Calcium Depletion Modifies the Structure of the Photosystem II O_2-Evolving Complex†

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ABSTRACT: A 5 min exposure of photosystem II to a pH 3 citric acid solution is a simple method for selective removal of Ca^{2+} from the O_2-evolving complex. The resulting preparation retains the 23 and 17 kDa extrinsic polypeptides, but the activity of this material is only 10−20% of that of an untreated control sample. Biochemical characterization of citrate-treated photosystem II reveals that some reaction centers lose the extrinsic proteins during citrate treatment. Furthermore, a comparison of photosystem II preparations treated with citrate, or depleted of 23 and 17 kDa extrinsic polypeptides by high-salt treatment, shows that low concentrations of a small reductant, NH_2OH, which has little effect on the activity of intact photosystem II, can reduce and inhibit the Mn cluster in both types of preparations. In contrast, a large reductant, hydroquinone, cannot access the majority of O_2-evolving centers in citrate-treated preparations, while 23 and 17 kDa-depleted material is rapidly inactivated by the reductant. Incubation of the citrate-treated samples in high (∼60 mM) concentrations of $CaCl_2$ restores 50% of the lost activity; this Ca^{2+}-reconstituted activity is chelator-insensitive, indicating that rebinding of Ca^{2+} restores the structural integrity of the O_2-evolving complex. A characterization of Ca^{2+} and Cl^- affinities in steady-state activity assays shows that citrate-treated preparations exhibit a Cl^- requirement similar to that of polypeptide-depleted photosystem II, while Ca^{2+} reactivation of O_2 evolution appears to occur at two structurally distinct sites. One site exhibits a high Ca^{2+} affinity, similar to that found in polypeptide-depleted samples, but a second, lower-affinity site also exists, with a K_M that is approximately 10 times greater than that of the high-affinity site, which is associated with centers that retain the extrinsic polypeptides. These data indicate that citrate-induced Ca^{2+} depletion causes release of the 23 and 17 kDa extrinsic polypeptides from some photosystem II reaction centers, and also modifies the structure of the polypeptide-retaining O_2-evolving centers so that the Mn cluster is exposed to small, but not large, reductants. This change may be due to subtle modifications to the structure of the photosystem II extrinsic proteins that produces a new pathway between the solvent and the Mn cluster or, alternatively, to the opening of an existing channel in the intrinsic lumenal polypeptide domain, between the solvent and the Mn cluster, that is normally occluded by a bound Ca^{2+} atom.

The active site of O_2 evolution in photosystem II (PSII),[1] called the O_2-evolving complex or OEC, is made up of a tetranuclear Mn cluster as well as one atom each of Ca^{2+} and Cl^-, as well as intrinsic and extrinsic polypeptides (*1*, *2*). Optimal stability and efficient functioning of the OEC depends on the presence of three extrinsic polypeptides whose masses are estimated to be 33, 23, and 17 kDa from SDS−PAGE experiments, but whose actual masses, derived from DNA sequences, are 26.5, 20, and 17 kDa, respectively (*3*). The largest of these subunits, called manganese-stabilizing protein (MSP), is required to stabilize the ligation of the Mn cluster in the dark, and to promote rapid redox cycling of the OEC in the light. In the absence of MSP, the dark-adapted Mn cluster dissociates, releasing two Mn^{2+} ions into solution (*4*). In the light, MSP-depleted PSII catalyzes H_2O oxidation at slower rates (∼40% of the control), and is sensitive to inhibition by high light intensities (*4*, *5*). In contrast, the 23 and 17 kDa polypeptides have not been shown to directly affect the stability or catalytic activity of PSII Mn. Instead, these proteins are necessary for the high-affinity retention of Ca^{2+} and Cl^- within the OEC (*6*). However, removal of the 23 and 17 kDa polypeptides, or of the functionally related subunits, called psbU and psbV, from cyanobacteria has been shown to lead to the formation of a novel low-field ($g = 12$) EPR multiline signal associated with the dark-adapted S_1 state of the enzyme (*7*, *8*). These spectroscopic results have been useful in providing direct evidence that the smaller extrinsic subunits of the OEC can have a regulatory effect on the magnetic properties of the PSII Mn cluster, even though they do not appear to control its reactivity.

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; OEC, O_2-evolving complex; PS, photosystem; SDS, sodium dodecyl sulfate; SMN, buffer composed of 0.4 M sucrose, 50 mM MES (pH 6), and 10 mM NaCl.

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Exposure of PSII to high ionic strengths (≥1 M NaCl) is often used to release the 23 and 17 kDa polypeptides (*6*). In the resulting polypeptide-depleted preparations, O_2 evolution rates are lowered to ~5−10% of the control activity assayed with intact PSII. Substantial rates of O_2 evolution activity are recovered by these preparations upon addition of high (millimolar) concentrations of Ca^{2+} and Cl^- (*6*, *9*). Neither of these inorganic cofactors is retained with high affinity by the salt-washed preparations (*6*, *10*, *11*). Rebinding of the 23 and 17 kDa polypeptides to PSII in the absence of added Ca^{2+} does not restore activity, but O_2 evolution is recovered upon long-term incubation of the reconstituted preparation with Ca^{2+} (*12*). When polypeptide-depleted PSII was exposed to reagents (NH_2OH and hydroquinone) that reduce the Mn cluster to Mn^{2+} and destroy O_2 evolution activity, it was found that low (~100 μM) concentrations of NH_2OH were much more effective than was the case with intact PSII preparations, where 1 mM NH_2OH was required to produce the same rate and extent of inactivation of the OEC (*13*). Intact PSII is unaffected by exposure to hydroquinone, in contrast to the strong activity inhibition and Mn^{2+} release obtained when the polypeptide-extracted enzyme is exposed to the reductant (*13*). These results are interpreted to indicate that the 23 and 17 kDa polypeptides form part of the larger structure that promotes high-affinity retention of Ca^{2+} and Cl^- within the OEC (*13*), and that protects the PSII Mn cluster from inhibition by exogenous reductants such as quinones. Characterization of the properties of PSII samples that have been reconstituted with the 23 kDa polypeptide alone has led to the conclusion that this polypeptide is involved specifically in Ca^{2+} retention (*14*) and shielding the Mn cluster (*13*). Selective resolution and reconstitution studies with the 17 kDa subunit indicate that this species promotes high-affinity Cl^- binding by PSII (*15*).

An alternate, rapid method for depletion of Ca^{2+} from PSII does not appear to detach the 23 and 17 kDa polypeptides from their binding sites on PSII (*16*). This method, exposure of PSII to pH 3 using buffered citrate solutions, has been shown to inhibit O_2 evolution activity; the resulting preparation can be reactivated by incubation in the dark with $CaCl_2$ (*16*). Recovery of O_2 evolution is not instantaneous, but requires incubation times of up to 50 min for maximal reconstitution of activity. This finding is similar to the result obtained with high-salt-extracted PSII samples to which the 23 and 17 kDa polypeptides have been rebound. In both preparations, the activities that are recovered after $CaCl_2$ incubation resemble those associated with intact PSII preparations in terms of their lack of dependence on added Ca^{2+} in the assay buffer, and in their insensitivity to EDTA or EGTA (*12*, *16*), which would chelate any unbound Ca^{2+} in the assay system. This behavior toward the chelators indicates that Ca^{2+} has rebound to its native site in PSII, where it is screened from the chelators by the enzyme's extrinsic subunits.

In the case of citrate-induced Ca^{2+} release from PSII, a study of the effect of lowered pH on polypeptide binding to PSII found that the 23 and 17 kDa polypeptides were labile upon long-term exposure to low pH, and actually dissociated from their binding sites in PSII (*17*). In light of this finding, it appears that the pH 3 treatment may facilitate Ca^{2+} removal from PSII by two means: metal chelation by a tricarboxylic acid and temporary dislocations of the extrinsic polypeptides to facilitate release of Ca^{2+} from its binding site in the OEC. Tso et al. (*18*) conducted an EPR study of structural changes in the PSII Mn cluster in which they used a 30, rather than a 5 min, exposure to citrate to prepare Ca^{2+}-depleted samples. The PSII samples treated in this way exhibited somewhat enhanced (~10−15% more than an untreated sample) levels of Mn^{2+} formation when exposed to NH_2OH; activity of the preparation before or after inhibitor treatment was not reported. In this paper, we show that after a short [5 min (*16*)] period of citrate treatment, the polypeptide structure of the OEC has been modified to permit more rapid access of a small reductant, NH_2OH, to the Mn cluster. Inhibition studies on citrate-treated PSII using the large reducing agent hydroquinone produce a heterogeneous response; while some centers are sensitive to hydroquinone inhibition, the majority of centers are resistant to inhibitory Mn reduction. Consistent with this finding, we have discovered that Ca^{2+} binding to reactivate the O_2-evolving reaction exhibits biphasic behavior, with low- and high-affinity sites that reflect the mixed integrity of the OEC population created by citrate treatment. In contrast, the kinetics of Cl^- binding in the light are monophasic, indicating the presence of a single type of binding site, independent of the presence or absence of the 23 and 17 kDa extrinsic components of the OEC.

MATERIALS AND METHODS

Isolation of PSII Membranes. Photosystem II preparations were isolated from market spinach according to the method of Berthold et al. (*19*) as modified by Ghanotakis et al. (*20*). PSII membranes were resuspended in a final buffer (SMN) containing 400 mM sucrose, 50 mM MES (pH 6.0), and 10 mM NaCl and were stored at −70 °C.

Salt Wash and Citrate Treatment Procedures. The 17 and 23 kDa extrinsic proteins were removed by incubation of PSII samples in 2 M NaCl (*21*). Following an SMN wash, these samples were again stored in SMN. Citrate treatment was performed essentially as described in ref *16*. Samples were washed in a 400 mM sucrose, 20 mM NaCl solution, and then resuspended to a concentration of 4 mg/mL in the same solution. An equal volume of 400 mM sucrose, 20 mM NaCl, and 20 mM citric acid (pH 3.0) was then added with stirring, and this mixture was allowed to incubate for 5 min, after which the pH was returned to 6.0 by dilution with SMN buffer.

SDS−PAGE and Protein Analysis. SDS−PAGE was performed using a Neville buffer system described by Piccioni et al. (*22*). Prior to loading, PSII membranes were incubated at 250 μg of Chl/mL in a denaturing solution of 2.6 M urea, 2% SDS, 0.04% bromophenol blue, 60 mM DTT, and 50 mM Na_2CO_3 at 37 °C for 15 min, and 16 μg of Chl was loaded per lane. Concentrations of individual protein bands were determined, after staining with Coomassie Blue, using an LKB laser densitometer and Gel Scan XL. Lanes were normalized relative to one another using the 47 kDa protein band (whose intensity was a function of Chl content only) as a standard to account for small variations in protein loading.

Other Assays. Chlorophyll concentrations were assayed in 80% acetone (v/v) according to the method of Arnon (*23*). PSII reaction center concentrations were based on a stoichiometry of 250 Chls per PSII complex (*20*). O_2 evolution

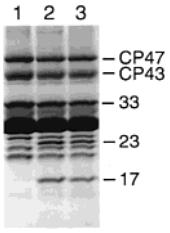

FIGURE 1: Effects of washing with 2 M NaCl or of exposure to pH 3 citrate on the extrinsic polypeptide composition of PSII: lane 1, salt-washed PSII; lane 2, native PSII; and lane 3, citrate-treated PSII.

Table 1: Extrinsic Polypeptide Composition of PSII Samples[a]

	estimated content (%)		
	33 kDa	23 kDa	17 kDa
intact PSII	100	100	100
salt-washed PSII	90	0	0
citrate-treated PSII	83	77	80

[a] As described in Materials and Methods, Coomassie Blue staining intensities of proteins in the individual lanes of the gel were normalized to the staining intensity of the CP47 band prior to analyses to estimate extrinsic protein contents. Loss of the 33 kDa protein from salt-washed PSII membranes is taken from ref *39*.

measurements were carried out under continuous saturating illumination with a Clark-type O_2 electrode, using 2,6-dichloro-*p*-benzoquinone (DCBQ) as the electron acceptor. Rates of fully intact PSII preparations were ~600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. Unless otherwise indicated, assays were performed in a buffer containing 50 mM MES (pH 6.0) and 15 mM NaCl. For data analysis and presentation, we utilized Origin version 6.0 (Microcal Software, Inc.).

RESULTS

Figure 1 presents representative results from experiments aimed at analyzing the polypeptide contents of intact, salt-washed, and citrate-treated PSII preparations. Qualitatively, the Coomassie-stained lanes of intact (lane 2) and citrate-treated (lane 3) preparations appear to be very similar. This was examined in greater detail using laser scanning densitometric analyses of these gels; the results are presented in Table 1. In these experiments, the Coomassie-stained protein band of CP47 was used as an internal control. The staining intensity of this intrinsic membrane protein was set to 100% for all samples as a means of adjusting for small deviations in sample concentration that are due to pipetting errors during application of the sample to the gel. The data presented in Table 1 show that citrate treatment leads to the loss of approximately 20% of each of the three extrinsic proteins. This result may be a consequence of the observation that binding of the 33, 23, and 17 kDa polypeptides is pH-sensitive. These polypeptides were shown to be 50% dissociated from PSII as a result of 30 min incubations at pH 3.6 (33 kDa), 4.1 (23 kDa), and 5.0 (17 kDa) (*17*). In light of these findings, it seems likely that exposure to citrate at pH 3 can also result in limited dissociation of extrinsic polypeptides, some of which are released from PSII during sample manipulation. If this is so, it is also likely that such a partial dissociation of the extrinsic polypeptides may facilitate citric acid access to the Ca^{2+} site.

Biochemical investigations of the integrity of citrate-treated preparations provided additional information about the structural heterogeneity of citrate-treated PSII. Incubation of citrate-treated preparations in $CaCl_2$ can restore O_2 evolution activity to ~50% of the activity found in intact PSII (*16*). Figure 2A shows the effect of increasing Ca^{2+} concentrations on reactivation of the OEC under conditions where the incubation time was fixed at 60 min. These data show that our citrate-treated preparations require a somewhat higher (~60 mM) $CaCl_2$ concentration for optimal restoration of activity than that reported in ref *16*, where 50 mM $CaCl_2$ gave maximal reconstitution of activity. The sigmoidal nature of the data in Figure 2A has not been previously observed. Such behavior is indicative of the possible existence of multiple Ca^{2+} binding sites that are involved in the reactivation process. A Hill plot of these data (Figure 2A, inset) yields a slope of 2, a result that can be taken to indicate that a minimum of two Ca^{2+} binding sites are involved in the

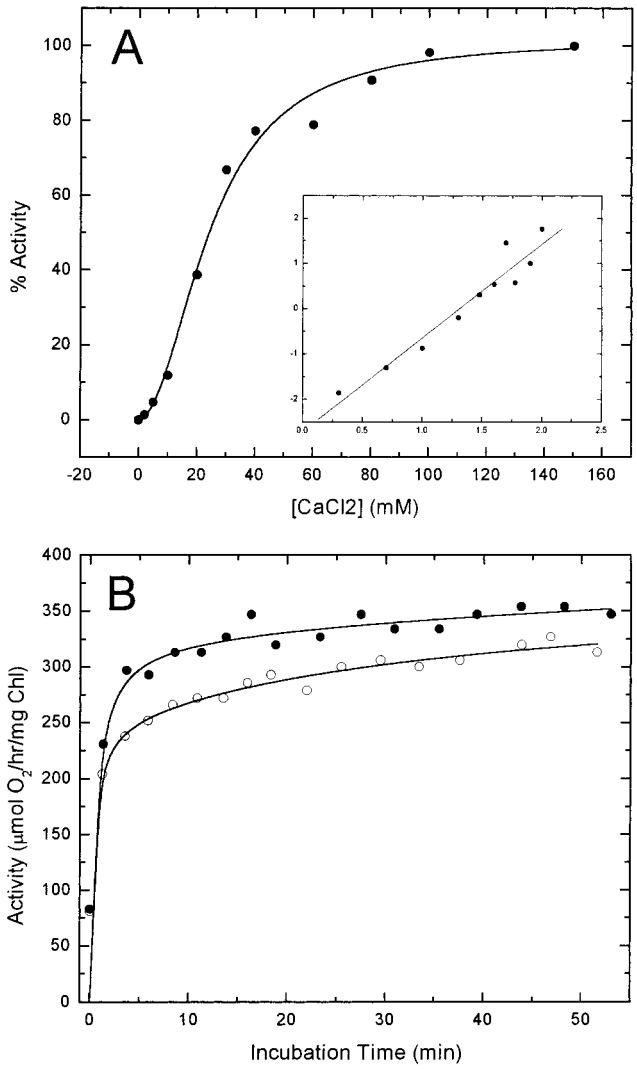

FIGURE 2: Recovery of O_2 evolution activity of citrate-treated PSII following incubation in $CaCl_2$. Samples were incubated at 4 °C for various times, and then assayed in a Ca^{2+}-free medium as described in Materials and Methods. (A) Determination of the optimal $CaCl_2$ concentration for reconstitution of O_2 evolution activity. The data shown were obtained by varying the Ca^{2+} concentration as shown at a constant (1 h) incubation time. The inset shows a Hill plot of the data. The 100% activity was 350 μmol h^{-1} (mg of Chl)$^{-1}$. (B) Effect of incubation time in 60 mM $CaCl_2$ on reconstitution of EDTA-insensitive O_2 evolution activity: (●) assays performed in 50 mM MES and 15 mM NaCl and (○) assays performed in the same medium containing 1 mM EDTA.

reactivation process (*24*). The origin of this result is discussed below.

The effect of the time of incubation on Ca^{2+} rebinding and reactivation of a citrate-treated PSII sample (Figure 2B) exposed to 60 mM $CaCl_2$ shows that a relatively short incubation time (5−10 min) is required to restore ∼85% of the maximum activity; longer (∼40−50 min) incubation allows complete equilibration of Ca^{2+}, in agreement with the results in ref *16*. To examine the nature and structural consequences of Ca^{2+} binding, the activity assays following incubation were performed in the presence or absence of 1 mM EDTA. In intact PSII preparations, bound Ca^{2+} is not readily accessible to EDTA, but the chelator removes reconstituted Ca^{2+} bound to preparations that possess an exposed OEC as a consequence of polypeptide extraction (*6*, *16*). It can be seen that while the majority of incubated oxygen-evolving centers rebind Ca^{2+} and become EDTA-insensitive almost immediately, a small population of centers remain EDTA-labile. The former centers are rather unique in that they appear to be able to bind Ca^{2+} fairly rapidly and then retain the metal under steady-state turnover conditions, whereas the latter centers, because they are sensitive to EDTA, must be modified in such a way that Ca^{2+} bound to these sites is accessible to chelators.

Two hallmarks of PSII integrity are the resistance of the OEC Mn cluster to reduction by exogenously added reagents, like hydroquinone (at millimolar concentrations) and NH_2-OH (at micromolar concentrations), which can rapidly reduce and destroy the Mn cluster in 23 and 17 kDa-depleted PSII (*13*). These structure-linked effects of Mn-specific inhibitory reductants were used to probe the integrity of citrate-treated PSII samples; intact preparations and salt-washed PSII lacking the 23 and 17 kDa polypeptides were used as control samples to fully assess the effects of reductants on protected and exposed preparations of the OEC, respectively. This comparison was made possible by the fact that citrate-treated PSII preparations could be assayed for O_2 evolution in a medium containing 20 mM $CaCl_2$, with no prior incubation. The activities obtained by this method are substantial, approximately 35% of those of an intact PSII preparation, and likely arise from some modification in the binding of PSII extrinsic proteins, after the pH 3 treatment, that facilitates Ca^{2+} access to its OEC binding site.

Figure 3A presents the results of experiments showing the effect on O_2 evolution of incubation of intact, citrate-treated, or salt-washed PSII preparations in increasing concentrations of NH_2OH. The time course of inhibition caused by exposure to a fixed (40 μM) NH_2OH concentration is shown in Figure 3B. Surprisingly, although the majority of centers in a citrate-treated sample retain the extrinsic polypeptides (Table 1), the ability of NH_2OH to inhibit this preparation is similar with respect to reductant concentration to what is observed in polypeptide-depleted preparations. As the graph shows, only minimal inhibition is observed if an intact PSII sample is exposed to the same NH_2OH concentration. These comparative data suggest that citrate treatment has modified the structure of the intact OEC to create a pathway between the external medium and the Mn cluster in this preparation. The results shown in Figure 3B indicate that the citrate-treated preparation may be inhibited more rapidly by NH_2-OH, although both preparations are ultimately inhibited to the same extent by this concentration of the reductant. The

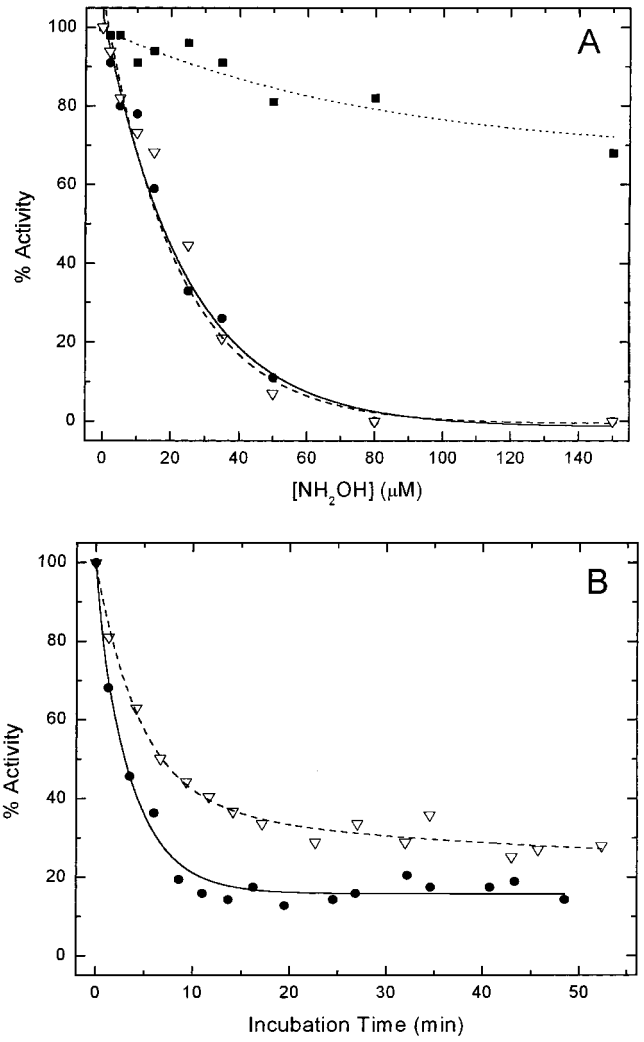

FIGURE 3: Inhibitory effects of NH_2OH on native, polypeptide-depleted, and citrate-treated PSII preparations. Samples were incubated at 4 °C for various times, and then assayed in a medium containing 50 mM MES and 20 mM $CaCl_2$. The 100% activities were 600, 290, and 200 μmol of O_2 h^{-1} (mg of Chl)$^{-1}$, respectively. (A) Comparison of NH_2OH sensitivities of PSII preparations. All samples were incubated for 1 h in increasing concentrations of NH_2-OH, and then assayed in Ca^{2+}-containing buffer, except for the intact PSII sample: (■) intact PSII, (●) citrate-treated PSII, and (▽) polypeptide-depleted PSII. (B) Effect of exposure time on NH_2-OH inhibition of O_2 evolution. Citrate-treated and salt-washed PSII samples were exposed to 40 μM NH_2OH for the times shown, and then assayed for O_2 evolution activity: (●) citrate-treated PSII and (▽) polypeptide-depleted PSII.

inhibitory effects of *N*-methylated derivatives of NH_2OH (*N*-methyl- and *N*,*N*′-dimethylhydroxylamines) on citrate-treated PSII were also examined. *N*-Methylhydroxylamine was a more effective inhibitor of PSII activity after citrate treatment [60% inhibition with 100 μM CH_3NHOH, compared to ∼5% inhibition in intact PSII (data not shown; *24*)]. The *N*,*N*′-dimethyl derivative gave a more complex result; although the majority of the activity of citrate-treated PSII was insensitive to the reductant, a 10% inhibition of activity was observed (data not shown). In contrast, intact PSII is completely insensitive to inhibition by this NH_2OH derivative (*24*; data not shown).

The partial inhibition of O_2 evolution activity by *N*,*N*′-dimethylhydroxylamine in citrate-treated PSII, coupled with the analyses of the extrinsic polypeptide content of various

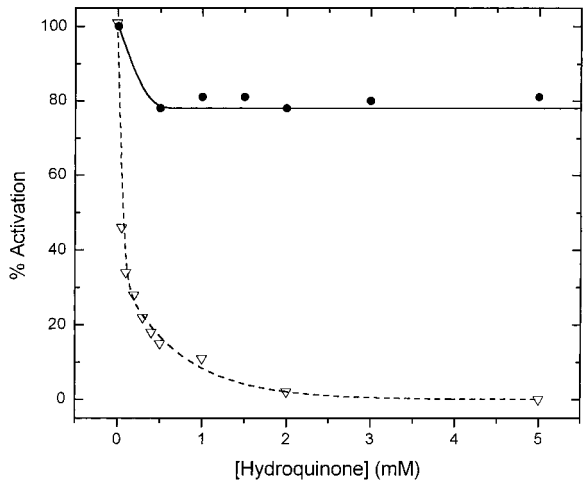

FIGURE 4: Inhibitory effects of hydroquinone on polypeptide-depleted and citrate-treated PSII preparations. Samples were incubated at 4 °C for 1 h with the indicated hydroquinone concentrations and then assayed in a buffer containing 50 mM MES and 20 mM $CaCl_2$: (●) citrate-treated PSII and (▽) polypeptide-depleted PSII. The 100% activities were 275 (salt-washed) and 220 (citrate-treated) μmol of O_2 h^{-1} (mg of Chl)$^{-1}$.

PSII preparations, suggested that some release of extrinsic polypeptides might have occurred during the pH 3 treatment step. This possibility was probed in greater detail using the large reductant hydroquinone; Figure 4 shows the concentration dependence of activity inhibition by this reductant in both salt-washed and citrate-treated PSII membrane preparations. These data confirm the existence of a major difference in the accessibility of the Mn complex of the OEC in the two PSII preparations. Retention of the 23 and 17 kDa proteins in the citrate preparation acts to severely limit access of hydroquinone to the Mn site (~20% of the maximum inhibition), in contrast to the polypeptide-depleted preparation (100% inhibition at 1 mM hydroquinone). The limited inhibition of the former preparation must therefore reflect structural heterogeneity; those reaction centers that are susceptible to inhibitory reduction by hydroquinone and *N*,*N*′-dimethylhydroxylamine are probably the same centers that have suffered loss of their complement of the 23 and 17 kDa polypeptides (Table 1), and which cannot regain EDTA-resistant O_2 evolution activity following incubation in $CaCl_2$ (Figure 2).

If citrate-treated PSII preparations are more susceptible to NH_2OH inhibition, then it is possible that they might exhibit unusual kinetics with respect to rebinding of Ca^{2+} and Cl^-. This possibility was explored in a series of steady-state assays in which concentrations of Ca^{2+} and Cl^- were systematically varied in assay reaction mixtures without a prior dark incubation period. Figures 5 and 6 present the results of these experiments. Figure 5 shows the results obtained when the Ca^{2+} concentration was varied at a constant (40 mM) Cl^- concentration. The plot of O_2 evolution rate versus Ca^{2+} concentration, shown in Figure 5A, reveals two notable features of Ca^{2+} reactivation of the OEC in citrate-treated PSII. First, at the lowest concentrations of Ca^{2+} used in these experiments, there is no sigmoidal behavior associated with Ca^{2+} reactivation in the assay system, in contrast to the results presented in Figure 2A for activation by dark incubation in Ca^{2+} prior to steady-state assays. The conditions of incubation and assay (room temperature rather

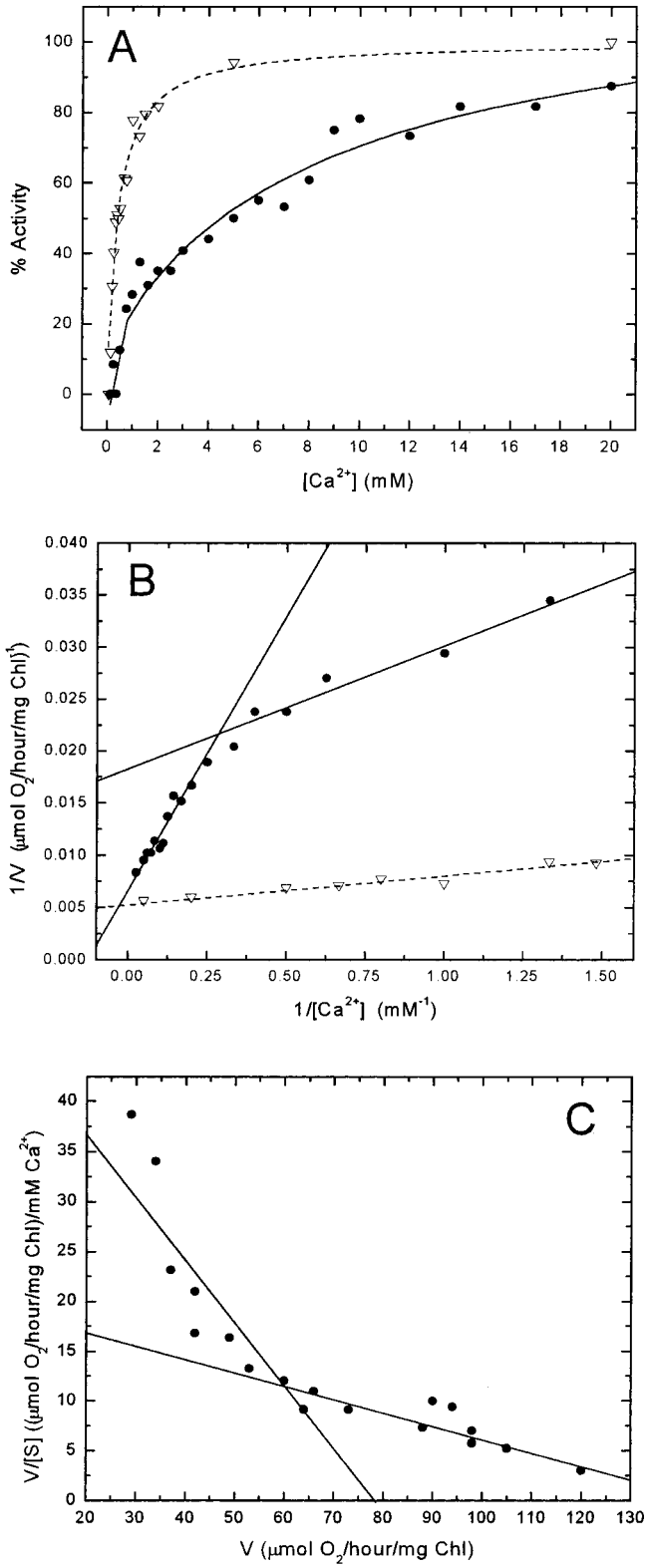

FIGURE 5: Comparative analysis of Ca^{2+} affinities in polypeptide-depleted and citrate-treated PSII preparations. Samples were assayed in a medium containing 40 mM Cl^- and increasing concentrations of Ca^{2+}. The Cl^- concentration was held constant by additions of the appropriate amounts of $(CH_3)_4N^+Cl^-$ and $CaCl_2$: (●) citrate-treated PSII and (▽) polypeptide-depleted PSII. Maximum (100%) restored activities were 233 (citrate-treated) and 280 (salt-washed) μmol of O_2 h^{-1} (mg of Chl)$^{-1}$. (A) Effect of Ca^{2+} concentration on O_2 evolution. Activities are presented as percentages of the maximum reactivation that was attained. (B) Lineweaver−Burk plot of the data in panel A. (C) Eadie−Scatchard plot of the data from the citrate-treated preparation (panel A).

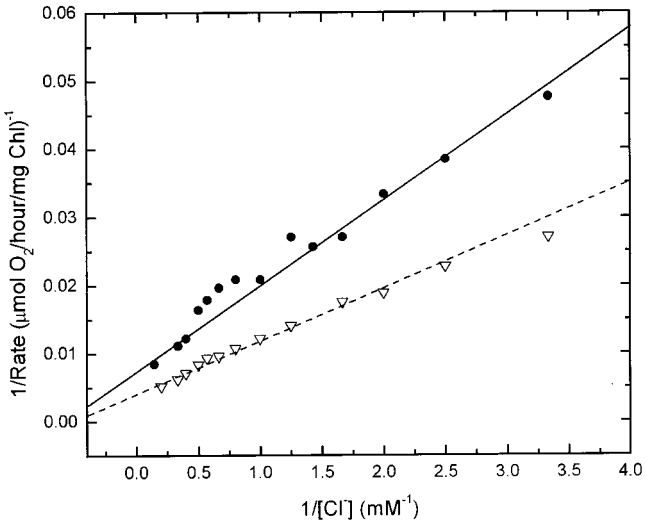

FIGURE 6: Comparison of the Cl^- requirements for O_2 evolution activity in salt-washed and citrate-treated PSII preparations. Samples were assayed in a medium containing 25 mM $Ca(MES)_2$ and varied concentrations of $(CH_3)_4N^+Cl^-$: (●) citrate-treated PSII and (▽) polypeptide-depleted PSII. The 100% activities were 200 (citrate-treated) and 240 (salt-washed) μmol of O_2 h^{-1} (mg of Chl)$^{-1}$.

than 4 °C) used in Figure 5A are responsible in part for this difference (data not shown). Second, the plot shown in Figure 5A yields an irregular curve (a break between 1 and 2 mM Ca^{2+}) for activation of the OEC in citrate-treated preparations. In theory, this can occur if Ca^{2+} binding occurs at two different types of sites, both of which activate the OEC but which exhibit different affinities for the ion (*25*). This was examined by two different methods of plotting steady-state kinetic data. As shown in Figure 5B, the standard Lineweaver−Burk double-reciprocal plot of the data obtained with citrate-treated preparations is nonlinear, which could be indicative of multiple sites of Ca^{2+} action. Figure 5B also shows the results from a salt-washed preparation, where the linear plot of the data reveals the presence of a single type of Ca^{2+} site. The existence of two sites of Ca^{2+} binding was confirmed by modeling the kinetic results from the citrate preparation as a reaction catalyzed by two enzymes with different K_M and V_{max} values (*25*). This can be assessed using an Eadie−Scatchard plot (Figure 5C), which places less weight on the data points of least confidence than does the standard Lineweaver−Burk plot (*25*). Figure 5C clearly shows the presence of two separate types of Ca^{2+} sites that activate the OEC in citrate preparations. These plots can only be used to provide approximate values for the kinetic constants of the separate activities (*25*). This is because the rate (v) of the reaction at any substrate ($[Ca^{2+}]$) concentration is given by the expression

$$v = V_{max1}[Ca^{2+}]/K_{M1} + [Ca^{2+}] + V_{max2}[Ca^{2+}]/K_{M2} + [Ca^{2+}]$$

which illustrates the difficulty in obtaining exact V_{max} and K_M values for reactions 1 and 2. The data points at various Ca^{2+} concentrations on the Eadie−Hofstee plot are distorted by the presence of a mixture of two reactions with different K_M and, possibly, V_{max} values. For this reason, the kinetic parameters obtained from the Eadie−Scatchard plot must be taken as approximations of the true values. These values, as well as those from the Lineweaver−Burk plot for a salt-washed PSII preparation, are presented in Table 2.

Table 2: K_M and V_{max} Values for Ca^{2+} and Cl^- Rebinding to Citrate Preparations and Polypeptide-Depleted PSII

	23 and 17 kDa-depleted PSII		citrate-treated PSII	
cofactor	K_M (μM)	V_{max} [μmol h^{-1} (mg of Chl)$^{-1}$]	K_M (μM)	V_{max} [μmol h^{-1} (mg of Chl)$^{-1}$]
Ca^{2+}	500	170	800[a]	60[a]
Ca^{2+}			6500[a]	140[a]
Cl^-	1900	208	1800	125

[a] This number does not reflect the actual K_M and V_{max} values of these activities. See the text for details.

Several points are revealed by the data in Table 2. First, for citrate-treated preparations, two Ca^{2+} binding sites are resolved, with widely differing estimates for their associated K_M and V_{max} values. The site with an estimated K_M of 800 μM exhibits a Ca^{2+} affinity on the order of that observed in some salt-washed preparations lacking the 23 and 17 kDa polypeptides (*6*, *17*). The lower estimated V_{max} [60 μmol h^{-1} (mg of Chl)$^{-1}$] associated with this site suggests that this type of binding site would be present at a lower concentration than the other site. The lower affinity of the second site (estimated K_M of 6500 μM) suggests that Ca^{2+} access to its OEC binding site is strongly impeded in these centers, most likely by the retained extrinsic polypeptides that shield the Mn cluster from destruction by hydroquinone. The estimate for V_{max} determined for this site [140 μmol h^{-1} (mg of Chl)$^{-1}$] would suggest that these centers are more abundant than the "exposed" centers detected by their reactivity with hydroquinone. These estimates of relative concentrations of polypeptide-sufficient and -depleted centers require the assumption that both types of centers exhibit the same k_{cat}; this is not strictly true because polypeptide-sufficient centers may not be completely reconstituted by short-term Ca^{2+} incubation. Thus, the apparent number of polypeptide-sufficient centers present in a citrate preparation represents an underestimate, and the actual number of centers is higher. This outcome is nevertheless consistent with the results from the experiments probing citrate preparations with reductants. Hydroquinone-sensitive, polypeptide-depleted centers represent ~20−25% of the active Ca^{2+} sites in the preparation (see Figure 4). It is also apparent that the estimated Ca^{2+} K_M for polypeptide-sufficient centers is a function of reconstitution conditions. The K_M obtained from assays without long incubation periods yields an estimated K_M of ~6.5 mM, whereas protracted dark incubation with the ion (Figure 2A) yields an estimated $K_{0.5}$ value for Ca^{2+} of ~25 mM. Possible explanations for the approximately 4-fold difference in these results are that the steady-state assays were performed in the light, at room temperature. One or both of these factors may be involved in facilitating Ca^{2+} binding. An alternative possibility is that, in the O_2 evolution assays involving additions of Ca^{2+} to the assay medium, the ion is not tightly bound to its site, but is nevertheless able to restore OEC function; if this were true, it might explain the lower maximum O_2 evolution rates obtained in these assays.

Figure 6 presents results from a similar set of kinetic experiments in which the level of Ca^{2+} was held constant in O_2 evolution assays using 25 mM $Ca(MES)_2$, and the

concentration of Cl^- was increased using $(CH_3)_4N^+Cl^-$ (*26*). As the Lineweaver−Burk plot shows, citrate-treated and polypeptide-depleted PSII preparations possess very similar Cl^- requirements; the K_M and V_{max} values are reported in Table 2. While fully intact PSII preparations have a very low [$K_M \sim 100$ μM (*9*, *27*)] Cl^- requirement, polypeptide depletion creates an elevated demand for the anion because the absence of the polypeptides allows rapid exchange between the PSII Cl^- binding site and the surrounding medium (*11*). The fact that citrate-treated preparations show a Cl^- requirement similar to that of salt-washed rather than intact PSII membranes would indicate that the structural changes induced by Ca^{2+} loss affect Cl^- retention by the OEC, as well as reductant access to the PSII Mn cluster.

DISCUSSION

Although the role of the 23 and 17 kDa extrinsic polypeptides in regulating Ca^{2+} and Cl^- binding by the OEC has been examined in several studies, any changes in the protein structure of the OEC that might arise from removal of Ca^{2+} by citrate treatment have been difficult to uncover because retention of extrinsic polypeptides screens the OEC from probes added to the external medium. The experiments of Shen and Katoh (*17*) showed that use of acidification for Ca^{2+} removal could lead to a loss of extrinsic proteins by PSII, and the data we present here provide new evidence that subtle changes in OEC structure are caused by acid-induced Ca^{2+} removal. An unusual result comes from Ca^{2+} reconstitution data that reveal an anomalous sigmoidal rather than hyperbolic binding behavior of the metal (Figure 2A) associated with long-term dark incubation experiments. A Hill plot (inset of Figure 2A) indicates that at least two Ca^{2+} ions are required for reactivation of the OEC. Convincing evidence (*10*, *28*) that the OEC ligates a single Ca^{2+} atom (*10*, *28*) makes it probable that the additional Ca^{2+} required for activation of the citrate-treated preparation plays a secondary role in reassembly of the native OEC. In fact, short-term ($\sim$30 s) incubation with Ca^{2+} prior to the assay at 25 °C abolishes sigmoidal Ca^{2+} binding behavior (Figure 5A); preliminary evidence suggests that loss of this behavior is due to an increased ionic strength and/or temperature (data not shown).

When comparisons of Mn cluster sensitivity to an exogenously added reductant in citrate-treated PSII are made with intact or salt-washed forms of the enzyme (Figure 4), it can be seen that acid-induced Ca^{2+} extraction modifies OEC structure. Figure 4 shows that citrate-treated PSII exhibits a limited sensitivity to hydroquinone inhibition under conditions where the intact enzyme is insensitive to the reductant (*13*), and where the salt-washed, polypeptide-depleted enzyme is very sensitive to inhibition (Figure 4). Loss of $\sim$20% of the O_2 activity to hydroquinone reduction in citrate-treated PSII is in reasonable agreement with the data in Table 1, which show that a comparable fraction of centers have lost extrinsic polypeptides. This same polypeptide loss probably accounts for the presence of the small fraction of EDTA-sensitive activity reconstituted by Ca^{2+} incubation in citrate PSII (Figure 2B); Ca^{2+} binding to 23 and 17 kDa-depleted PSII centers is sensitive to chelation (*6*, *10*, *12*, *16*). Steady-state assays also reveal Ca^{2+} binding heterogeneity in the citrate preparations (Figure 5). Probing citrate-treated PSII with a smaller reductant, NH_2OH, yielded additional information about structural modification to the Ca^{2+}-depleted enzyme. As the results of these experiments show (Figure 3), despite extensive retention of the smaller extrinsic proteins, the citrate preparation is approximately as sensitive to inhibition by NH_2OH as is a salt-washed preparation depleted of the 23 and 17 kDa proteins, where extensive inhibition is obtained with either hydroquinone or NH_2OH.

Information about the relationship between Ca^{2+} extraction and alterations in the OEC can also be obtained from a comparison of citrate-treated samples with PSII preparations depleted of Ca^{2+} by high-salt treatment and then reconstituted with the 23 and 17 kDa polypeptides in the absence of Ca^{2+} (*12*). Like the citrate preparation, polypeptide-reconstituted samples require periods of dark incubation (up to $\sim$50 min) for optimal reactivation by dark incubation in $CaCl_2$. In these experiments, the polypeptide-reconstituted samples exhibited a Ca^{2+} K_M of $\sim$500 μM, which is much lower than the value observed for the PSII centers that retain the extrinsic polypeptides after citrate treatment, where a K_M of 6.5 mM is obtained (Table 2). However, the Ca^{2+} incubation conditions are not comparable; 23 and 17 kDa-reconstituted samples were incubated at 25 °C, whereas the citrate samples were incubated with Ca^{2+} at 4 °C to reproduce the conditions originally used with these preparations (*16*). Both types of samples can also be partially reactivated by brief incubation with Ca^{2+} prior to assay under continuous illumination. Although illumination might be hypothesized to play a role in rapid Ca^{2+} reincorporation into citrate-treated PSII (*29*−*31*), retaining the extrinsic polypeptides (work in progress, noted above) points to temperature and/or ionic strength as the agents that accelerate Ca^{2+} rebinding to citrate PSII.

Although a reinvestigation of Ca^{2+} binding to PSII is not a primary aim of this work, it is instructive to compare the results presented here with those from other characterizations of citrate-treated or intact PSII (*10*, *31*) preparations. Calcium binding properties of citrate-treated PSII in the dark at equilibrium (incubation for up to 20 h with $^{45}Ca^{2+}$) show two sites (K_d = 60 μM and 1.3 mM) in a ratio of $\sim$3:1, which were attributed to a possible heterogeneity of Ca^{2+} binding by the dark-stable S_1 state (*10*). The existence of more than one binding site in the light cannot be confirmed with these data. Our K_M values from illuminated citrate-treated samples cannot be compared directly with the K_d values from dark binding experiments; the short incubation times we use to assess inhibition of the OEC by reductants preclude complete equilibration with added Ca^{2+}, because of the barrier imposed by the 23 and 17 kDa proteins (*6*, *10*), and under illumination, K_M values are greater than the K_d values obtained by dark incubation. In contrast to the $^{45}Ca^{2+}$ binding experiments with dark-adapted, salt-washed PSII preparations, which also identified two Ca^{2+} binding sites (K_d = 25 μM and 2.65 mM), our results from steady-state assays detect a single site (K_M = 500 μM). In studies of competitive Ca^{2+} displacement from intact, dark-adapted PSII by a number of metal ions, Vrettos et al. (*32*) found only one site, or K_d. Although metal equilibration was conducted under conditions (darkness, long-term incubations to ensure equilibrium) similar to those used in ref *10*, the analysis of binding was based on activities taken from steady-state activity assays of O_2 evolution. Collectively, these considerations indicate that differences in incubation or assay conditions, and in PSII Ca^{2+} affinities in darkness and in

the light (*30*), are factors contributing to the differences in estimates of the affinity of Ca^{2+} for PSII.

The significant points of the data that we present here are that it can be demonstrated that after citrate treatment (1) some PSII extrinsic polypeptides have been removed by acidification, (2) a small (~20%) population of PSII centers is created that are sensitive to destruction by hydroquinone, and (3) in comparison to salt-washed PSII preparations depleted of the 23 and 17 kDa polypeptides, these citrate-treated preparations exhibit two classes of Ca^{2+} binding sites, which appear on the basis of EDTA sensitivity and K_M values to be similar to the sites found in either polypeptide-depleted PSII or polypeptide-retaining preparations created by citrate treatment. With this information about the structural effects of citrate treatment in hand, the consequences of acidification on OEC structure can be divided into two categories. First, the polypeptide-depleted PSII centers in citrate-treated PSII, as far as can be determined, are structurally similar to those in salt-washed PSII membranes. That is, these centers are readily accessible to inhibition by NH_2OH and hydroquinone and exhibit facile equilibration of Ca^{2+} and Cl^- between the solvent and the binding sites for these activators. This limited loss of the 23 and 17 kDa polypeptides is probably an unavoidable consequence of the efficient Ca^{2+} removal produced by citrate at pH 3. The second consequence of citrate treatment, a more subtle structural modification of the OEC, is revealed by the finding that Ca^{2+}-depleted PSII centers retaining the extrinsic 23 and 17 kDa proteins are sensitive to inhibition by low concentrations of NH_2OH. This finding indicates that these PSII centers contain an opening through which NH_2OH, but not hydroquinone, can gain access to the Mn cluster. The similarity of the Cl^- K_M values for illuminated, salt-washed, and citrate-treated PSII preparations (Figure 6) is also a probable consequence of this alteration to OEC structure.

There are at least two plausible explanations for the results we have obtained on structurally modified PSII centers that retain their extrinsic polypeptides. The first is that Ca^{2+} extraction has removed an obstruction from the pathway by which H_2O and other small, uncharged molecules (for example, NH_2OH or NH_3) normally gain access to the OEC. In many Ca^{2+} cofactor proteins, the metal incorporates H_2O into its coordination shell (*33*), and it has already been hypothesized that in PSII one function of Ca^{2+} in the OEC would be to ligate substrate H_2O or ^-OH (*32*, *34*−*36*). Ligation of ^-OH is supported by the observation (*32*) that Ca^{2+} and its only known active surrogate ion, Sr^{2+}, are stronger Lewis acids than any of the competing ions that can displace Ca^{2+}, but which do not restore O_2 evolving activity. Proposals have also appeared which suggest that H_2O gains access to its site of oxidation through a channel in the PSII protein structure that extends from the bulk solvent to the OEC (*37*, *38*). If this is so, and if Ca^{2+} were to be ligated into a H_2O channel in the OEC, then removal of the metal from PSII might easily produce the effect on NH_2OH access to the Mn cluster that is revealed by our data. The second consequence of this hypothesis is that it predicts that the inhibitory effect of Ca^{2+} extraction on H_2O oxidation arises from loss of the substrate-binding site in the OEC. An alternate explanation for the results we have obtained must also be considered, namely, that Ca^{2+} extraction by citrate creates a global alteration to OEC structure that results in a nonspecific permeability to small ligands, as well as to the activating inorganic ions. Such an alteration might be necessary to explain the enhanced Cl^- permeability of citrate-treated PSII, which is similar to that of the salt-washed enzyme. At this point, we cannot distinguish between the pathway specific and global structural alteration models. Experiments are in progress to define conditions for, and consequences of, reincorporation of Ca^{2+} into the OEC.

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