Calcium Binding Studies of Photosystem II Using a Calcium-Selective Electrode†

Geoffrey N. Grove and Gary W. Brudvig*

Department of Chemistry, Yale University, New Haven, Connecticut 06520-8107

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ABSTRACT: The identification of Ca^{2+} as a cofactor in photosynthetic O_2 evolution has encouraged research into the role of Ca^{2+} in photosystem II (PSII). Previous methods used to identify the number of binding sites and their affinities were not able to measure Ca^{2+} binding at thermodynamic equilibrium. We introduce the use of a Ca^{2+}-selective electrode to study equilibrium binding of Ca^{2+} to PSII. The number and affinities of binding sites were determined via Scatchard analysis on a series of PSII membrane preparations progressively depleted of the extrinsic polypeptides and Mn. Untreated PSII membranes bound approximately 4 Ca^{2+} per PSII with high affinity ($K = 1.8$ μM) and a larger number of Ca^{2+} with lower affinity. The high-affinity sites are assigned to divalent cation-binding sites on the light-harvesting complex II that are involved in membrane stacking, and the lower-affinity sites are attributed to nonspecific surface-binding sites. These sites were also observed in all of the extrinsic polypeptide- and Mn-depleted preparations. Depletion of the extrinsic polypeptides and/or Mn exposed additional very high-affinity Ca^{2+}-binding sites which were not in equilibrium with free Ca^{2+} in untreated PSII, owing to the diffusion barrier created by the extrinsic polypeptides. Ca^{2+}-depleted PSII membranes lacking the 23 and 17 kDa extrinsic proteins bound an additional 2.5 Ca^{2+} per PSII with $K = 0.15$ μM. This number of very high-affinity Ca^{2+}-binding sites agrees with the previous work of Cheniae and co-workers [Kalosaka, K., et al. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) pp 721−724, Kluwer, Dordrecht, The Netherlands] whose procedure for Ca^{2+} depletion was used. Further depletion of the 33 kDa extrinsic protein yielded a sample that bound only 0.7 very high-affinity Ca^{2+} per PSII with $K = 0.19$ μM. The loss of 2 very high-affinity Ca^{2+}-binding sites upon depletion of the 33 kDa extrinsic protein could be due to a structural change of the O_2-evolving complex which lost 2−3 of the 4 Mn ions in this sample. Finally, PSII membranes depleted of Mn and the 33, 23, and 17 kDa extrinsic proteins bound approximately 4 very high-affinity Ca^{2+} per PSII with $K = 0.08$ μM. These sites are assigned to Ca^{2+} binding to the vacant Mn sites.

Ca^{2+} has been identified as a cofactor (*1*) essential for the proper functioning of photosystem II (PSII[1]). Studies showing that the removal of Ca^{2+} inhibits the O_2-evolving capability of PSII (*2*−*4*) have fueled an interest in determining the role of Ca^{2+} in PSII. Several groups have done research on the number, location, and binding affinities of Ca^{2+} in PSII with varying results (for a review, see refs *5*−*8*). Past work has shown that Ca^{2+} binding to PSII depends on the 33, 23, and 17 kDa extrinsic proteins (*9*) as well as on the presence and redox state of the Mn cluster (*10*, *11*). Previous studies have assayed Ca^{2+} binding either by measuring the reconstitution of O_2-evolving activity or by measuring the Ca^{2+} which remains bound after washing away free and rapidly dissociating Ca^{2+}. However, as described below, these methods fail to provide a measure of total Ca^{2+} bound at equilibrium. In this work, we introduce the use of a Ca^{2+}-selective electrode to detect levels of free Ca^{2+} in titration experiments of PSII samples. This electrode has been used successfully to study the binding of Ca^{2+} to another membrane protein, bacteriorhodopsin (*12*, *13*). We have examined the binding of Ca^{2+} to a series of PSII membrane samples that were progressively depleted of the extrinsic polypeptides and Mn in order to evaluate the total equilibrium binding of Ca^{2+} to specific PSII complexes.

A key aspect in measuring Ca^{2+} binding to PSII is the Ca^{2+}-depletion procedure. There are a variety of methods used to deplete Ca^{2+} in PSII. A concern about substantial residual contaminating Ca^{2+} in some of the Ca^{2+}-depleted samples (*2*, *14*) led us to choose one of the more rigorous Ca^{2+}-depletion procedures, which includes the use of NaCl washing at lowered pH, with A23187 (calcium ionophore) and EGTA (*15*). This procedure yields a sample lacking the 23 and 17 kDa extrinsic proteins and containing much less than 1 Ca^{2+} per reaction center; yet, it allows for the

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* Author to whom correspondence should be addressed. Telephone: (203) 432-5202. Fax: (203) 432-6144. E-mail: gary.brudvig@yale.edu.

[1] Abbreviations: Ca^{2+}-depleted PSII, PSII depleted of Ca^{2+} and the 23 and 17 kDa extrinsic polypeptides; Chl, chlorophyll; DCBQ, 2,5-dichloro-*p*-benzoquinone; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ex-depleted PSII, PSII depleted of the 33, 23, and 17 kDa extrinsic polypeptides which retain 1−2 Mn per 200 Chl; Ex-Mn-depleted PSII, PSII depleted of the 33, 23, and 17 kDa extrinsic polypeptides and Mn; LHCII, light-harvesting complex II; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Mn-depleted PSII, PSII depleted of Mn and the 23 and 17 kDa extrinsic polypeptides; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PSII, photosystem II; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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reconstitution of >90% of the O_2-evolving activity upon the addition of Ca^{2+}. This procedure also avoids the use of light, which may photodamage the samples and affects the redox state of the Mn cluster (*16*, *17*). When considering the possible contamination of Ca^{2+} and/or a variety of Mn redox states in various preparations, it is not surprising that different numbers of Ca^{2+}-binding sites ($n = 1-3$) and different affinities ($K = 0.001$ to >1 mM) for Ca^{2+}-depleted samples have been reported (*8*). In the Ca^{2+}-depleted PSII preparation of Kalosaka et al. (*15*), which was depleted of all but 0.26 Ca^{2+} per reaction center, three apparent K values (1−4 μM, 67−97 μM, and 2.7−7 mM) were observed by analysis of the reconstitution of O_2-evolution activity and attributed to 3 distinct Ca^{2+}-binding sites. The 2 high-affinity sites characterized in this experiment were also identified by atomic absorption measurements which showed that approximately 2 Ca^{2+} remained bound after six washes with buffer containing 1 mM EGTA and A23187 (*15*). Other studies have found fewer Ca^{2+}-binding sites (*2*, *9*, *14*); however, this may be due to incomplete Ca^{2+} depletion. It is known that the highest-affinity Ca^{2+} site is not depleted when less rigorous Ca^{2+} depletion methods are used (*18*, *19*). It has also been suggested that a single Ca^{2+}-binding site could have different affinities owing to an S-state dependent variation in the ligand environment (*10*). This interpretation was used by Ädelroth et al. (*9*) who found 1 Ca^{2+}-binding site per Ca^{2+}-depleted PSII and two dissociation constants (60 μM and 1.7 mM). Although the dissociation constants are similar to the results for two of the sites found by Kalosaka et al. (*15*), it should be noted that a different Ca^{2+}-depletion procedure was used which retains the 23 and 17 kDa extrinsic proteins (*20*) and which may retain the highest-affinity Ca^{2+} ion (*15*, *21*).

Three techniques have been employed so far in the detection of Ca^{2+}. The first involves a measurement of O_2-evolution activity restored upon the addition of Ca^{2+} to a Ca^{2+}-depleted sample (*22*). By varying the concentration of Ca^{2+} added to the Ca^{2+}-depleted samples and measuring the O_2-evolution rates for each concentration, a Woolf-Hofstee plot [rate of O_2 evolution vs (rate of O_2 evolution)/[Ca^{2+}]] is generated. From such a plot, values for the numbers of sites and apparent binding affinities are determined. The concentration of bound Ca^{2+} is not measured directly. This technique may not be used on samples which have been depleted of Mn because the O_2-evolving activity has been destroyed. A light-induced O_2-evolution assay will only measure the equilibrium binding of Ca^{2+} to PSII if the affinity of Ca^{2+} does not change with S state. However, the results of Boussac and Rutherford (*10*) show that this is not the case. Therefore, measurements of Ca^{2+} binding by using steady-state activity assays may not yield equilibrium binding affinity data because the kinetics of binding/dissociation, as well as the equilibrium thermodynamics of binding, may contribute to the measured values. Also, any Ca^{2+} which binds but does not affect the O_2-evolution rate will be undetectable. The second technique, atomic absorption, measures the concentration of Ca^{2+} directly (*15*). In this technique, the sample is washed into Ca^{2+}-free buffer, and the Ca^{2+} content is measured using atomic absorption; however, any Ca^{2+} that has a low affinity and/or rapid exchange rate would be lost when the samples are washed. The third technique, scintillation counting using $^{45}Ca^{2+}$ (*9*), measures the Ca^{2+} concentration directly; as in atomic absorption, the sample is washed in Ca^{2+}-free buffer to remove the potentially large background signal. During this washing technique, any Ca^{2+} that is low-affinity and/or rapidly exchanging will be washed away. The advantages of using a Ca^{2+}-sensitive ion-selective electrode are that it does not require activity assays or the washing away of free Ca^{2+} to assess the level of Ca^{2+} bound and can, thereby, detect Ca^{2+} binding at thermodynamic equilibrium.

The presence or absence of the extrinsic polypeptides affects Ca^{2+} binding in PSII and was the principal reason we chose to study a series of samples which had varying polypeptide compositions. It has been shown that the 23 and 17 kDa extrinsic proteins slow the rate of exchange of Ca^{2+} bound to PSII with bulk solution (*16*, *17*, *23*, *24*). Samples depleted of the 23 and 17 kDa extrinsic proteins will, therefore, reach thermodynamic equilibrium more quickly than if these proteins are present. Since we wish to measure Ca^{2+} binding at equilibrium, the 23 and 17 kDa extrinsic polypeptides were depleted from all of the samples except the untreated PSII samples where we were interested in looking at the binding to rapidly equilibrating sites in an intact sample. Less clearly defined in the literature is the role of the 33 kDa extrinsic protein in Ca^{2+} binding. A sequence in the 33 kDa extrinsic protein has been found to be similar to that of ICaBP (intestinal calcium binding protein) which suggests that it may have a Ca^{2+}-binding site (*25*, *26*). However, functional EPR studies (*27*) have suggested that the 33 kDa protein does not play a direct role in the binding of the Ca^{2+} needed to produce O_2. This does not rule out the possibility that it may still play a role in binding Ca^{2+} which does not restore O_2 evolution.

The oxidation state of the Mn cluster has also been found to affect the Ca^{2+}-binding affinity (*10*). In our work, the sample preparation and [Ca^{2+}] determination were done in the dark. Therefore, the Mn cluster remained in the S_1 state (*28*). In addition, it has been found that an intact Mn complex is required for Ca^{2+} to bind with high affinity (*11*, *29*). It has been suggested that Ca^{2+} binding in PSII may require the presence of Mn as in concanavalin A (*30*), where Mn^{2+} must bind prior to Ca^{2+} binding (*31*).

In an effort to understand photoactivation of PSII better, several research groups have studied the binding of Mn^{2+} to Mn-depleted PSII (for a review, see ref *5*). In photoactivation studies, it has been found that binding of the first Mn^{2+} to Mn-depleted PSII may require displacement of Ca^{2+} (*32*) and that light is required to facilitate the binding of the second Mn atom to Mn-depleted PSII samples (*33*). In other work, using chemical modification of histidine residues and assays of Mn^{2+} photooxidation, it was suggested that 4 Mn may bind to a Mn-depleted PSII sample without the need for light activation (*34*). Ca^{2+}-binding studies may be relevant to the photoactivation process because the added Ca^{2+} may bind to empty Mn^{2+}-binding sites in Mn-depleted PSII.

In this work, we have measured Ca^{2+} binding to a series of PSII membrane samples which were progressively depleted of the extrinsic polypeptides and Mn. We have run experiments on five different preparations of PSII. These include untreated PSII membranes, Ca^{2+}-depleted PSII membranes, Ex-depleted PSII membranes, Mn-depleted PSII membranes, and Ex-Mn-depleted PSII membranes. By

comparing these various types of samples, we have obtained a more complete picture of calcium binding in PSII.

EXPERIMENTAL PROCEDURES

PSII membranes were isolated from market spinach by the procedure of Berthold et al. (*35*), using the modifications of Beck et al. (*36*). However, while the procedure of Beck et al. (*36*) calls for the freezing of the sample as thylakoid membranes, we found that the rates of O_2-evolution activity were better if the PSII membranes were prepared directly from spinach. The PSII membranes were stored at 77 K in 20 mM MES (pH 6.0−NaOH), 15 mM NaCl, and 30% (v/v) ethylene glycol and used for all other preparations.

Ca^{2+}-depleted PSII membranes were prepared using the high-salt procedure of Kalosaka et al. (*15*). PSII membranes depleted of the 33, 23, and 17 kDa extrinsic proteins were prepared according to the procedure of Ono et al. (*37*), with the following modifications: the concentration of $CaCl_2$ was increased to 1.2 M $CaCl_2$/10 mM NaCl/25 mM PIPES (pH 6.5)/30% (v/v) ethylene glycol as in Kuwabara et al. (*38*) for the first incubation, and then the sample was resuspended in the same buffer with 1 M $CaCl_2$ and pelleted. The sample was then washed three times into buffer containing 200 mM NaCl/25 mM PIPES (pH 6.5)/30% (v/v) ethylene glycol (*38*), and then twice into 15 mM NaCl/20 mM MES (pH 6.0−NaOH)/30% (v/v) ethylene glycol. Mn-depleted PSII membranes were prepared by treatment with hydroxylamine as in Tamura and Cheniae (*39*).

All preparations and titrations were completed in the dark at 0 °C. Chlorophyll concentration assays were done using the method of Arnon (*40*). PSII concentrations were calculated assuming 200 chlorophylls per PSII reaction center in the PSII membrane samples as measured by the spin-quantitation of the tyrosine D ($Y_D^{\bullet}$) EPR signal (*41*, *42*). The O_2-evolution rates were measured with a Clark-type O_2 electrode at 25 °C in 20 mM MES (pH 6.0)/15 mM NaCl/10 mM $CaCl_2$ containing 250 μM DCBQ and 1 mM $K_3Fe(CN)_6$. An Oriel 1000 W (model 66187) tungsten lamp, fitted with a liquid filter (Oriel 6123) filled with distilled water, a 610 nm cutoff filter (Oriel LP610), and a heat filter (Schott KG5), was used to supply saturating light. O_2-evolution activities were 400−600 μmol of O_2 h^{-1} (mg of Chl)$^{-1}$ for untreated PSII membranes. For assays without Ca^{2+}, buffers were treated with Chelex 100 and contained 20 mM MES (pH 6.0)/45 mM $N(CH_3)_4Cl$ with 250 μM DCBQ and 1 mM $K_3Fe(CN)_6$. For the handling of Ca^{2+}-depleted samples, all utensils and containers were treated with 2 N nitric acid and all buffers were treated with Chelex 100.

For Ca^{2+}-titration experiments, a Na_2EDTA sample was prepared at pH 10.8 to provide better solubility and tighter binding of Ca^{2+}; the PSII samples were prepared at pH 6.0, which is the optimal pH for O_2-evolution activity. PSII samples at <1 mg Chl/mL were homogenized with a tissue homogenizer and incubated for 6−8 h in 15 mM NaCl/10 mM EDTA/20 mM MES (pH 6.0) to remove any divalent cations, while maintaining 15 mM NaCl in solution. The samples were then pelleted (40000*g*) for 10 min and resuspended seven times into Chelexed 15 mM NaCl/20 mM MES (pH 6.0), and 800 μL of the sample was added to the titration cell (constructed of Lucite). Based on the serial dilutions made, we estimate the residual EDTA or free divalent cation concentration to be <1 nM. The cell geometry was a flat-bottomed cylindrical hole with a radius large enough to allow the Orion Calcium electrode (model 93-20) to fit inside. A second cylindrical hole adjacent to the first snugly fit a Teflon tube (Alpha TFT 200 14) filled with 1 M KNO_3 in Agar support to serve as a salt bridge for the Double Junction Reference Electrode (Orion model 90-02). The entire cell was kept on ice, and the sample was stirred using a micro stir bar. Readings were taken using a Corning 125 pH meter set to read relative millivolts. Each addition of calcium chloride solution took approximately 15 s followed by approximately an additional 30 s to allow the millivolt reading to stabilize before a reading was made. Calcium chloride hydrate (99.99$^+$% from Aldrich) was dissolved in Chelexed 15 mM NaCl/20 mM MES (pH 6.0) to prepare the standard stock solutions used for making Ca^{2+} additions. The standard curves were generated by incremental Ca^{2+} additions that were small enough that points were taken at ≤1 mV steps; 1 mV is the resolution of the Corning 125 meter. The free Ca^{2+} concentration for each PSII sample was obtained from the point on the standard curve with the same millivolt reading. Standard curves were quite reproducible for repeated titrations after a given electrode pretreatment, but were somewhat variable after each electrode pretreatment which involved incubating the electrode in 1 mM calcium chloride standard solution for more than 1 h. Therefore, a standard curve was run along with each PSII titration.

The O_2-evolving activity of the various PSII preparations was measured before and after the samples were incubated with EDTA to determine whether the treatment resulted in the loss of activity. Samples having undergone any type of depletion (i.e., Ca^{2+}, Mn, or extrinsic polypeptides) exhibited activities of <2% of the original untreated samples. The O_2 evolution of untreated PSII membranes was measured before and after a complete titration experiment was run. It was found that 93−97% of the O_2-evolving activity was retained. Ca^{2+}-depleted samples assayed after a completed titration experiment retained 70−80% of their original activity when assayed in buffer containing 10 mM $CaCl_2$.

Protein composition was determined via SDS−PAGE, using the buffer system of Chua (*43*) with the modifications of Ikeuchi (*44*) and a slab gel containing a 6% acrylamide (w/v) stacking gel and a 12.5% acrylamide (w/v) resolving gel. The gel was stained with Coomassie Blue R-250. Densitometry was taken on a Bio-Rad Scanning Densitometer (model 1650).

Manganese abundance was determined using a Varian SpectrAA-20 flame atomic absorption spectrometer. PSII membranes were wet ashed using 0.8 M HCl to release manganese. Atomic absorption measurements showed that extrinsic 33, 23, and 17 kDa polypeptide-depleted samples, which had not been treated with hydroxylamine to remove Mn, retained 1−2 Mn per 200 Chl.

RESULTS

The binding of Ca^{2+} to various PSII membrane preparations was studied by using a Ca^{2+}-selective electrode. The electrode detects Ca^{2+} by means of an ion-selective resin surface. The composition of the resin is proprietary.

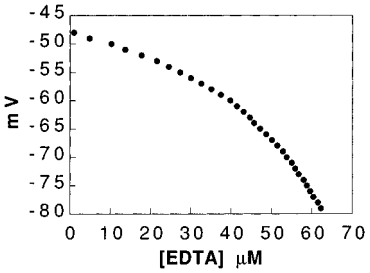

FIGURE 1: Titration of EDTA into Ca^{2+}-free buffer (1 mM ammonia/ammonium chloride, pH 10.8).

However, we will briefly describe the functioning of this surface.

Pretreatment by equilibrating the electrode in a standard Ca^{2+} solution is required to load the ion-exchange resin with a specific amount of Ca^{2+}. The concentration of this standard solution is varied depending on the range of Ca^{2+} concentrations to be measured. Then the electrode is immersed in the sample and allowed to establish an equilibrium between Ca^{2+} bound to the ion-exchange resin and to sites in the sample. Binding or dissociation of Ca^{2+} from the ion-exchange resin changes the voltage response of the electrode. If the sample contains free Ca^{2+}, the resin will bind extra Ca^{2+} and give a positive voltage reading (relative to a Ca^{2+}-free solution). However, if the sample binds Ca^{2+} more tightly than the electrode, it will strip Ca^{2+} out of the electrode and yield a negative voltage reading (relative to a Ca^{2+}-free solution). Readings of the free Ca^{2+} concentration can only be made once enough Ca^{2+} has been added to replace the Ca^{2+} stripped out of the electrode. Once this amount of Ca^{2+} has been added, so that the electrode readings overlap those of a standard curve, the free Ca^{2+} concentration can be determined from the standard curve. Then the bound Ca^{2+} is calculated from the difference between the total Ca^{2+} added and the free Ca^{2+}.

The effect of a chelator with a high-affinity Ca^{2+}-binding site is illustrated in Figure 1. In this control experiment, EDTA was added to a Ca^{2+}-depleted buffer. As higher concentrations of chelator were added, the potential of the electrode was driven to lower values. Our interpretation of this result is that as higher concentrations of chelator were added, Ca^{2+} that was bound to the resin during the pretreatment of the electrode was stripped away by EDTA. Similarly, this means that if a protein sample has Ca^{2+}-binding sites with higher affinities than the resin, then the initial millivolt readings will be lower than the reading from a Ca^{2+}-free buffer used to obtain the zero-Ca^{2+} point in the standard curve. In both the standard curve and the protein curve, as the Ca^{2+} concentration is increased, the millivolt reading will increase owing to binding of additional Ca^{2+} to the ion-exchange resin. We note, however, that in the portion of the titration where the added Ca^{2+} is replacing the Ca^{2+} that was stripped off the electrode by a high-affinity chelator, the behavior is non-Nernstian and cannot be readily analyzed quantitatively.

Since various preparations of PSII membranes bind Ca^{2+}, it is expected that lower initial millivolt readings would be observed from the PSII samples, as compared with their respective standard curves which contain no Ca^{2+} and no chelator. We would also expect from our EDTA experiments that this initial negative offset between the control and protein

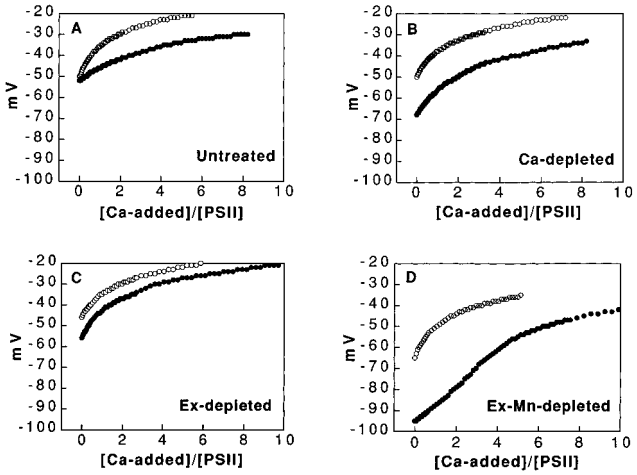

FIGURE 2: Raw data from Ca^{2+}-selective electrode titrations. (A) Untreated PSII membranes (29 μM), (B) Ca^{2+}-depleted PSII membranes (25 μM), (C) Ex-depleted PSII membranes (28 μM), and (D) Ex-Mn-depleted PSII membranes (15 μM). Open circles are data for the standard samples (Chelexed 15 mM NaCl/20 mM MES, pH 6.0) to which aliquots of $CaCl_2$ standard solution were added. Closed circles are data for protein samples.

curves would increase if the concentration of PSII increased or if its ability to bind Ca^{2+} was increased by having a greater number of binding sites, a higher binding affinity, or both. As shown in Figure 2, the initial offset between the standard curve and the experimental curve varied between sample types. Given our interpretation of the initial offset in millivolt reading, we can qualitatively assess the Ca^{2+} binding in the various sample types.

The untreated sample, which contained Mn and all of the extrinsic proteins, showed the smallest offset between the standard curve and the protein curve. This corresponds to the least binding or lowest-affinity binding. Greater than 90% of the O_2-evolving activity of this sample was still present after a titration experiment which indicates that the sample was largely intact throughout the titration. We would expect, therefore, that the Mn and Ca^{2+} sites necessary for O_2 evolution were filled and not available for Ca^{2+} binding owing to slow equilibration when the extrinsic proteins were bound, which is consistent with the small offset we observed.

The Ca^{2+}-depleted sample contained Mn and the 33 kDa extrinsic protein, but was lacking Ca^{2+} and the 23 and 17 kDa extrinsic proteins. The Ex-depleted sample was the same as the Ca^{2+}-depleted sample except that the 33 kDa extrinsic protein was also removed. These two samples showed similar initial millivolt readings which could mean that they had either similar numbers of binding sites or similar affinities. Qualitatively, we can say that both of these samples have higher affinity and/or more Ca^{2+} binding than the untreated sample.

The Ex-Mn-depleted sample which lacked Mn, Ca^{2+}, and the 33, 23, and 17 kDa extrinsic proteins showed the largest offset which corresponds to the highest number of sites and/or the highest-affinity sites. The Mn-depleted sample (data not shown), which was the same as the Ex-Mn-depleted sample except that it retained the 33 kDa protein, was indistinguishable within error from the Ex-Mn-depleted sample. SDS−PAGE densitometry of the Mn-depleted samples showed that they retained 70−80% of the 33 kDa, 18−20% of the 23 kDa, and 12−17% of the 17 kDa extrinsic protein, as compared to the untreated samples.

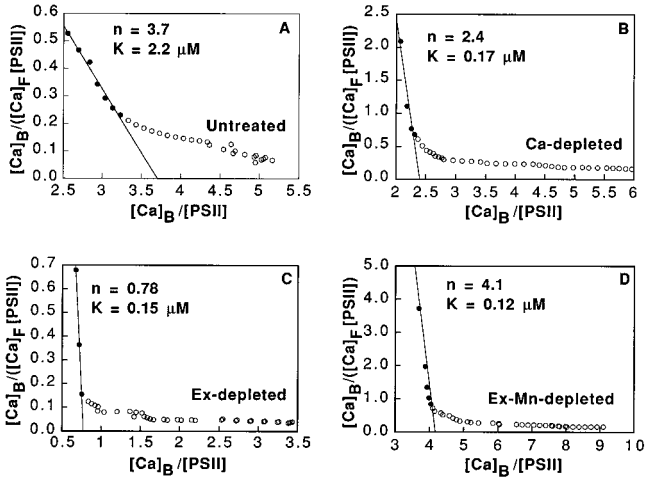

FIGURE 3: Scatchard plots of Ca^{2+}-selective electrode titrations, where $[Ca^{2+}]_B$ = concentration of Ca^{2+} bound to PSII, $[Ca^{2+}]_F$ = concentration of Ca^{2+} free in solution, n = number of Ca^{2+}-binding sites, and K = dissociation constant. (A) Untreated PSII membranes, (B) Ca^{2+}-depleted PSII membranes, (C) Ex-depleted PSII membranes, and (D) Ex-Mn-depleted PSII membranes. Closed circles are data points used for the linear fit to determine affinities and stoichiometries for binding to high-affinity sites. Open circles are the remaining data points from lower-affinity binding sites.

Affinities and numbers of binding sites may be more precisely determined by generating Scatchard plots (Figure 3) using the information contained in the standard and protein titration curves. To obtain values for free concentrations of Ca^{2+}, the millivolt readings from the protein curves were compared to the corresponding standard curve by direct point-to-point correlation. Since the PSII samples bound Ca^{2+} tightly, the initial readings were lower than for the standard titration curve. These initial millivolt values were not used in generating the Scatchard plot because there was no corresponding point on the standard curve from which to determine the free Ca^{2+} concentration. When enough Ca^{2+} was added to restore the Ca^{2+} stripped away from the ion-exchange resin, the standard and protein curves began to overlap and the free Ca^{2+} concentration for the protein curve could be obtained. Since the concentration of added Ca^{2+} was known, a simple subtraction yielded the concentration of bound Ca^{2+}. These data were used for the Scatchard plots. We note that the points used for the Scatchard analysis are only those on the tail end of the titration of the high-affinity sites. As a result, we can determine the number of high-affinity sites accurately, but cannot distinguish whether they all have the same dissociation constant; the measured dissociation constant will also reflect the weakest binding of the high-affinity sites.

Figure 3 shows the results of the Scatchard analysis with the fits giving n (number of binding sites) and K (dissociation constant) for the highest-affinity sites found in each type of sample studied. Table 1 summarizes the results for our determinations of the number of binding sites and their affinities for all of the samples studied. Errors were assigned by taking the standard deviation of results from at least two experiments on different PSII samples in each of which at least four points were used to define the highest-affinity Ca^{2+} binding in the Scatchard analysis. We have analyzed only the highest-affinity Ca^{2+}-binding to each type of sample, but lower-affinity Ca^{2+}-binding was observed for all the samples. As noted below, the highest-affinity Ca^{2+}-binding sites in untreated PSII membranes are assigned to sites on the LHCII. These sites should also be present in all the other samples. In each of the treated samples, fitting the intermediate-affinity data to a second straight line gave a K value in the range of 1−8 μM. This value is the same as that for the highest-affinity Ca^{2+}-binding sites in the untreated sample, consistent with binding to the LHCII in all cases.

Table 1: Results from Scatchard Analysis of the Highest-Affinity Ca^{2+} Binding Sites Observed in Each Type of PSII Membrane Preparation

PSII membrane sample type	number of sites, n	dissociation constant K (μM)
untreated	4.0 ± 0.4	1.8 ± 0.5
Ca^{2+}-depleted	2.5 ± 0.2	0.15 ± 0.03
Ex-depleted	0.7 ± 0.1	0.19 ± 0.06
Mn-depleted	3.5 ± 0.6	0.05 ± 0.03
Ex-Mn-depleted	4.3 ± 0.2	0.08 ± 0.04

Table 2: Summary of Ca^{2+} Binding in PSII[a]

PSII membrane sample type	calcium binding sites		
	LHCII	Mn	Ca
untreated	4	*b*	*c*
Ca^{2+}-depleted	4	*b*	2−3
Ex-depleted	4	*b*	1
Mn-depleted	4	4	0
Ex-Mn-depleted	4	4	0

[a] LHCII represents binding sites on the LHCII proteins of PSII. Mn represents vacant Mn sites where calcium may bind. Ca represents the Ca^{2+}-binding sites in PSII samples containing an intact or partially intact O_2-evolving complex. [b] Not observed because the sites are occupied by the Mn cluster. [c] Not observed because the sites are not in equilibrium with free Ca owing to the diffusion barrier created by the extrinsic polypeptides.

DISCUSSION

We have used a Ca^{2+}-sensitive ion-selective electrode to study the binding of Ca^{2+} to PSII membranes. Figure 2 shows examples of the raw data collected in our titration experiments. Qualitatively, Figure 2 indicates that there is no very high-affinity Ca^{2+} binding in equilibrium with free Ca^{2+} in untreated PSII membranes; note that untreated PSII does have Ca^{2+} bound to very high-affinity sites, but that these sites are not in equilibrium with free Ca^{2+} (see below). Figure 2 also suggests that the affinities of Ca^{2+} for Ex-depleted PSII membranes and for Ca^{2+}-depleted PSII membranes may be similar, while Mn depletion makes available sites which bind Ca^{2+} with a higher affinity than in any of the other sample types. The affinities we have measured for the various Ca^{2+}-binding sites are given in Table 1. They are higher than values reported in the literature for Ca^{2+} binding to PSII membrane preparations (*5*); however, they are within the range of affinities found in Ca^{2+}-binding proteins (*45*, *46*).

In Table 2, we summarize our interpretation of the results of our Ca^{2+}-binding studies. There are three types of Ca^{2+}-binding sites presented in Table 2. These include LHCII sites (present in all samples), Mn sites (present only when Mn is depleted), and Ca sites (the very high-affinity Ca^{2+}-binding sites present when the O_2-evolving complex is intact or partially intact).

We observed that there are no very high-affinity Ca^{2+}-binding sites in untreated PSII membranes (in comparison

to the results for other sample types), which seems to conflict with the results of Ädelroth et al. (*9*) who found one very high-affinity site in intact PSII membranes. However, the titration experiments we ran were completed within 1−2 h, and Ädelroth et al. (*9*) reported an exchange rate on the order of 80 h. We conclude that Ca^{2+} remained bound to the very high-affinity Ca^{2+}-binding sites in untreated PSII during our titration experiments. We interpret the Ca^{2+}-binding sites available in untreated PSII membranes to be on the LHCII and on the surface of the other PSII proteins. The four sites with *K* values of 1.8 ± 0.5 μM (Table 1) have a high enough affinity to allow us to argue that these are the sites which specifically bind divalent cations on the LHCII rather than nonspecific sites on the protein surface. Furthermore, the affinities we measured are in excellent agreement with the equilibrium dialysis binding studies done by Davis et al. (*47*) on the LHCII (K_d = 2.5 μM). Since there are approximately 200 chlorophylls per PSII reaction center in our PSII membrane samples as measured by the spin-quantitation of the tyrosine D ($Y_D^{\bullet}$) EPR signal (*41*, *42*) and there are about 50 chlorophylls in the core reaction center which has been depleted of the LHCII proteins (*48*, *49*), we can estimate that there are approximately 150 chlorophylls contained in the LHCII. Since each LHCII complex contains 15 chlorophylls (*50*), we would expect about 10 LHCII complexes per reaction center. As the PSII membranes stack, 2 LHCII complexes may bind a Ca^{2+} ion after the LHCII is phosphorylated (*51*). This means that we should see 1 Ca^{2+}-binding site per 2 LHCII complexes and approximately 5 Ca^{2+} sites per PSII complex. Our number of 4 Ca^{2+}-binding sites in untreated PSII membranes is in reasonable agreement with the expected number of specific divalent cation-binding sites on the LHCII. Binding to the LHCII sites should occur in each of the samples used in our studies. Analysis of the intermediate-affinity Ca^{2+}-binding sites in the treated samples gave *K* values that were in the same range as that assigned to the LHCII in the untreated sample, consistent with Ca^{2+} binding to the LHCII in all the samples studied. Weaker Ca^{2+} binding than that assigned to the LHCII was also observed in all sample types and is probably due to nonspecific Ca^{2+} binding to protein surfaces.

The Mn-depleted and Ex-Mn-depleted PSII samples gave similar results. Within error, both yield 4 binding sites per PSII (Mn sites, see Table 2), as well as equivalent binding constants (K = 0.05 ± 0.03 and 0.08 ± 0.04 μM, respectively). In these samples, the binding is stronger by a factor of 2 than in Ca^{2+}-depleted samples. The finding that approximately 4 Ca^{2+} bind per Mn-depleted PSII suggests that 1 Ca^{2+} may bind in each of the vacated Mn sites. This is consistent with previous work showing that Mn^{2+} and Ca^{2+} act as competitive inhibitors in photoactivation studies (*52*, *53*) and with the conclusion that Ca^{2+} is displaced when Mn^{2+} binds to Mn-depleted PSII during photoactivation (*32*). The model showing that only 1 Mn is bound and photooxidized in the rate-limiting step of photoactivation (*33*) does not imply that only 1 Mn^{2+} or Ca^{2+} ion should bind in the dark, since the study of Mn in these experiments measured binding stoichiometries and dissociation constants for Mn^{2+} in the rate-limiting step of photoactivation, whereas we measure Ca^{2+} binding directly and at thermodynamic equilibrium. Ädelroth et al. (*9*) found that only 0.1 Ca^{2+} bound per PSII after washing with 0.8 M Tris (pH 8). Since it is known that this procedure only partially depletes Mn (*54*), it is possible that the residual Mn precluded Ca^{2+} binding to the Mn sites, as we also observed in Ex-depleted PSII. It is also possible that rapid dissociation of Ca^{2+} from the Mn sites in Tris-washed PSII caused loss of Ca^{2+} from the sample during the washing step before the scintillation measurement of $^{45}Ca^{2+}$.

Chemical modification of amino acid residues has been employed in an effort to identify ligands to the Mn cluster. The results of these studies have shown that histidyl and carboxyl residues are important for proper functioning of the electron-transport mechanism from the Mn cluster and that there are 4 ligands implicated in Mn binding (*55*). If each of these ligands binds a separate Mn, then this would imply 4 sites; however, the authors indicate that these 4 ligands may also bind to the same Mn atom. In more recent studies using chemical-modification techniques and assays of Mn^{2+} photooxidation, Mn^{2+} dissociation constants of 0.1 and 10 μM were found at pH 6.0 (*56*). The lower value agrees within error with the dissociation constant we find for Ca^{2+} binding at pH 6.0 in Ex-Mn-depleted PSII samples. The binding affinities of different ions depend on the charge of the ion, the ionic radius, and the rigidity of the site (*46*). Because Mn^{2+} and Ca^{2+} have the same charge, and their ionic radii only differ by 0.1 Å, it is not unreasonable that they could bind with similar affinity to PSII. This is yet another indication that the Ca^{2+} binding we observe in Mn-depleted and Ex-Mn-depleted PSII is occurring at the vacated Mn binding sites. In both of these previous studies, two separate binding affinities for Mn were found. This may be because the measured binding affinities were based on an electron-transfer assay.

It also follows from our data that the presence or absence of the 33 kDa extrinsic protein has no effect on the Ca^{2+}-binding affinity at the Mn sites. If Ca^{2+} binding to the Mn sites behaves like Mn^{2+} binding, then these results are consistent with the findings of Miller et al. (*57*), who showed that the 33 kDa extrinsic protein does not contain ligands to the Mn atoms in PSII. We would, therefore, expect that the 33 kDa extrinsic protein would not contain ligands to Ca^{2+} when Ca^{2+} binds in the Mn sites and that the loss of the 33 kDa extrinsic protein would not have a direct effect on Ca^{2+} binding in Mn-depleted PSII samples. Another result which supports the idea that the 33 kDa does not affect the environment of Mn binding to PSII is the work of Seidler and Rutherford (*27*), whose experiments show that the presence of the 33 kDa extrinsic protein is not necessary for the production of several of the characteristic Mn EPR signals obtained in Ca^{2+}-depleted PSII.

The Ex-depleted samples have been treated with 1.5 M $CaCl_2$ to remove the 33, 23, and 17 kDa extrinsic proteins. These samples contain approximately 1−2 Mn per PSII, as assayed by flame atomic absorption. In comparing the Ca^{2+} binding in the Ex-depleted sample to that of the Ex-Mn-depleted sample, we find that there are fewer Ca^{2+}-binding sites in the Ex-depleted sample. This agrees with Ädelroth et al. (*9*) who also found fewer Ca^{2+}-binding sites upon removal of the 33, 23, and 17 kDa extrinsic polypeptides (0.3 Ca^{2+}/PSII). The residual binding of 1−2 Mn per PSII appears to prevent Ca^{2+} binding to the Mn sites that were available in the Mn-depleted and the Ex-Mn-depleted samples. This finding is also consistent with the idea that

Ca^{2+} is binding to the Mn sites that are made available upon Mn depletion.

The Ca^{2+}-depleted samples, which are also depleted of the 23 and 17 kDa extrinsic proteins, show 2−3 very high-affinity binding sites (Ca, see Table 2). The binding to these sites is stronger than to the sites found in the untreated PSII membranes by 1 order of magnitude ($K = 0.15 \pm 0.03$ μM, from Table 1). In a previous study of Ca^{2+}-depleted samples prepared by the same procedure we have used, 2 high-affinity Ca^{2+}-binding sites were found (*15*). By using scintillation counting, Ädelroth et al. (*9*) found 1.5 Ca^{2+} per PSII in samples of untreated PSII membranes which were isolated from spinach that had been grown in medium with $^{45}Ca^{2+}$. These two studies, in combination, indicate that there are 2 high-affinity Ca^{2+}-binding sites in an intact PSII complex and that these 2 binding sites are retained when the 23 and 17 kDa extrinsic polypeptides are depleted. We can correlate 2 of the very high-affinity Ca sites that we have found in our Ca^{2+}-depleted samples with the ∼2 high-affinity Ca^{2+}-binding sites previously found (*9*, *15*). However, we measure much higher affinities for Ca^{2+} than found by using a Woolf−Hofstee analysis of reconstitution of O_2-evolution activity (*15*). It is possible that the lower affinities measured using the Woolf−Hofstee analysis are an indication that these sites are exchangeable; the differentiation in binding constants previously measured could be due to differences in exchange rates for each of the sites. It is also possible, as has been suggested by Boussac and Rutherford (*10*), that the different Ca^{2+}-binding affinities are a result of variations in the Ca^{2+} affinity with S state; the Ca^{2+}-binding affinity has been found to be highest when the O_2-evolving complex is in the S_1 state. Our samples were dark adapted for several hours at 0 °C before the titration experiments were run and were not exposed to light during the course of the titrations. Therefore, we expect that all centers should be in the S_1-resting state (*36*) and, since the S_1-resting state binds Ca^{2+} more tightly than the other S states, this could explain the higher Ca^{2+}-binding affinities we find.

Although Ädelroth et al. (*9*) have found ∼2 high-affinity Ca^{2+} sites in an intact PSII complex, we have found 2.5 very high-affinity Ca^{2+} sites in Ca^{2+}-depleted PSII. Our larger value may be due to experimental error. However, it is possible that the depletion of the 23 and 17 kDa extrinsic polypeptides exposes a new, very high-affinity site that is not present in an intact PSII complex. For example, ionic protein−protein interactions between the 23 and/or 17 kDa extrinsic polypeptides and the PSII core could be substituted by Ca^{2+}−protein interactions in the polypeptide-depleted sample.

When 2−3 of the 4 Mn atoms are lost from the O_2-evolving complex, ∼2 very high-affinity Ca^{2+}-binding sites are lost. This requirement for the presence of the Mn cluster for high-affinity Ca^{2+} binding correlates with the results of Tamura and Cheniae (*11*) who found that assembly of the Mn complex is necessary to create 1−2 Ca^{2+}-binding sites. Interestingly, our results show that partial Mn binding is not sufficient to create the very high-affinity Ca^{2+}-binding sites. However, partial Mn binding is sufficient to prevent Ca^{2+} binding to the Mn sites.

Since the removal of the 33 kDa extrinsic polypeptide caused our samples to lose Mn, we cannot say definitively whether the 33 kDa extrinsic protein is directly involved in creating Ca^{2+}-binding sites or whether it is only the presence of an intact Mn cluster which is required. It would be useful to run a titration experiment on a sample depleted of the 33 kDa extrinsic polypeptide, but with an intact Mn cluster. Unfortunately, once the 33 kDa extrinsic protein is removed, a high concentration of chloride (approximately 200 mM) is required to maintain the stability of the Mn cluster. Since the ionic strength of the solution will also affect the binding of the extrinsic polypeptides and Ca^{2+}, it would to be necessary to run a complete set of samples at the high ionic strength in order to make comparisons between different PSII complexes.

In this work, we have established the use of a Ca^{2+}-selective electrode to study ion binding in PSII. Due to the ability of the electrode to measure binding at thermodynamic equilibrium, we have found binding sites previously undetected and measure dissociation constants for Ca^{2+} that are higher than previously measured. In the future, this technique may be used to study the competition of Mn^{2+} and Ca^{2+} binding to PSII samples.

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