Studies of Ca^{2+} Binding in Spinach Photosystem II Using $^{45}Ca^{2+}$ †

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ABSTRACT: The Ca^{2+}-binding properties of photosystem II were investigated with radioactive $^{45}Ca^{2+}$. PS II membranes, isolated from spinach grown on a medium containing $^{45}Ca^{2+}$, contained 1.5 Ca^{2+} per PS II unit. Approximately half of the incorporated radioactivity was lost after incubation for 30 h in nonradioactive buffer. About 1 Ca^{2+}/PS II bound slowly to Ca^{2+}-depleted membranes in the presence of the extrinsic 16- and 23-kDa polypeptides in parallel with restoration of oxygen-evolving activity. The binding was heterogeneous with dissociation constants of 60 μM (0.7 Ca^{2+}/PS II) and 1.7 mM (0.3 Ca^{2+}/PS II), respectively, which could reflect different affinities of the dark-stable S-states for Ca^{2+}. The reactivation of oxygen-evolving activity closely followed the binding of Ca^{2+}, showing that a single exchangeable Ca^{2+} per PS II is sufficient for the water-splitting reaction to function. In PS II, depleted of the 16- and 23-kDa polypeptides, about 0.7 exchangeable Ca^{2+}/PS II binds with a dissociation constant of 26 μM, while 0.3 Ca^{2+} binds with a much weaker affinity (K_d > 0.5 mM). The rate of binding of Ca^{2+} in the absence of the two extrinsic polypeptides was significantly higher than with the polypeptides bound. The rate of dissociation of bound Ca^{2+} in the dark, which had a half-time of about 80 h in intact PS II, increased in the absence of the 16- and 23-kDa polypeptides and showed a further increase after the additional removal of the 33-kDa protein and manganese. The rate of dissociation was also significantly faster in weak light than in the dark regardless of the presence or absence of the 16- and 23-kDa polypeptides. Removal of the 33-kDa donor-side polypeptide together with the two lighter ones led to a reduction in the amount of bound Ca^{2+}, while practically no Ca^{2+} bound after treatments to dissociate also the manganese of the water-oxidizing site.

There is a wealth of information showing that Ca^{2+} is essential for photosynthetic oxygen evolution [see Ghanotakis and Yocum (1990), Yocum (1991), Debus (1992) and Rutherford et al. (1992) for recent reviews]. During recent years, new details of the involvement of the ion in the water-splitting process have accumulated. For example, in the absence of Ca^{2+}, tyrosine Z appears to be unable to transfer an electron to oxidized reaction center dimer P680 once the S_3 state has been reached (Boussac et al., 1992). EPR studies have shown that depletion of Ca^{2+} leads to changes in the water-oxidation center which alter the properties of the S_2 state and stabilize an organic radical, most likely an amino acid residue, in the S_3 state (Boussac et al., 1989; Sivaraja et al., 1989; Ono & Inoue, 1990).

Quantitative determinations have shown that about two Ca^{2+} are associated with photosystem II, one of which can be easily and reversibly dissociated after labilization of the binding. This usually involves permanent or transient removal of the extrinsic 16- and 23-kDa proteins by treatment with high concentrations of NaCl (Ghanotakis et al., 1984a), usually in combination with the chelating agent EGTA[1] and an ionophore (Cammarata & Cheniae, 1987) or by low-pH treatment (Ono & Inoue, 1988). The polypeptide dependence of the binding of Ca^{2+} has led to proposals that at least the 23-kDa protein provides conditions for high-affinity binding of Ca^{2+} (Ghanotakis et al., 1984b; Homann, 1988).

Previous studies of Ca^{2+}-binding affinities have been based on the reactivation of oxygen evolution activity after readdition of Ca^{2+} to depleted PS II preparations. Here we have examined the binding directly by measuring the incorporation and release of radioactive $^{45}Ca^{2+}$ under a range of experimental conditions. A preliminary account of some of the results presented herein has been published in the proceedings from the annual meeting of the British Biochemical Society (Andréasson et al., 1994).

MATERIALS AND METHODS

Photosystem II membranes (250 chlorophyll/PS II) were prepared from spinach as described in Franzén et al. (1985) and suspended in 20 mM Mes−NaOH, pH 6.5, 20 mM NaCl, and 0.4 M sucrose. To avoid contamination of the PS II membranes with extraneous Ca^{2+}, a buffer containing ultrapure sucrose (BHD Aristar, Ca < 1 ppm) and Mes (Sigma Ultra) was used ("Ca^{2+}-free" buffer). All buffers were prepared with deionized water, which was further purified by glass distillation.

Removal of Extrinsic Proteins and Manganese. Removal of the 16- and 23-kDa extrinsic proteins was accomplished by washing with 1.2 M NaCl as described in Kuwabara and Murata (1983). To remove also the 33-kDa protein, 1 M $MgCl_2$ was used (Ono & Inoue, 1983a). Manganese and the three extrinsic proteins were extracted either with 0.8 M Tris, pH 8.3 (Tamura & Cheniae, 1987) or with 1 M $MgCl_2$ and 0.5 mM *p*-benzhydroquinone (Ghanotakis et al., 1984c).

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[1] Abbreviations: Chl, chlorophyll; PS II, photosystem II; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)- *N,N,N',N'*-tetraacetic acid.

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Removal of Ca^{2+}. PS II membranes depleted of Ca^{2+} and retaining the 16- and 23-kDa proteins were prepared with low pH/citrate washing as described in Ono and Inoue (1988). To avoid contamination of the depleted membranes with Ca^{2+}, the last washing step to remove citrate was made with Ca^{2+}-free buffer (see above).

Depletion of Ca^{2+} with NaCl/EGTA was accomplished essentially as described in Boussac et al. (1990), but the depleted membranes were finally washed twice and suspended in buffer containing 20 μM EGTA or in Ca^{2+}-free buffer.

Labeling Experiments. Spinach was cultivated hydroponically on a medium containing $^{45}Ca^{2+}$ (Amersham, England) ($^{40}Ca^{2+}/^{45}Ca^{2+} = 1.4 \times 10^8$). Labeled PS II membranes were isolated as described in Lindberg et al. (1990). To study the release of Ca^{2+} from PS II membranes, isolated from spinach cultivated in the presence of $^{45}Ca^{2+}$, the membranes were dialyzed for 24 h in darkness against a Mes buffer without added Ca^{2+}, and the residual amount of $^{45}Ca^{2+}$ was determined by scintillation counting.

To determine the time course of binding of Ca^{2+}, PS II membranes were depleted of Ca^{2+} by one of the methods mentioned above and suspended at 1.5 mg Chl/mL in a buffer containing various concentrations of Ca^{2+} ($^{40}Ca^{2+}/^{45}Ca^{2+} = 10^6-10^7$). At different incubation times in the labeling medium, samples were withdrawn, washed 3−4 times (dilution factor range, 3.5×10^5 to 2.4×10^7), depending on the Ca^{2+} concentration, in Ca^{2+}-free buffer, and examined for bound Ca^{2+} by scintillation counting as described in Lindberg et al. (1993). During the incubation and washing procedures the PS II samples were kept in darkness at 0 °C.

For studies of the dissociation of Ca^{2+}, PS II membranes were first depleted of Ca^{2+} by one of the methods described above and then allowed to bind $^{45}Ca^{2+}$ by incubation in darkness in a medium with a total concentration of Ca^{2+} of 5 mM. The time required to reach equilibrium was estimated from the binding studies. The labeled membranes were washed in Ca^{2+}-free buffer to remove excess Ca^{2+} and then dialyzed against a large volume of Ca^{2+}-free buffer (>100 times the sample volume) as an alternative to the washing procedure used to remove released Ca^{2+}. At different times after the start of dialysis, samples were withdrawn and the residual amount of bound Ca^{2+} was determined by scintillation counting. The amount of Ca^{2+} associated with the membranes as a result of the labeling was calculated from the incorporation of $^{45}Ca^{2+}$ and the fractional amount of the radioisotope in the labeling medium.

To study the exchange of bound Ca^{2+} with the medium, PS II samples were incubated in a buffer containing $^{45}Ca^{2+}$ without prior removal of bound Ca^{2+}. After different times samples were withdrawn, washed to remove free Ca^{2+}, and examined for labeling as described above.

Oxygen Evolution Measurements. Oxygen evolution rates at saturating white light were measured with a Clark electrode (Hansatech) at 20 °C with 1 mM phenyl-*p*-benzoquinone (PPBQ) present as an electron acceptor in the Mes buffer.

RESULTS

PS II Membranes from Spinach Cultivated in the Presence of $^{45}Ca^{2+}$. PS II membranes isolated from spinach cultivated with $^{45}Ca^{2+}$ in the medium were found to contain 1.5 moles of Ca^{2+}/250 mol of chlorophyll. The Ca^{2+} content was measured 4 h after the start of the preparation, beginning with the isolation the chloroplasts. After dialysis for 24 h against a medium without added Ca^{2+}, the Ca^{2+} content of the PS II membranes had decreased to about half of the original value (Table 1).

Table 1: Ca^{2+} Content in PS II Membranes Isolated from Spinach Cultivated on a $^{45}Ca^{2+}$-Containing Medium

time of incubation in cold medium (h)[a]	rel $^{45}Ca^{2+}$ abundance	Ca^{2+}/PS II
4[b]	100	1.5
28[c]	47	0.7

[a] Measured from the start of isolation of the chloroplasts. [b] Freshly isolated PS II membranes. [c] After 24 h of dialysis.

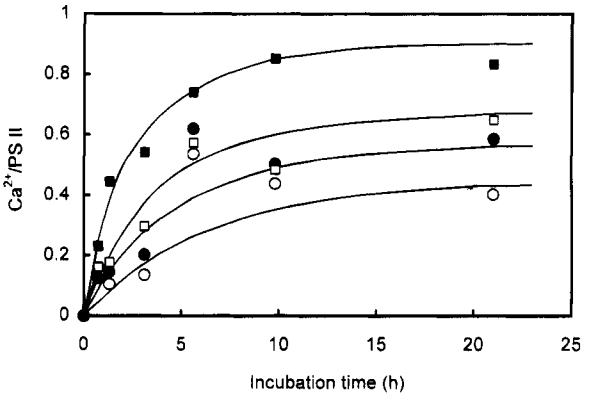

FIGURE 1: Binding of Ca^{2+} to PS II membranes previously depleted of Ca^{2+} by washing with citrate. PS II membranes were labeled in the dark with $^{45}Ca^{2+}$ at different total concentrations of Ca^{2+} as described in Materials and Methods, and the amount of bound Ca^{2+} was measured by scintillation counting at the times indicated. The following concentrations of Ca^{2+} were used: 0.124 (○), 0.49 (●), 1.01 (□), 5.03 mM (■).

PS II Membranes with the 16- and 23-kDa Subunits (Intact PS II). The depletion of Ca^{2+}, either by the low-pH/citrate (Ono & Inoue, 1988) method or by washing with NaCl/EGTA (Boussac et al., 1990) (see below), resulted in loss of oxygen-evolving activity amounting to 75−80% of the control activity, in agreement with earlier reports for these depletion methods. For the different types of Ca^{2+}-depleted material, the rebinding of Ca^{2+} was studied together with the effect on oxygen evolution.

With the three extrinsic proteins (16, 23, and 33 kDa) bound to PS II, the rate of binding and the amount of Ca^{2+} bound at equilibrium in the dark depended on the concentration of Ca^{2+} in the medium (Figure 1). Incorporation of Ca^{2+} leveled out only after several hours even at the highest concentrations of Ca^{2+} used. Both the rate of binding and the final amount of incorporated Ca^{2+} showed saturation at high concentrations, which indicates that the binding is an equilibrium reaction involving a rate-limiting step. A Scatchard plot of the amount of Ca^{2+} bound at equilibrium indicated a heterogeneity in the binding with K_d values of 60 μM (corresponding to 0.7 Ca^{2+}/PS II) and 1.7 mM (0.3 Ca^{2+}/PS II), respectively (Figure 2).

To measure the effect of rebinding of Ca^{2+} on the rate of oxygen evolution, small portions of the samples taken for scintillation counting were assayed for oxygen evolution activity. When measured in an assay medium without supplementing Ca^{2+}, the restoration of oxygen-evolving

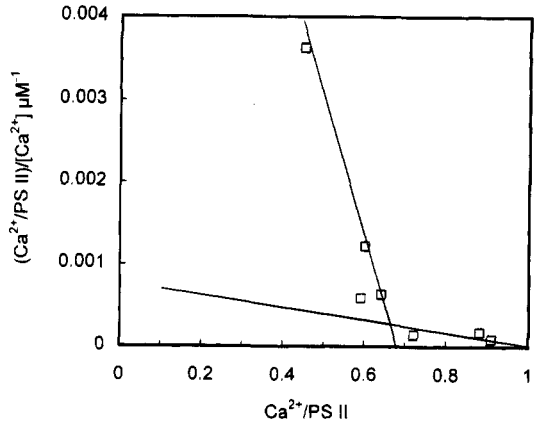

FIGURE 2: Scatchard plot of the binding of Ca^{2+} to citrate-washed PS II. The plotted values were taken at equilibrium in binding experiments with Ca^{2+} concentrations from 124 μM to 10 mM. The two lines correspond to K_d values of 60 μM and 1.7 mM, respectively.

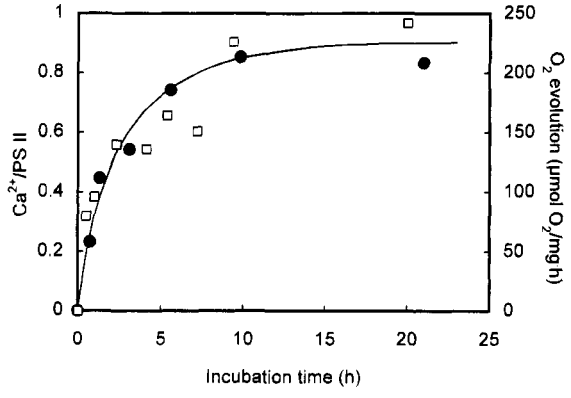

FIGURE 3: Reactivation of oxygen evolution by Ca^{2+} binding in PS II membranes depleted of Ca^{2+} by citrate washing. Depleted PS II membranes were incubated at 5.03 mM Ca^{2+} in the dark, and the amount of bound Ca^{2+} was measured after different times (●). The corresponding increase in oxygen evolution activity (□) was measured by adding 10 μL of the incubation suspension to 1 mL of an assay medium containing 20 mM Mes−NaOH, pH 6.5, 0.4 M sucrose, 20 mM NaCl, and 2 mM EDTA.

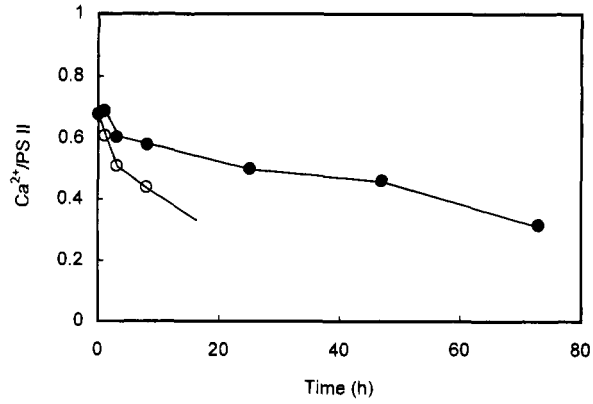

FIGURE 4: Dissociation in the dark of Ca^{2+} from PS II membranes. PS II membranes were treated with citrate to remove Ca^{2+} and then labeled with $^{45}Ca^{2+}$ as described in Materials and Methods. The amount of Ca^{2+} bound to the membranes after incubation for various times in Ca^{2+}-free buffer in darkness (●) or in room light (○), respectively, is shown.

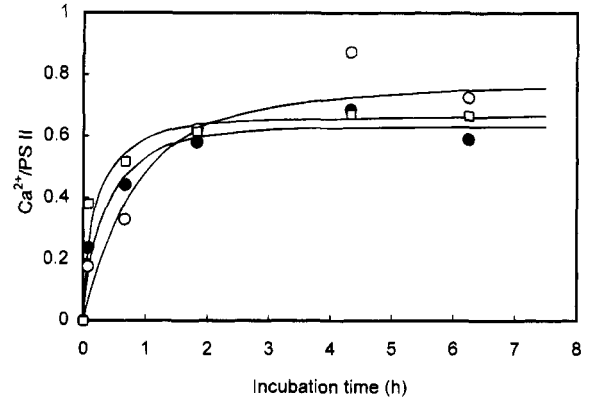

FIGURE 5: Binding of Ca^{2+} to PS II membranes depleted of Ca^{2+} by washing with NaCl/EGTA. PS II membranes were labeled in the dark with $^{45}Ca^{2+}$ at different total concentrations of Ca^{2+} as described in Materials and Methods, and the amount of bound Ca^{2+} was measured by scintillation counting at the times indicated. The following concentrations of Ca^{2+} were used: 0.15 (○), 0.51 (●), and 2.48 mM (□).

activity closely followed the incorporation of Ca^{2+} (Figure 3).

When we repeated the low-pH/citrate treatment on PS II membranes, which had previously been depleted of Ca^{2+} by the low-pH/citrate procedure and then labeled with $^{45}Ca^{2+}$ (exchange of about 1 Ca^{2+}/PS II), more than 80% of the labeled Ca^{2+} was removed, showing that the labeled site is susceptible to this depletion method.

The dissociation of bound Ca^{2+} from PS II membranes complete with the extrinsic protein subunits, measured as described in Materials and Methods, was dominated by a very slow reaction phase in the dark with a half-time of about 80 h and with indications of a faster, initial transient. In room light there was a significant acceleration of the dissociation reaction to a half-time of about 20 h (Figure 4).

PS II Membranes Depleted of the 16- and 23-kDa Subunits. The rebinding of Ca^{2+} to PS II membranes treated with NaCl/EGTA to dissociate the 16- and 23-kDa extrinsic proteins and Ca^{2+} followed a behavior similar to that observed in the presence of the subunits with the rate of incorporation depending on the concentration of Ca^{2+} in the medium (Figure 5). However, at the same concentrations of Ca^{2+}, the rate of binding was faster by a factor of 10−20 compared to PS II membranes with the two extrinsic protein subunits bound. The amount of Ca^{2+} bound at equilibrium was similar for concentrations of Ca^{2+} from 150 μM to 2.5 mM (Figure 5), an indication of saturation of the binding and that the dissociation constant must be significantly lower than 150 μM. A closer examination of the binding properties of PS II membranes depleted of the 16- and 23-kDa polypeptides (Figure 6) showed that the binding occurred at two sites with different affinities, as was observed with PS II membranes with the two subunits bound, with a major site corresponding to 0.6−0.7 Ca^{2+} per PS II unit and a dissociation constant of 26 μM and a minor site with a dissociation constant on the order of 0.5 mM, assuming a total incorporation of 1 Ca^{2+} per PS II unit.

When NaCl/EGTA-treated and labeled membranes were again treated with EGTA , the labeled Ca^{2+} was lost from the membranes. In addition, this loss was accelerated in light.

The dissociation of Ca^{2+} from NaCl-washed PS II membranes was considerably faster than in the presence of the extrinsic 16- and 23-kDa subunits and could be resolved into two transients, a major phase encompassing about 75% of the total reaction with a half-time of about 20 h in the

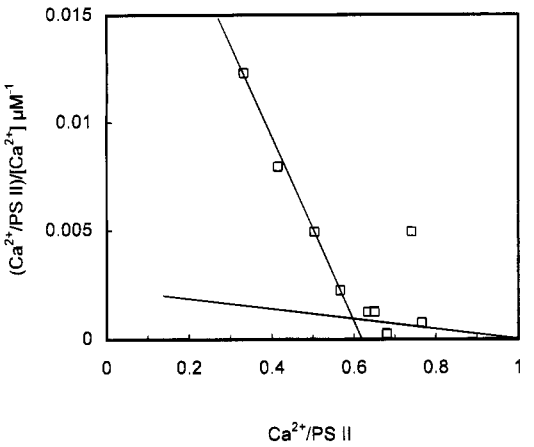

FIGURE 6: Scatchard plot of the binding of Ca^{2+} to NaCl/EGTA-treated PS II membranes. The plotted values were taken at equilibrium in binding experiments with Ca^{2+} concentrations from 25 μM to 2.48 mM. The two lines correspond to K_d values of 26 μM and 0.5 mM, respectively.

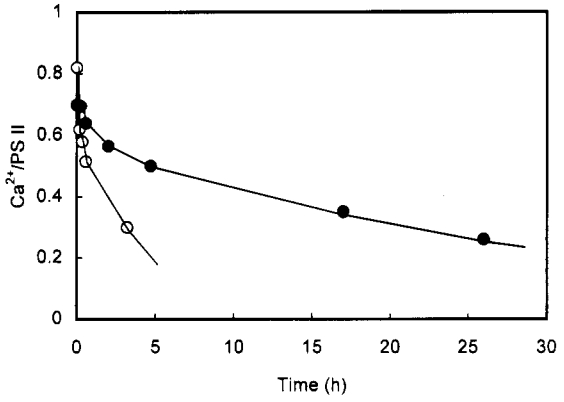

FIGURE 7: Dissociation in the dark of Ca^{2+} from PS II membranes. PS II membranes were treated with NaCl/EGTA to remove Ca^{2+} and then labeled with $^{45}Ca^{2+}$ as described in Materials and Methods. The amount of Ca^{2+} bound to the membranes after incubation for various times in Ca^{2+}-free buffer in darkness (●) or in room light (○), respectively, is shown.

dark and a lesser (25%) phase with a half-time of about 2 h. In room light the half-time of dissociation was about 3 h with no clear indications of biphasic behavior (Figure 7). Thus, removal of the extrinsic protein subunits resulted in a considerably faster equilibration of Ca^{2+} with its binding site at PS II rather than a major change in the dissociation constant.

With PS II membranes depleted of the 16- and 23-kDa polypeptides, the rate of oxygen evolution showed no apparent correlation with the amount of bound Ca^{2+} when measured in a medium without supplementing Ca^{2+}, but remained at 15−20% of the activity before removal of Ca^{2+} regardless of the amount of incorporated Ca^{2+} (Figure 8, lower curve). The residual activity probably corresponds to intact PS II centers complete with polypeptides and Ca^{2+}. NaCl-washed PS II membranes seemed to require additional Ca^{2+} for activation of oxygen evolution, since, at all stages of the rebinding process, the activity could be restored to 70−80% of that of the Ca^{2+}-containing control with 10 mM Ca^{2+} in the assay medium (Figure 8, upper curve).

As EGTA has been recently reported to bind to PS II (Zimmermann et al., 1993), a dissociation study was made with PS II membranes depleted of Ca^{2+} by treatment with 1 M NaCl to remove the extrinsic 16- and 23-kDa subunits

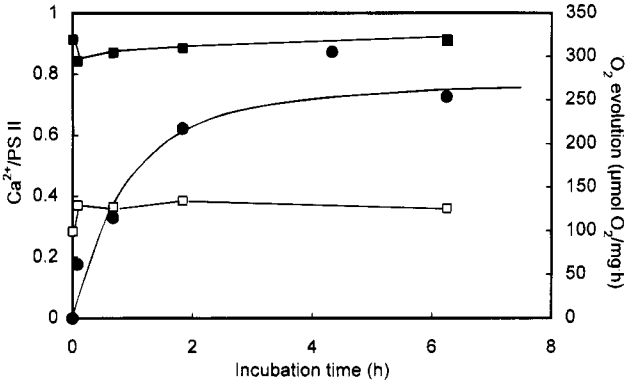

FIGURE 8: Effect of the binding of Ca^{2+} and reactivation of oxygen evolution in PS II membranes depleted of Ca^{2+} by treatment with NaCl/EGTA. The extent of binding was measured in membranes incubated at 0.15 mM Ca^{2+} in the dark (●) for the designated times after washing to remove free Ca^{2+}. The effect of incorporation of Ca^{2+} on the activity in the washed samples was measured in a medium containing 25 mM Mes−NaOH, pH 6.5, 0.3 M sucrose, and 25 mM NaCl without addition of Ca^{2+} (□) or with 10 mM Ca^{2+} present (■).

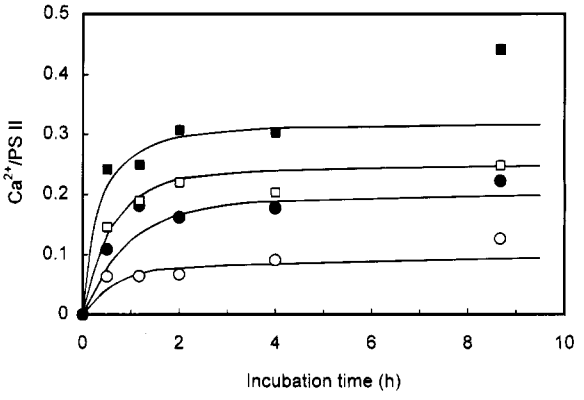

FIGURE 9: Binding of Ca^{2+} to PS II membranes treated with 1 M $MgCl_2$ to remove the 16-, 23-, and 33-kDa polypeptides. The labeling of the $MgCl_2$-washed membranes was carried out in the dark in media containing 0.1 (○), 1 (●), 5 (□), and 10 mM Ca^{2+} (■).

followed by extensive washing with Ca^{2+}-free buffer without EGTA and then labeled with $^{45}Ca^{2+}$. No difference in the dissociation rate from the labeled membranes could be detected in comparison with EGTA-treated material (results not shown). This indicates that bound EGTA does not seriously interfere with Ca^{2+} binding to PS II.

PS II Membranes Lacking the 16-, 23-, and 33-kDa Subunits. The binding of Ca^{2+} to PS II membranes treated with 1 M $MgCl_2$ to remove the 33-kDa protein in addition to the 16- and 23-kDa subunits, was studied by exchange with $^{45}Ca^{2+}$ in the medium, i.e., without prior depletion of Ca^{2+}. The rate of labeling was faster than in material with the 33-kDa protein bound, and equilibrium was reached within 1 h at concentrations of Ca^{2+} ranging from 0.1 to 10 mM (Figure 9). However, only about 0.3 Ca^{2+}/PS II was bound at the highest concentration, indicating a weakening of the binding after removal of the 33-kDa protein or an increase in the exchange rate for Ca^{2+} which would allow the bound Ca^{2+} to dissociate to some extent during the washing procedure used to remove the excess Ca^{2+}. Assuming a weakening of the binding, the final values for the bound Ca^{2+} are consistent with a K_d value of 100 μM (0.2 Ca^{2+}/PS II). Some additional binding was observed at high concentrations of Ca^{2+} which may indicate the presence of

Table 2: Effect of Removal of the 33-kDa Protein and Manganese on the release of $^{45}Ca^{2+}$ from Labeled PS II Membranes from Spinach

material	bound $^{45}Ca^{2+}$ (%)
NaCl/EGTA-treated and labeled[a] (−16, 23 kDa)	100
+1 M $MgCl_2$ (−33 kDa)	59
+1 M $MgCl_2$ + 0.5 mM hydroquinone (−33 kDa, Mn)	22

[a] Labeled in the presence of 1 mM Ca^{2+}. The labeled membranes contained about 0.67 Ca^{2+}/PS II.

a site with much lower affinity. With the assumption that a binding of maximally 1 Ca^{2+}/PS II takes place, the K_d value for this site may be estimated to >30 mM.

PS II Membranes Depleted of the 16-, 23-, 33-kDa Polypeptides and Mn. Exchange studies after removal of manganese in addition to the three extrinsic protein subunits by washing with 0.8 M Tris, pH 8, showed a binding of about 0.1 Ca^{2+}/PS II after 3.5 h in the presence of 4 mM Ca^{2+} with a binding half-time of less than 30 min (not shown). Since Tris is known not to remove all manganese efficiently (Yocum et al., 1981), the observed binding may be related to centers with some manganese still bound, while the Mn-depleted PS II centers were unable to bind Ca^{2+} or bound either with a much lower affinity or with high exchange rates. The influence of manganese binding on the binding of Ca^{2+} was studied by comparing how much radioactivity was retained by PS II membranes first treated with NaCl/EGTA to remove Ca^{2+} and then labeled, with either 1 M $MgCl_2$ to remove the 33-kDa subunit or with $MgCl_2$ plus 0.5 mM benzhydroquinone to release also bound manganese (Boussac et al., 1990) (Table 2). After treatment for 30 min in darkness, 60% of the originally bound Ca^{2+} was retained in the material depleted of all polypeptides, whereas only 20% remained when the manganese had also been removed. The observation that substantial amounts of Ca^{2+} remain bound 30 min after the removal of the 33-kDa polypeptide suggests that a drastic rise in the rate of exchange of Ca^{2+} with the medium is not the only reason for the low incorporation of Ca^{2+} in the binding experiment (Figure 9) but that a decrease in affinity, induced by the depletion of this polypeptide, may contribute to the less efficient labeling. The further loss of label after reductant-induced release of manganese suggests that there is interaction between the binding sites of the two metal ions.

DISCUSSION

Our measurement of the Ca^{2+} content in PS II membranes isolated from spinach grown on radioactive $^{45}Ca^{2+}$ (Table 1) agrees with earlier results showing that two Ca^{2+} are firmly bound. One binding site has recently been localized to the LHCII antenna complex, conceivably with a structural role, whereas a second site, suggested to be functional in water oxidation, was found associated with the PS II core (Han & Katoh, 1993). Furthermore, after 24 h of dialysis against a Ca^{2+}-free medium about half of the original amount was lost. This agrees with earlier studies showing that one of the ions is more easily liberated than the other, although in those cases the removal of the Ca^{2+} usually involved treatments to remove extrinsic polypeptides and chelation of the liberated Ca^{2+}. Since less than 1 Ca^{2+} remained after isolation and dialysis of the membranes, it is likely that Ca^{2+} was spontaneously lost from both sites. About one Ca^{2+} per PS II unit was lost during the 24-h dialysis, which exceeds the contribution expected from release from the activity-activating site (Figure 4), from which Ca^{2+} is lost with a half-time about 80 h. This may indicate that a fraction of the Ca^{2+} released during the 24-h period originates in the site proposed to be located in the light-harvesting complex.

There have been reports in the literature that the methods commonly used to remove Ca^{2+} from PS II, athough they reduce the water-splitting activity, do not result in a reduction in the amount of bound Ca^{2+} (Shen et al., 1988; Enami at al., 1989; Shen & Katoh, 1991). For several reasons these reports have been subjected to criticism (Boussac & Rutherford, 1992). Our results support the commonly held view that low-pH/citrate treatment results in the removal of 1 Ca^{2+}/PS II with a corresponding loss of activity (Ono & Inoue, 1988). First, as observed by others, application of the method resulted in the loss of most of the activity, which was subsequently recovered by readdition of Ca^{2+}. Second, maximally about 1 Ca^{2+}/PS II reassociated with the depleted membranes, suggesting that reactivation involved a single site. The close correlation between reincorporation of Ca^{2+} and reactivation of oxygen evolution provides additional evidence that binding occurs at the "functional" site. Third, reapplication of the depletion procedure on the labeled membranes resulted in the removal of most of the labeled Ca^{2+}, showing that this depletion method specifically attacks one specific site, i.e., the site related to oxygen-evolving activity. Although the evidence concerning the correlation between loss of water-splitting activity and depletion of Ca^{2+} using the NaCl/EGTA method is less complete, the stoichiometry of Ca^{2+} binding (1 Ca^{2+}/PS II) to depleted and inactivated membranes indicates that a specific site is involved. Other evidence comes from the observation that when NaCl/EGTA-treated and labeled membranes again were treated with EGTA , the labeled Ca^{2+} was lost from the membranes in a process which was accelerated in light. Since it is known that the deactivation of water splitting by NaCl/EGTA is also stimulated by light, this strongly supports the general view that the deactivation is coupled to removal of Ca^{2+} from PS II. Additional evidence for a correlation beween water-splitting activity and binding of Ca^{2+} may be found in the presence of two binding affinities for Ca^{2+} and in the values of their magnitudes both from activity measurements and from our observations of Ca^{2+} binding.

Previous measurements of the affinity for Ca^{2+} in PS II [reviewed in Ghanotakis and Yocum (1990), Yocum (1991), Debus (1992), and Rutherford et al. (1992)], based on activation of oxygen evolution in NaCl-washed material, have indicated the existence of at least two classes of binding sites with dissociation constants of about 100 μM and 2 mM, respectively, with some indications of an additional site with much higher affinity in a more resolved type of PS II preparation (Kalosaka et al., 1990). The two classes of binding sites with high and low affinity for Ca^{2+} and proportions of 3:1, which we have found when the binding of Ca^{2+} to NaCl-washed PS II membranes was measured directly, show an apparent similarity to the binding sites observed in the previous activity studies.

It is intriguing that dissociation constants of the high- and low-affinity sites and their proportions are practically unaffected by the measuring conditions. It has been suggested that the high- and low-affinity sites, found in activity studies,

might be associated with the S_1 and S_0 states, respectively (Boussac & Rutherford, 1988), which display a similar 3:1 distribution in the dark. However, this assignment is ambiguous since the population of the S-states under conditions of turnover, where the affinities were previously measured, is expected to deviate significantly from the distribution in the dark. Furthermore, although we observe a similar 3:1 population of the two classes of sites in the dark, it is unlikely that these are associated with the S_1 and S_0 states, since extended dark-adaptation, comparable to that used in our binding experiments, is expected to predominantly populate the S_1 state (Styring & Rutherford, 1987), which should then result in only one class of binding site. Therefore, one may have to search for other causes of the binding heterogeneity. For example, since it has been observed that low-pH treatment may convert high-affinity Ca^{2+} binding to low affinity (Ono & Inoue, 1988; Homann, 1988), differences in protonation state among the PS II centers could give rise to the observed heterogeneity in affinity. Variations in protonation were invoked to account for the different affinities for Ca^{2+} of the various S-states in the hypothesis presented in Boussac and Rutherford (1988).

In the present work we demonstrate similar affinities in the dark in PS II membranes complete with the 16- and 23-kDa polypeptides and in membranes where these are absent. The sizes in the dark of the two populations of binding sites, about 70% high-affinity sites and 30% at low affinity, are similar to those in the NaCl-washed centers, which suggests a common cause of the binding heterogeneity. In both cases, the biphasic dissociation kinetics (Figures 4 and 7) may reflect the difference in affinity for Ca^{2+} of two separate populations of centers, where the faster, minor reaction transient is likely to correspond to the smaller population of low-affinity sites, whereas the majority of the centers, i.e., those with high affinity, display the slower dissociation kinetics.

One important conclusion which may be drawn from the present study is that removal of the 16- and 23-kDa extrinsic proteins does not reduce the affinity for Ca^{2+} but rather causes a noticeable increase. Instead, the polypeptides dramatically affect the rate of exchange of Ca^{2+} with the medium, which is evident if one compares the rates of binding and dissociation of Ca^{2+} in intact with those in NaCl-washed PS II centers (Figures 1, 4, 5, and 7).

As can be seen from the dissociation experiments in Figures 4 and 7, the rate of dissociation of Ca^{2+} in both types of centers is clearly stimulated by illumination. The accelerating effect of light on the dissociation of Ca^{2+} in NaCl-washed PS II centers fully agrees with earlier observations that the inhibition of electron transport, induced by salt washing and ascribed to loss of Ca^{2+}, was stimulated in light (Decker et al., 1984). The results in Figures 4 and 7 also imply that dissociation of Ca^{2+} from higher S-states (S_2 and S_3), which are likely to be present during illumination, is faster than in the lower, dark-stable S-states which are responsible for the dissociation in the dark and give direct experimental support for the suggestion (Boussac & Rutherford, 1988) that the binding of Ca^{2+} in the S_2 and S_3 states is different from that in the S_0 and S_1 states.

The results of our studies also explain why earlier experiments have been interpreted to show that at least the 23-kDa subunit creates a high-affinity site for Ca^{2+} (Ghanotakis et al., 1984b; Homann 1988). This interpretation was mainly based on observations that PS II membranes devoid of the 16- and 23-kDa proteins and incubated with Ca^{2+} lost their activity when diluted in a Ca^{2+}-free assay medium. When we repeated this experiment, we observed that, despite about one bound Ca^{2+} (in addition to the Ca^{2+} ion likely to be present in the antenna), NaCl-washed, polypeptide-depleted PS II centers were unable to sustain oxygen evolution (disregarding the residual activity of intact PS II centers) on dilution in the Ca^{2+}-free assay medium (Figure 8). In agreement with previous observations, we found that the activity could be restored by adding Ca^{2+} to the assay medium. The virtually immediate loss of activity after illumination of polypeptide-deficient PS II centers can be understood if the dissociation of Ca^{2+} is much faster in the strong light used in the oxygen evolution assay than in weak light (cf. Figure 7). This is reasonable since strong light should promote population of the higher S-states and consequently a faster release of Ca^{2+}. Without Ca^{2+} supplementation, the total concentration of Ca^{2+} in the assay medium, based on the Ca^{2+} content of the NaCl-washed membranes, was about 0.2 μM, far below the concentration needed to saturate the high-affinity site found previously to be associated with the reactivation of oxygen evolution (about 100 μM).

Furthermore, since Ca^{2+} in the assay medium restores the activity immediately in polypeptide-deficient PS II membranes (Figure 8), not only dissociation but also reassociation of Ca^{2+} with its binding site must be much faster than observed in the dark (cf. Figure 5), leading to the conclusion that the rate of association of Ca^{2+} with the higher S-states must be higher than with the dark-stable S_0 and S_1 states.

The magnitude of the effect of strong light on the rate of exchange of Ca^{2+} appears to be quite different in intact and in NaCl-washed, polypeptide-deficient PS II centers, which is evident if one compares the relation between bound Ca^{2+} and electron transport activity in the two types of preparation. With the extrinsic subunits present, the activity closely follows the amount of incorporated Ca^{2+} when measured in a Ca^{2+}-free assay medium (Figure 3). This shows that, even if the dissociation of Ca^{2+} is accelerated in the higher S-states (Figure 4), the loss of Ca^{2+} in these states is not fast enough to influence the activity significantly in the assay of oxygen evolution.

From this analysis one may conclude that one important function of the 23-kDa protein and possibly also the 16-kDa protein is to severely restrict exchange of Ca^{2+} with the environment rather than to increase the affinity for Ca^{2+}. This might be accomplished if the 23-kDa protein constitutes a physical barrier for Ca^{2+} exchange between the binding site with intrinsically high affinity and the medium. The consequences of removal of this barrier for the activity appear to be particularly pronounced in the higher S-states where the intrinsic exchange rate for Ca^{2+} is rapid. An additional conclusion which might be drawn is that possible structural effects on the intrinsic proteins, induced by binding of the 23-kDa extrinsic protein (Ono et al., 1992), do not seem to have a large influence on the affinity for Ca^{2+}, at least not in the lower S-states. The picture of a Ca^{2+} -binding site shielded by the 23 kDa polypeptide, which follows from the present results, closely adheres to the hypothesis put forward in Homann (1988) and in Miyao and Murata (1987).

The reduced binding of Ca^{2+}, after the additional alterations in the donor-side structure induced by removal of the

33-kDa protein, may be explained by a genuine lowering of the affinity for Ca^{2+}, possibly in combination with a rise in the exchange rate. Although the high ionic strength (1 M $MgCl_2$) used to remove the 33-kDa protein (Table 2) may have influenced the loss of Ca^{2+}, it is clear from the results in Figure 9, where the binding was done at low ionic strength, that the extent of binding of Ca^{2+} to membranes without the 33-kDa protein is intrinsically much lower than to NaCl/EGTA-treated membranes. Therefore it is likely that removal of the 33-kDa protein is the main factor responsible for the release of Ca^{2+}. The reduced binding of Ca^{2+} in the absence of the 33-kDa protein may result from a further increase in accessibility of the Ca^{2+}-binding site, compared to the situation in NaCl-washed membranes, suggesting that the binding site may be located at a position where exchange with the environment normally is restricted by the 33-kDa protein. The location may be close to the manganese center. Evidence for manganese-dependent binding of Ca^{2+} is indicated by the lower labeling after Tris treatment (Figure 9), in agreement with some earlier studies (Tamura & Cheniae, 1988). This dependence is augmented further by the additional apparent decrease in affinity, relative to that in polypeptide deficient PS II centers, after removal of the manganese by treatment of membranes with the reductant hydroquinone (Table 2). A binding site for Ca^{2+} functionally or physically associated with that of manganese has been implied in earlier photoactivation studies (Ono & Inoue, 1983b; Tamura & Cheniae, 1988), from the effect of Ca^{2+} removal on the EPR properties of the S_2 and S_3 states (Boussac et al., 1989; Sivaraja et al., 1989; Ono & Inoue, 1990) and from X-ray absorption measurements (Yachandra et al., 1993).

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