the signal. The signals were somewhat weak because the PSII centers were not all converted to the form showing the dark-stable multiline signal, as will be shown below.

The dark-stable multiline signals observed at pH 5.5 and moderately high NaCl concentration were very similar to that observed in NaCl-washed PSII, which lacks the PsbP (23 kDa) and PsbQ (17 kDa) extrinsic subunits, in the presence of EDTA (Figure 1, trace a). This treatment results in Ca^{2+} depletion of the oxygen evolving complex. Some differences in the higher field hyperfine lines were observed for the signals from PSII at pH 5.5/moderate NaCl compared with those from NaCl-washed PSII treated with EDTA. The signal intensity of the EDTA-treated sample was much stronger because the PSII centers were fully converted to the Ca^{2+}-depleted form. This was evident because no normal S_2-state multiline or $g = 4.1$ signal was observed after illumination at 195 K (data not shown).

Using MES as the buffer, it was found that both pH 5.5 and moderately high NaCl concentration were required to observe the dark-stable multiline signal in intact PSII (Figure 2A).

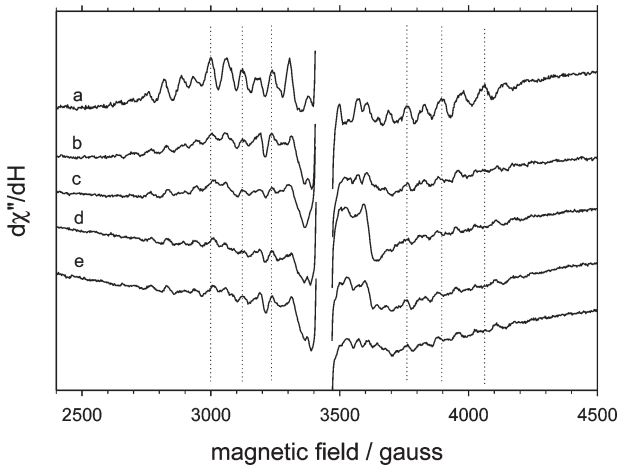

FIGURE 1: Formation of the dark-stable multiline EPR signal in PSII at pH 5.5 and moderate NaCl concentration. Spectra shown are from the preilluminated, dark-adapted states for (a) PSII lacking the PsbP and PsbQ subunits at pH 6.3 in the presence of 0.1 mM EDTA, (b, c) intact PSII at pH 5.5 in MES buffer plus 250 or 150 mM NaCl, respectively, and (d, e) intact PSII at pH 5.5 in itaconic acid buffer plus 100 mM NaCl. All samples except (d) also contained 2 mM PPBQ. EPR conditions included 20 mW microwave power, 20 G modulation amplitude, and temperature of 10 K. Dashed lines are included to facilitate comparison of line positions.

In particular, the signal was observed neither in PSII prepared at pH 5.5 with 15 mM NaCl nor in PSII prepared at pH 6.3 with 150 mM NaCl (Figure 2A, traces b and d). The normal S_2-state EPR signals, produced by illumination at 195 K, were examined in the same samples (Figure 2B). It was found that in the sample at pH 5.5/150 mM NaCl the normal multiline signal reached about 59% of its intensity in the control sample at pH 6.3/15 mM NaCl. A decrease in pH to 5.5 alone resulted in 86% of the control signal, while 150 mM NaCl alone led to 75% of the control signal. Thus at pH 5.5/150 mM NaCl, about 30% of the normal multiline signal was lost compared with the sample at pH 5.5/15 mM NaCl and about 40% was lost compared with the control at pH 6.3/15 mM NaCl. This suggests that formation of the dark-stable multiline signal was correlated with loss of the normal multiline signal. The $g = 4.1$ signal decreased to 81% of the control height at pH 5.5/150 mM NaCl and to 78% of the control height at pH 5.5/15 mM NaCl. Moderately high NaCl concentration alone did not appear to affect the $g = 4.1$ signal intensity (97%). This indicates that, unlike the normal multiline signal, the $g = 4.1$ signal was not significantly reduced by the increase in NaCl concentration and suggests that formation of the dark-stable multiline signal was not correlated with loss of the $g = 4.1$ signal. The line width of the $g = 4.1$ signal was not significantly affected by pH 5.5 and/or moderate NaCl concentration. The error in the signal height measurements can be taken to be about 10%.

The formation of the $S_2Y_Z^{\cdot}$ signal, produced by illumination at 0 °C in the presence of an electron acceptor, was examined in the same samples as are shown in Figure 2A,B, since this signal is also associated with Ca^{2+} depletion of the OEC. The $S_2Y_Z^{\cdot}$ signal was observed in the sample prepared at pH 5.5 in the presence of 150 mM NaCl and showed a line width of about 200 G (Figure 2C). Because a large portion of the sample was not Ca^{2+} depleted, the spectra are presented after subtraction of 65% of the S_2-state spectrum to reduce interference from the normal S_2-state multiline signal. The PSII sample at pH 6.3/150 mM NaCl appeared to show a small amount of the $S_2Y_Z^{\cdot}$ signal, but the two 15 mM NaCl samples did not.

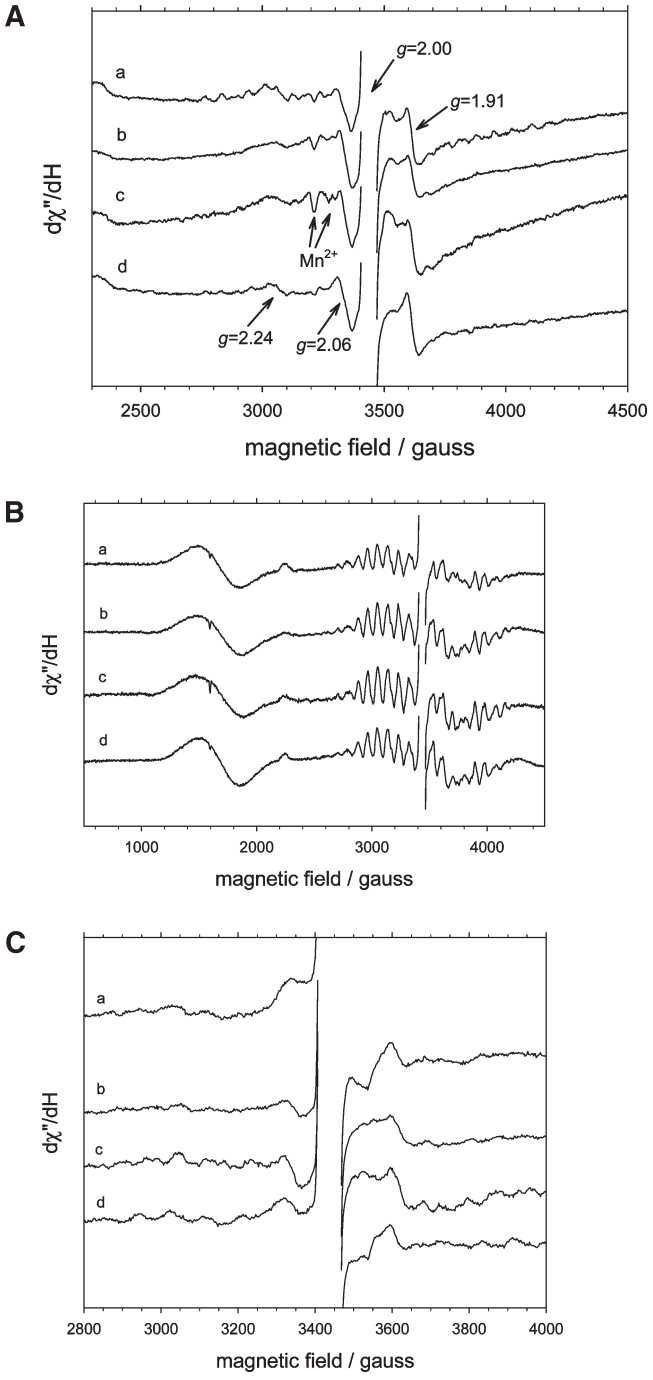

FIGURE 2: Effect of pH 5.5 and moderate NaCl concentration on EPR signals in intact PSII: (A) spectra of the preilluminated, dark-adapted state; (B) difference spectra of the 195 K illuminated minus dark-adapted state; (C) difference spectra of the 273 K illuminated minus 65% of the 195 K illuminated state. The three panels show signals from the same four samples, which were prepared using MES buffer: (a) pH 5.5/150 mM NaCl; (b) pH 5.5/15 mM NaCl; (c) pH 6.3/15 mM NaCl; (d) pH 6.3/150 mM NaCl. Trace a is the same as trace c in Figure 1. EPR conditions were as in Figure 1. Several signals commonly observed in the dark-adapted state are indicated in panel A, including Y_D (and other radicals) at $g = 2.00$, Rieske center at $g = 1.91$, g_y of cyt b_{559} at $g = 2.24$, Mn^{2+} features, and an unknown Cu^{2+} or Fe^{3+} signal at $g = 2.06$.

Reversal of Formation of the Dark-Stable Multiline Signal in Intact PSII. Since the formation of the dark-stable multiline signal at pH 5.5 plus moderate NaCl concentration was suspected to arise from loss of calcium at the OEC, the effect of adding Ca^{2+} under these conditions was tested (Figure 3). Intact PSII was

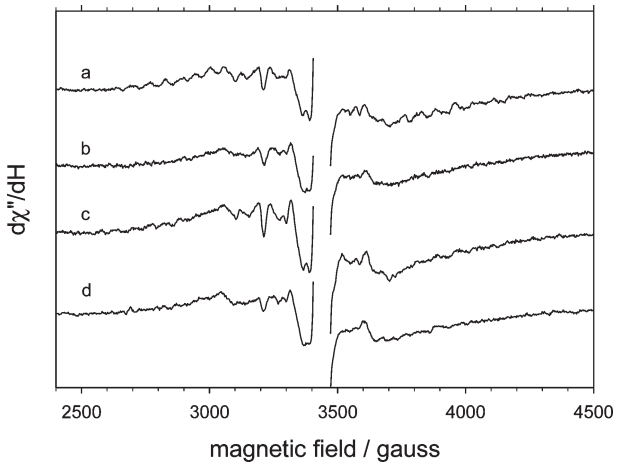

FIGURE 3: Reversal of formation of the dark-stable multiline signal in intact PSII. PSII samples were kept at pH 5.5 (MES buffer) throughout the experiment and treated with (a) 250 mM NaCl, (b) 25 mM NaCl, (c) 250 mM NaCl and then transferred to 25 mM NaCl, and (d) 250 mM NaCl and then transferred to 220 mM NaCl plus 15 mM $CaCl_2$. The preilluminated, dark-adapted state is shown. Trace a is the same spectrum as that shown in Figure 1, trace b. EPR conditions were as in Figure 1.

treated with 250 mM NaCl at pH 5.5 to produce the dark-stable multiline signal (trace a); no dark-stable multiline signal was observed in the control, which was kept at 25 mM NaCl (trace b). Another intact PSII sample treated at pH 5.5/250 mM NaCl was subsequently transferred to a buffer at the same pH with 15 mM $CaCl_2$ and 220 mM NaCl (trace d). No dark-stable multiline signal was observed in the presence of 15 mM Ca^{2+}, although the NaCl concentration remained moderately high. Since Ca^{2+} was able to reverse the formation of the dark-stable multiline signal, this confirmed that the signal was due to the loss of Ca^{2+}. A third intact PSII sample treated at pH 5.5/250 mM NaCl was transferred to buffer containing 25 mM NaCl. This sample also showed little or no dark-stable multiline signal, indicating that moderately high NaCl concentration was required to maintain the signal even though Ca^{2+} had presumably already been removed from the OEC. This is probably because once the NaCl concentration was lowered, the trace amount of Ca^{2+} present in the buffer was sufficient to allow rebinding of Ca^{2+}. It should be noted that no special Ca^{2+} removal procedures were used for glassware or buffer preparation.

As for the intact PSII samples studied in Figure 2, those of Figure 3 showed an inverse relationship between the dark-stable multiline signal and the normal multiline signal (spectra not shown). After illumination at 195 K, the sample kept at 250 mM NaCl showed about 57% of the normal multiline signal compared with the control that was kept at 25 mM NaCl, while the two samples that lost the dark-stable multiline signal due to the addition of Ca^{2+} or due to return to low NaCl showed about normal multiline signal intensity (96% and 92%, respectively). The $g = 4.1$ signal showed a loss of 5% or less due to the moderately high NaCl conditions. When the sample kept at pH 5.5/250 mM NaCl was illuminated at 273 K in the presence of an electron acceptor, an $S_2Y_Z\cdot$ signal with line width of about 160 G was observed (not shown).

Extrinsic Subunit Loss in Intact PSII. The intact PSII samples treated at pH 5.5 and moderately high NaCl from Figures 2 and 3 were inspected for loss of the PsbP and PsbQ (23 and 17 kDa) extrinsic subunits using SDS−PAGE (Figure 4).

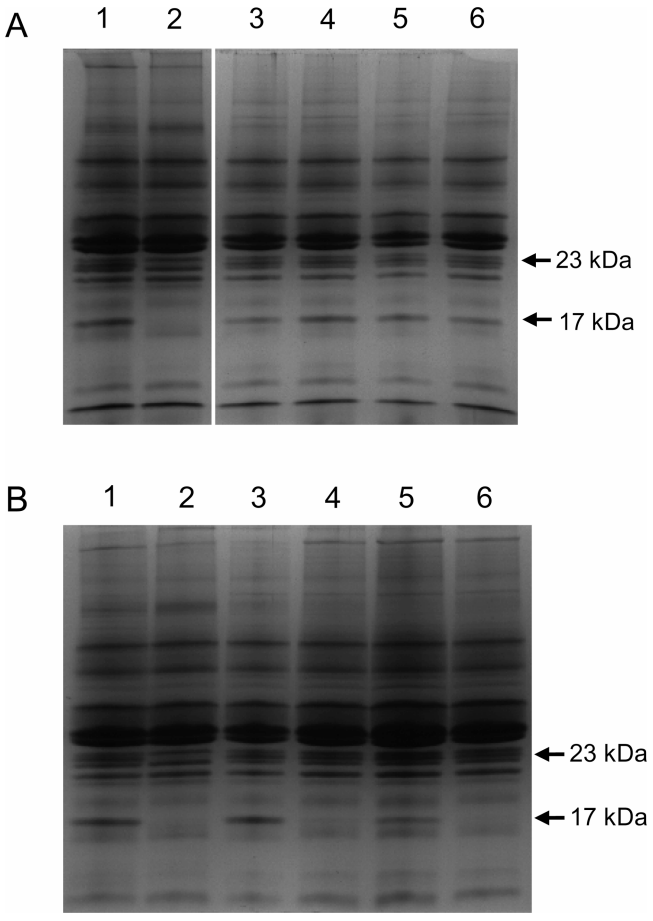

FIGURE 4: SDS−PAGE of PSII in the presence of pH 5.5 buffer and/or moderate NaCl concentration. Each panel shows for comparison (1) intact PSII and (2) PSII lacking PsbP and PsbQ (1.5 M NaCl-washed). (A) Intact PSII in (3) pH 5.5/150 mM NaCl, (4) pH 5.5/15 mM NaCl, (5) pH 6.3/15 mM NaCl, and (6) pH 6.3/150 mM NaCl; samples were the same as those in Figure 2. (B) Intact PSII at pH 5.5 in (3) 25 mM NaCl, (4) 250 mM NaCl, (5) 250 mM NaCl and then transferred to 25 mM NaCl, and (6) 250 mM NaCl and then transferred to 220 mM NaCl plus 15 mM $CaCl_2$; samples were the same as those in Figure 3. The vertical gap in (A) represents a lane that was deleted. The PsbP (23 kDa) and PsbQ (17 kDa) subunits are indicated by arrows.

In each panel, intact PSII (lane 1) is compared with PSII treated with 1.5 M NaCl (lane 2), showing dissociation of the PsbP and PsbQ subunits. Loss of the PsbP subunit in particular is known to result in a requirement for Ca^{2+} for full activity (*30*, *31*). Given the moderately high NaCl concentrations (100−250 mM) used here to produce the dark-stable multiline signal, some loss of the PsbP subunit was considered to be a possibility. In Figure 4A, comparison of the sample treated at pH 5.5 and 150 mM NaCl (lane 3) with the control sample at pH 6.3/15 mM NaCl (lane 5) revealed some loss of the PsbQ (17 kDa) subunit but little or no loss of the PsbP (23 kDa) subunit. Quantification of the protein band intensities indicated 35−50% loss of the PsbQ subunit in the two samples treated with 150 mM NaCl at pH 5.5 and 6.3 (lanes 3 and 6) but no loss due to pH 5.5 treatment alone (lane 4). No loss of the PsbP subunit relative to the pH 6.3/15 mM NaCl control was observed in the other three samples. In Figure 4B, comparison of the sample treated at pH 5.5 and 250 mM NaCl (lane 4) with the control sample at pH 5.5/25 mM NaCl (lane 3) revealed complete loss of the PsbQ subunit and slight loss of the PsbP subunit. Quantification indicated no PsbQ subunit remained bound to the sample at pH 5.5/250 mM NaCl (lane 4) or to the sample that had been transferred into 15 mM $CaCl_2$ plus 220 mM NaCl (lane 6), while 35−45% of the PsbQ subunit evidently rebound to the sample that had been returned to 25 mM NaCl after treatment with 250 mM NaCl (lane 5). Treatment with 250 mM NaCl led to a loss of up to 10% of the PsbP subunit.

Appearance of the Dark-Stable Multiline Signal in NaCl-Washed PSII. The effect of moderately high NaCl concentration at pH 5.5 was tested in PSII from which the PsbP and PsbQ subunits had been removed by washing with 1.5 M NaCl. At pH 5.5 and 150 mM NaCl, after preillumination followed by dark adaptation, NaCl-washed PSII showed a strong dark-stable multiline signal (Figure 5A, trace a). Even at pH 6.3 in the presence of 15 mM NaCl, NaCl-washed PSII showed a noticeable dark-stable multiline signal (trace d). Samples at pH 5.5 in the presence of 75 and 15 mM NaCl were also observed to show dark-stable multiline signals with intermediate intensities (traces b and c). The normal S_2-state multiline signal was induced in the same samples by illumination at 195 K (not shown), resulting in signals that showed a pattern of relative intensities that was inversely related to that of the dark-stable multiline signal, as expected if the signals are mutually exclusive. None of the four samples showed a significant $g = 4.1$ signal. Assuming that the dark-stable multiline and the normal multiline signals accounted for 100% of the S_2 state in each sample and were mutually exclusive, it was possible to estimate the percentage of the sample that contributed to each signal. In this way it was found that the percentage of sample showing the dark-stable multiline signal was 75−85% of the PSII sample at pH 5.5/150 mM NaCl, 55−65% at pH 5.5/75 mM NaCl, 40−50% at pH 5.5/15 mM NaCl, and 25−35% at pH 6.3/15 mM NaCl.

The $S_2Y_Z\cdot$ signals were also observed in NaCl-washed PSII after illumination at 273 K (Figure 5B). All four of the samples showed a prominent $S_2Y_Z\cdot$ signal, with no apparent trend in intensity with sample treatment. The line widths of the signals were observed to be in the range of 120−125 G, with a possible narrower component for some of the signals. When the dark-stable multiline signal background was removed by subtracting the equivalent of 30% of the spectrum of the dark-adapted sample, the shape of the $S_2Y_Z\cdot$ signal became more clear (Figure 5C). This removed shoulders and the apparent narrower component, leaving a signal that was 120 G from peak to trough.

O_2 Evolution Activity. The effects of treatment at pH 5.5 and 150 mM NaCl on O_2 evolution activity were examined in intact PSII and in PSII from which PsbP and PsbQ had been removed by NaCl washing (Figure 6), using subsaturating light intensity. For these experiments, the control rates at pH 6.3 were measured using 15 mM NaCl for intact PSII and 7.5 mM $CaCl_2$ for NaCl-washed PSII. During treatments, samples were kept in the dark or under dim green light until the O_2 evolution assay was carried out. In intact PSII, a decrease in pH to 5.5 at low NaCl concentrations (15−25 mM) resulted in a decrease in the O_2 evolution activity to 65% compared to the pH 6.3 control. This is consistent with previous studies on the pH dependence of O_2 evolution activity (*32*, *33*). In NaCl-washed PSII, a similar decrease in activity to 73% was observed at pH 5.5 when 7.5 mM $CaCl_2$ was present. Intact PSII at pH 5.5 showed essentially the same activity in the presence of 7.5 mM $CaCl_2$ as in 15 mM NaCl, indicating a lack of Ca^{2+} dependence, whereas NaCl-washed PSII showed a drop in activity to 14% in the absence of Ca^{2+}, showing a strong Ca^{2+} dependence as is already well-known for this preparation. In 150 mM NaCl at pH 5.5, intact PSII showed a further decrease in activity to 42% of the control; however,

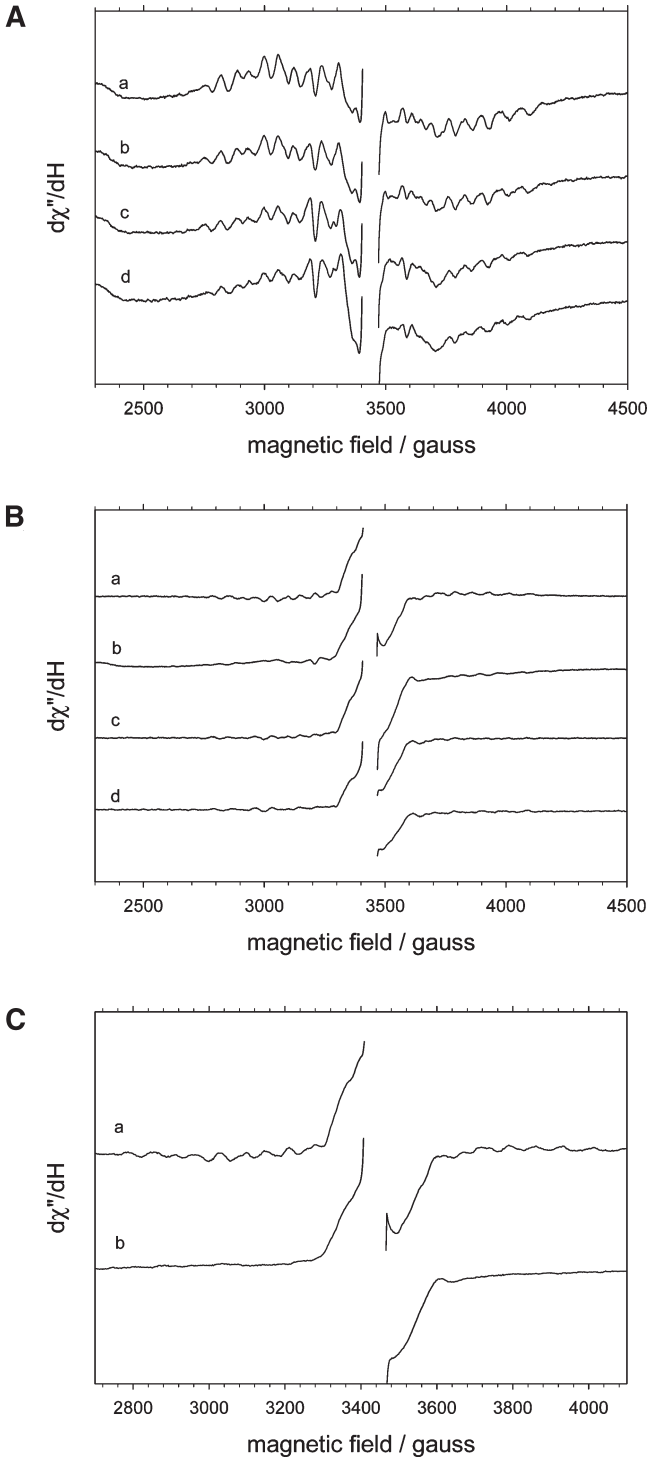

FIGURE 5: Effect of pH 5.5 and moderate NaCl concentration on EPR signals in NaCl-washed PSII lacking the PsbP and PsbQ subunits: (A) spectra of the preilluminated, dark-adapted state and (B) difference spectra of the 273 K illuminated minus dark-adapted state. In (A) and (B) signals are shown from NaCl-washed PSII in (a) pH 5.5/150 mM NaCl, (b) pH 5.5/75 mM NaCl, (c) pH 5.5/15 mM NaCl, and (d) pH 6.3/15 mM NaCl. Panel C shows removal of background dark-stable multiline signal from the $S_2Y_Z^{\cdot}$ signal for the sample in pH 5.5/150 mM NaCl: (a) same spectrum as trace a, panel B; (b) difference spectrum of the 273 K illuminated minus 30% of the dark-adapted state. Samples were prepared using MES buffer. EPR conditions were as in Figure 1.

addition of Ca^{2+} as 10 mM $CaCl_2$ with 130 mM NaCl was unable to promote any further activity. NaCl-washed PSII showed a strong Ca^{2+} dependence at the higher NaCl concentration, with

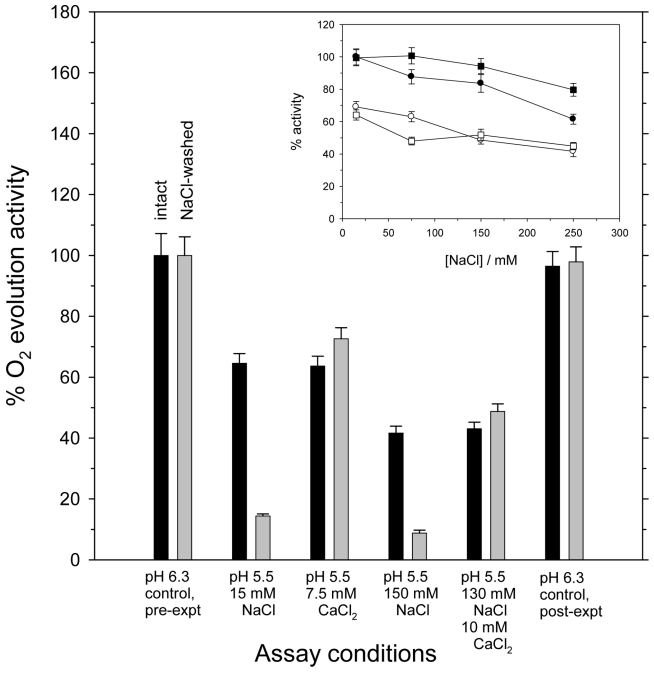

FIGURE 6: Suppression of O_2 evolution activity of intact PSII (black) and NaCl-washed PSII (gray) after pH 5.5/150 mM NaCl treatment. For the control assays ("pH 6.3 control, pre-expt"), intact and NaCl-washed PSII preparations were used without further treatment. For all other assays, intact PSII and NaCl-washed PSII was prepared in MES buffer at pH 5.5 with 150 mM NaCl, and assays were carried out in the buffer indicated. The control buffer at pH 6.3 contained 15 mM NaCl for intact PSII or 7.5 mM $CaCl_2$ for NaCl-washed PSII. All rates are given as percentages of the control rate measured before treatment. For the intact PSII after treatment ("pH 6.3 control, post-expt"), the buffer also contained 1 mM EDTA. Inset: Effect of increasing NaCl concentration on O_2 evolution activity of intact PSII at pH 5.5 and 6.3. Intact PSII was prepared prior to the assay by suspending in MES buffer at pH 5.5 (open symbols) or pH 6.3 (closed symbols) with the indicated concentration of NaCl. Measurements were carried out either without added Ca^{2+} (circles) or in the presence of 11 mM $CaCl_2$ (squares), which was added to the assay cell in addition to the given NaCl concentration. Rates are relative to the control rate at pH 6.3, 15 mM NaCl.

49% activity in the presence of 10 mM $CaCl_2$ and 130 mM NaCl, up from 9% in 150 mM NaCl alone. (It may be noted that the Ca^{2+} dependence of this NaCl-washed preparation was particularly high for such preparations, probably because of the successive washes in the absence of added Ca^{2+} or Cl^-.) For both preparations, the decrease in activity upon transferring to pH 5.5 and 150 mM NaCl took place within minutes (the time frame of the experiment), and the activity was fairly stable over the next hour or more. Both preparations showed restoration of approximately full activity when the sample treated at pH 5.5/150 mM NaCl was assayed in control buffer at pH 6.3, even after 2 h or more of treatment. It is particularly noteworthy that the treated intact PSII sample showed 96% activity in buffer that contained 15 mM NaCl and 1 mM EDTA, indicating that binding of Ca^{2+} from the medium was not responsible for the restoration of activity.

In a separate experiment, the activity of intact PSII at pH 5.5 was found to decrease as the NaCl concentration increased, from 69% at 15 mM NaCl to 41% at 250 mM NaCl relative to the control rate at pH 6.3 (Figure 6, inset). The relative decrease in activity due to increased NaCl concentration was greater at pH 5.5 than at pH 6.3. In this experiment as well as others, an increase from 15 to 150 mM NaCl resulted in a decrease in activity of 30−40% at pH 5.5 compared with 10−15% at pH 6.3. The loss in

activity at pH 6.3 may be partially accounted for by a partial loss of the PsbP subunit at higher NaCl concentrations, which is expected to lead to a dependence on Ca^{2+}. This can be seen in the partial recovery of activity upon the addition of $CaCl_2$ at various NaCl concentrations (solid squares vs circles), which accounted for about 18% activity at 250 mM NaCl. As found above, addition of Ca^{2+} at pH 5.5 did not lead to recovery of activity. There was no significant difference in the activity measured at pH 5.5 for the absence versus the presence of $CaCl_2$ (open squares vs circles) at various NaCl concentrations except for a possible decrease in activity at 75 mM NaCl in the presence of Ca^{2+}.

The suppression of O_2 evolution activity at pH 5.5/moderate NaCl was generally greater than the suppression of the normal multiline signal under similar conditions. For example, at pH 5.5/150 mM NaCl the activity was around 40% of the pH 6.3 control, while around 60% of the normal multiline signal was observed.

DISCUSSION

In this study, we have shown that the dark-stable multiline EPR signal was produced in PSII by treatment with moderately high concentrations of NaCl (100−250 mM) at the mildly acidic pH of 5.5 (Figures 1−3). In intact PSII, this treatment produced a somewhat weak signal, and both a decrease in pH and an increase in NaCl concentration together were required to produce an observable signal. In NaCl-washed PSII lacking the PsbP (23 kDa) and PsbQ (17 kDa) subunits, the dark-stable multiline signal produced by this treatment was much stronger, representing about 80% of the sample (Figure 5). In this preparation, this signal appeared in weaker form at lower NaCl concentrations and even at pH 6.3. It is more difficult to estimate the proportion of centers responsible for the dark-stable multiline signal in intact PSII than in NaCl-washed PSII. Although it is reasonable to assume that the dark-stable multiline signal arises from PSII centers that do not produce the normal S_2 state, correlating the appearance of the dark-stable multiline signal with the loss of the normal multiline signal is complicated by the presence of the $g = 4.1$ signal. Loss of the normal S_2-state multiline signal at pH 5.5 was about 30% in the presence of 150 mM NaCl and 40% in the presence of 250 mM NaCl, compared with conditions of low NaCl at the same pH. However, under the same conditions there was essentially no loss of the normal S_2-state $g = 4.1$ signal, which is generally acknowledged to arise from different PSII centers than the multiline signal. It is difficult to estimate accurately the percentage of the PSII centers that leads to the two normal S_2-state signals, but the spectra suggest that no more than half of the normal S_2 state was represented by the $g = 4.1$ signal. This suggests that at pH 5.5 the dark-stable multiline signal represents about 15% of the sample in 150 mM NaCl and 20% in 250 mM NaCl, although possibly more.

The presence of the dark-stable multiline signal indicates that Ca^{2+} was removed or disrupted from its functional site at the Mn cluster. This interpretation was supported by the appearance of the inhibited $S_2Y_Z^{\cdot}$ signal in the same samples after illumination at 273 K in the presence of an acceptor. The conclusion that Ca^{2+} was lost from its functional site was also supported by the observation that in intact PSII the dark-stable multiline signal did not appear in the presence of Ca^{2+} at moderately high NaCl concentration. Interestingly, the signal was also not observed when intact PSII was returned to lower NaCl concentrations, probably due to the binding of Ca^{2+} loosely associated with PSII or from trace amounts in the buffer. This indicates that the presence of a moderately high concentration of NaCl was necessary to maintain the Ca^{2+}-depleted or -disrupted S_2 state in intact PSII.

In the experiments presented here the dark-stable multiline signal was observed in PSII prepared in the absence of a Ca^{2+} chelator or other carboxylate-containing compound. The pH of 5.5 was chosen for the experiments since it is the lowest pH at which MES, which does not contain carboxyl groups, is usable as a buffer. This pH also gave us the opportunity to test whether the buffering species was important. Itaconic acid, a dicarboxylic acid with a pK_a near 5.5, was used as the buffer for comparison with MES. No significant difference in the character or amount of dark-stable multiline signal was observed when MES or itaconic acid was used as the buffering agent, further indicating that a chelator or polycarboxylic acid was not important for its formation.

In previous studies, the dark-stable multiline signal was produced by two major methods of Ca^{2+} depletion: treatment with EDTA or EGTA after removal of the PsbP and PsbQ subunits (sometimes with subsequent rebinding of the subunits) (*9*, *11*, *22*) and treatment of intact PSII with citrate at pH 3.0 (*10*, *22*), which leaves the extrinsic subunits in place. Both treatments involve the use of a polycarboxylic acid that can chelate or complex Ca^{2+}. These characteristics of dark-stable multiline signal formation combined with FTIR studies of Ca^{2+}-depleted PSII have supported the suggestion that direct binding of polycarboxylic acids such as EDTA and EGTA to the manganese cluster in Ca^{2+}-depleted PSII are responsible for the dark-stable multiline signal (*22*, *23*). The studies presented here show that the binding of polycarboxylic acid is not necessary for formation of the dark-stable multiline signal. Rather, the apparent requirement for a chelator or polycarboxylic acid is probably a result of the very high affinity of the OEC for Ca^{2+}, which is present in trace amounts in the absence of strong chelators such as EDTA or EGTA, as suggested by Yocum and co-workers (*4*).

The requirement for a moderately high NaCl concentration raised the question of whether the PsbP (23 kDa) and PsbQ (17 kDa) subunits were removed from intact PSII, thereby facilitating the loss of Ca^{2+} from the Mn_4Ca cluster. It was found that at pH 5.5 some PsbQ subunit (35−50%) was lost due to the presence of 150 mM NaCl, and essentially all PsbQ subunit was lost due to 250 mM NaCl (Figure 4). The PsbP subunit, which is associated with a requirement for Ca^{2+}, was not noticeably lost at 150 mM NaCl and only slightly lost ($\leq$10%) at 250 mM NaCl. The pH of 5.5 versus 6.3 did not appear to be a factor in the amount of subunit lost. In early experiments on the effects of NaCl concentration on the loss of the extrinsic subunits at pH 6.5, it was found that 100 mM NaCl removed about 30% of PsbQ and 10−15% of PsbP, while 250 mM NaCl removed about 90% of PsbQ and 40% of PsbP (*34*). In comparison, we observed similar loss of the PsbQ subunit but higher retention of the PsbP subunit. Our results suggest that dissociation of PsbP was not a requirement for formation of the dark-stable multiline signal at pH 5.5 and moderately high NaCl, but further studies would be needed to confirm this. The dark-stable multiline signal has been previously observed in intact PSII after citrate treatment, which removes Ca^{2+} without loss of the extrinsic subunits (*35*).

Although perhaps not a requirement, loss of the PsbP (23 kDa) subunit certainly promoted formation of the dark-stable multiline signal. In NaCl-washed PSII, which lacked PsbP and PsbQ, at pH 5.5 the signal appeared in 40−50% of the sample at 15 mM NaCl and in 80% of the sample at 150 mM NaCl (Figure 5). The stronger signal observed in NaCl-washed PSII relative to intact PSII indicates that removal of the PsbP and PsbQ subunits promoted signal formation, probably because Ca^{2+} is more likely

to be released from the OEC in their absence. The extrinsic subunits are generally recognized to present a diffusion barrier for ion movement into and out of the OEC. The greater affinity of Na^+ for the Ca^{2+} binding site when the extrinsic subunits are removed (*36*) probably promoted greater replacement of Ca^{2+} with Na^+. It is also possible that the removal of the PsbP and PsbQ subunits had some effect on the pK_a of the residues that coordinate Ca^{2+}, adjusting it upward. Even at pH 6.3/15 mM NaCl, a weak dark-stable multiline signal was observed. In this case, the subunit removal and buffer medium were not significantly different from many previous studies involving NaCl-washed PSII, but the observation of the dark-stable multiline signal under these conditions has not been previously reported. A likely reason for this difference in observation is that the preillumination protocol used here promoted formation of the signal. Preillumination would have ensured that the majority of centers without Ca^{2+} were in the S_2 state. It is also likely that loss of Ca^{2+} took place during the preillumination period, because of the lower affinity for Ca^{2+} in the higher S-states (*4*, *5*).

In intact PSII a fairly large loss in O_2 evolution activity resulted from treatment at pH 5.5 and 150 mM NaCl, but there was no evidence that part of the loss in activity was due to dependence on Ca^{2+}. In particular, the addition of Ca^{2+} to samples at pH 5.5 with 150−250 mM NaCl could not restore any activity, whereas it did at pH 6.3 for the same NaCl concentrations (Figure 6). The appearance of Ca^{2+} dependence with increasing NaCl concentration at pH 6.3 can be attributed to a partial loss of the PsbP (23 kDa) subunit. If the loss of PsbP were about the same at pH 5.5 as at pH 6.3, we would expect that the same amount of Ca^{2+} dependence would be observed at pH 5.5 as at pH 6.3. (We have not yet carefully compared the loss of PsbP at pH 5.5 and 6.3 using SDS−PAGE but believe it to be about the same at the two pH values.) It was also observed that essentially full activity was restored when intact PSII treated at pH 5.5/150 mM NaCl was returned to pH 6.3/15 mM NaCl (Figure 6). The restoration of full activity indicates that little or no Ca^{2+} was lost from the PSII space during the treatment, which did not include illumination prior to assay. Whether or not the sample was illuminated during the treatment at pH 5.5 and moderate NaCl concentration is likely to be important since illumination promotes loss of Ca^{2+} from NaCl-washed PSII. Under similar conditions of pH 5.5/moderate NaCl, the appearance of the dark-stable multiline signal indicated that at least 15−20% of the sample showed dissociation of Ca^{2+} from the Mn cluster in the S_2 state. However, when the sample was returned to pH 5.5/25 mM NaCl after treatment at pH 5.5/250 mM NaCl but before illumination, the Ca^{2+} remained bound or was able to rebind to the Mn cluster, leading to the absence of the dark-stable multiline signal (Figure 3, trace c). This result is consistent with the O_2 evolution findings.

When Ca^{2+} was added to intact PSII at pH 5.5/moderate NaCl, the dark-stable multiline signal did not form (Figure 3, trace d); however, there was no improvement in the O_2 evolution activity when Ca^{2+} was added under similar conditions (Figure 6). The EPR result implies that Ca^{2+} remained bound or was able to rebind in the S_2 state. However, the lack of improvement in O_2 evolution activity when Ca^{2+} was added would mean that either the suppression in activity was not related to Ca^{2+} loss or that Ca^{2+} was lost from the OEC but was unable to reassociate beyond the S_2 state of the catalytic cycle, even when present in excess. Despite the lack of direct evidence for Ca^{2+} dependence in the O_2 evolution assays, we consider the first possibility to be the less likely one of the two. This is because the EPR results show that Ca^{2+} partially dissociated from the Mn cluster in the S_2 state (after illumination). From previous studies it is known that the affinity for Ca^{2+} in the S_3 state is lower than in the S_2 state (*5*, *37*). This implies that Ca^{2+} would not be likely to rebind to the Mn cluster in the S_3 state after dissociation in the S_2 state. Hence we suggest that a more consistent explanation for the suppressed activity is that in a portion of the sample Ca^{2+} was *unable* to promote activity at pH 5.5/moderate NaCl, even when present in excess. This would mean that some of the lost activity may have indeed been due to dissociation or disruption of Ca^{2+}, although the defect could not be overcome by addition of Ca^{2+}. This would also provide an explanation for why loss of PsbP at pH 5.5/moderate NaCl apparently did not lead to a dependence on Ca^{2+}, namely, because added Ca^{2+} was unable to function.

In NaCl-washed PSII, there was a relatively large dependence of O_2 evolution activity on Ca^{2+}, whether at pH 5.5 or at pH 6.3, as is already well-known. It is interesting to note that upon addition of $CaCl_2$ the relative activity was similar to the activity of intact PSII under the same conditions (either with or without Ca^{2+}). This would be compatible with the interpretation above that the ability of Ca^{2+} to function was limited by the conditions of decreased pH and increased NaCl concentration, even in the presence of sufficient Ca^{2+}. It would also mean that whatever was affected by these conditions (e.g., protonation of coordinating residues) is similarly affected in both intact and NaCl-washed PSII.

The condition of pH 5.5 plus moderate NaCl concentration for producing the dark-stable multiline signal can be compared with the pH 3.0 citrate treatment method. The citrate method was proposed to release Ca^{2+} by protonation of a carboxylate group that ligates Ca^{2+} (*35*). We now know from XRD studies that the coordinating groups are D1-Glu189 and the C-terminal carboxylate of D1-Ala344 (*6*−*8*). Citrate treatment leads to complete conversion of the sample to the form that shows the dark-stable multiline signal, probably due to complete protonation of the coordinating residues. At pH 5.5 and moderate NaCl concentration, however, a relatively small portion (about 20% at 250 mM NaCl) of intact PSII showed the dark-stable multiline signal, indicating that Ca^{2+} had been removed or disrupted in only a portion of the sample. Thus the release of Ca^{2+} in intact PSII may have involved the protonation of one or more residues with an average pK_a around 5.5. This pK_a is close to that observed to be the apparent acid pK_a in pH dependence profiles of O_2 evolution activity in intact PSII (*32*, *33*, *35*, *38*). Using normal NaCl concentrations of 15−25 mM, the dark-stable multiline signal was not observed at pH 5.5, implying Ca^{2+} was still bound at the OEC. However, the moderately high NaCl concentration coupled with the pH of 5.5 resulted in partial loss of Ca^{2+} from the OEC as represented by the dark-stable multiline signal. The most likely explanation for this is that it was due to the displacement of Ca^{2+} with Na^+ via mass action. Alternatively, Na^+ may have disrupted access of Ca^{2+} to its binding site through less specific electrostatic effects.

In this study, the inhibited $S_2Y_Z\cdot$ signal, which is associated with Ca^{2+} depletion, was observed in samples that showed the dark-stable multiline signal. Since both signals are associated with Ca^{2+} loss, one might expect to observe the same intensity pattern for both signals in response to the same treatments. However, that was not found to be the case in NaCl-washed PSII, where the dark-stable multiline signal intensity increased with the NaCl concentration at pH 5.5 and was lowest at pH 6.3/15 mM NaCl. The $S_2Y_Z\cdot$ signals for these samples were all fairly large

and did not follow a similar trend (Figure 5). This could be due to the more poorly controlled illumination conditions for producing the signal. Alternatively, if Ca^{2+} loss occurs from the normal S_2 or higher states (*4*, *5*), it is possible that the multiple turnovers that can take place during illumination at 273 K led to further loss of Ca^{2+} than had occurred in the preilluminated, dark-adapted state.

It was observed that the line width of the $S_2Y_Z\cdot$ signal in intact PSII was significantly larger than the line width of the signal in NaCl-washed PSII. In intact PSII at pH 5.5, the line width was about 200 G at 150 mM NaCl (Figure 2) and 160 G at 250 mM NaCl, whereas in NaCl-washed PSII at pH 5.5 it was about 120 G at 150 mM NaCl (Figure 5). The line widths of the inhibited $S_2Y_Z\cdot$ signals have been reported to vary widely depending on the sample treatments, with peak-to-trough widths ranging from 100 to 230 G (*39*). For those treatments that also lead to the dark-stable multiline signal (EDTA/EGTA or citrate), the line widths have been found to be 130−165 G (*9*, *10*, *22*). It has also been reported previously that the narrower line width (130 G) was observed in PSII lacking the PsbP and PsbQ subunits while the wider line width (165 G) was observed in PSII retaining the extrinsic subunits (*22*), similar to the observation made here. Thus the line width of the $S_2Y_Z\cdot$ signal evidently reflects the influence of those subunits on the OEC conformation. Narrowing of the line width in the absence of PsbP and PsbQ might occur through an increase in the distance from the Y_Z radical to the Ca^{2+}-depleted Mn cluster or through a decrease in the magnetic dipole strength of the Mn cluster.

In conclusion, the dark-stable multiline signal, which is associated with loss of Ca^{2+} from the Mn_4Ca cluster of the OEC, has been induced without the use of polycarboxylic acids. In particular, the signal was observed in intact PSII at pH 5.5 and moderately high NaCl concentration of 100−250 mM. It was also observed in NaCl-washed PSII lacking the PsbP and PsbQ subunits without special treatment but was further enhanced by pH 5.5 and moderately high NaCl concentration. The conditions under which it was observed were milder than those used in past studies, without the use of Ca^{2+} chelators or citrate at pH 3, in particular. This simplifies the possible interpretations of how Ca^{2+} was removed, suggesting that it occurred through protonation of key residues that coordinate Ca^{2+}, followed by replacement of Ca^{2+} with Na^+ by mass action. Observation of the dark-stable multiline signal in NaCl-washed PSII was especially noteworthy since it answered the long-held question of why the signal was not present in this preparation, which is well-known to require the addition of Ca^{2+} to show full O_2 evolution activity. We now know that the signal is indeed found in this preparation but has probably not been previously reported because samples were not prepared by preillumination. This result allows us to conclude that the Ca^{2+}-stimulated activity in NaCl-washed PSII can be identified with replacement of Ca^{2+} that has been lost from the Mn_4Ca cluster itself.

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