Calcium depletion modifies the structure of the photosystem II O₂-evolving complex

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Introduction

Although Ca²⁺ is an essential cofactor for O₂ evolution (Ghanotakis, et al., 1984a) and has been shown to play a role in stabilizing the protein structure that ligates Mn (Mei and Yocum, 1991), it is probable that the metal plays additional role(s) in H₂O oxidation. In many Ca²⁺ cofactor proteins, H₂O is present as a ligand. In the case of PSII, the Lewis acidity of Ca²⁺ may be crucial to binding of either H₂O (Riggs-Gelasco, et al., 1996; Pecoraro, et al. 1998) or OH⁻ (Vrettos et al, 2001b) as substrate intermediates in the S-state cycle. The topological disposition of Ca²⁺ with respect to the Mn cluster has not been explored in detail. In this communication we present results showing that Ca²⁺ can impede access of small reducing agents (NH₂OH, N-methyl NH₂OH) to site(s) of Mn ligation in the O₂-evolving complex. We further show that Ca²⁺ extraction/restoration is a reversible process. Our results are consistent with the existence of a substrate access channel between the solvent and the PSII Mn cluster, which is partially occluded by bound Ca²⁺.

Materials and Methods

Photosystem II was isolated according to Ghanotakis, et al. (1984b) and small extrinsic proteins were removed by NaCl washing (Ghanotakis, et al. (1984a). Alternatively, Ca²⁺ was released from PSII using the pH 3 citrate method of Ono and Inoue (1988); the metal is extracted but the extrinsic polypeptides remain bound to the intrinsic core of the enzyme system. Reductant access to the Mn cluster was monitored by assaying inhibition of steady-state O₂ evolution rates with DCBQ as the electron acceptor, using samples exposed to hydroxylamines for varying periods of time in the dark. Maximal rates were obtained only when samples were incubated in Ca²⁺ before illumination.

Results

Table 1 presents the results of a series of assays to determine the K_M values for Ca²⁺ and Cl⁻ in salt-washed and citrate-treated PSII. The single Ca²⁺ K_M exhibited by the polypeptide-depleted sample reflects loss of the diffusion barrier between Ca²⁺ in solution and its PSII binding site, which correlates with extraction of the 23 and 17 kDa polypeptides (Ghanotakis, et al., 1984a). The two K_M values for Ca²⁺ that are detected in the citrate sample are consistent with the presence of structural heterogeneity created by low pH extraction of Ca²⁺; about 20% of the centers in this sample have lost Ca²⁺ and the 23 and 17 kDa polypeptides, and are therefore partially (ca. 20%) sensitive to inhibition with hydroquinone (data not shown). These centers exhibit a lower K_M than do the remaining, intact centers that retain bound extrinsic polypeptides and are insensitive to hydroquinone. The higher K_M in these
centers must be due to the presence of the 23 and 17 kDa polypeptides that form part of the
shield around the OEC. The Cl− Km for both PSII samples indicates that Ca2+ extraction,
regardless of the method, increases the Km above the level observed in intact PSII.

**Table 1:** Km and Vmax values for Ca2+ and Cl− in polypeptide-depleted and citrate-treated PSII
preparations.

<table>
<thead>
<tr>
<th>Photosystem II Preparation</th>
<th>23,17 kDa Depleted</th>
<th>Citrate-treated</th>
</tr>
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<tbody>
<tr>
<td>Cofactor</td>
<td>Km (µM) Vmax (µmol/hr/mg Chl)</td>
<td>Km (µM) Vmax (µmol/hr/mg Chl)</td>
</tr>
<tr>
<td>Ca2+</td>
<td>500 170</td>
<td>800 60</td>
</tr>
<tr>
<td>Ca2+</td>
<td>6,500 140</td>
<td></td>
</tr>
<tr>
<td>Cl−</td>
<td>1,900 208</td>
<td>1,800 125</td>
</tr>
</tbody>
</table>

The data shown in Fig. 1 below illustrate the contrasting responses of intact PSII and citrate-
treated samples to inhibition by hydroxylamines. The most prominent feature of the citrate-
treated sample, relative to the intact enzyme, is its enhanced sensitivity to NH2OH, relative to
that of intact PSII, which retains both Ca2+ and the small extrinsic polypeptides. As the data
display, N-methyl hydroxylamine, but not the dimethyl derivative, is able to reduce and destroy
the PSII Mn complex in citrate-treated PSII, but with less efficiency than NH2OH. Intact PSII
is unaffected by the N-methyl NH2OH derivatives under the conditions used here.

**Fig. 1.** Effect of hydroxylamines on inhibition of activity in citrate-treated and intact PSII. Symbols are:
□, NH2OH, intact PSII; ●, NH2OH citrate PSII; ▲,▼,citrate-treated PSII exposed to methyl and dimethyl hydroxylamines, respectively.

**Fig. 2.** Ca2+ reconstitution of citrate PSII re-
stores structural integrity to the OEC. Symbols:
○, NH2OH, citrate PSII; ●, same after Ca2+ re-
reconstitution; ▲,▼, same as Fig. 1, but after
constitution of citrate-treated PSII.
Figure 2 presents results of similar reductant probing experiments carried out on citrate-treated PSII preparations in which Ca\(^{2+}\) had been restored to its binding site. As the figure shows, \(\text{NH}_2\text{OH}\) resistance is partially restored in the Ca\(^{2+}\) incubated sample, as is insensitivity to Mn reduction by the methyl derivatives of \(\text{NH}_2\text{OH}\). Taken together, these data show that removal of Ca\(^{2+}\) exposes a sterically constrained pathway between the external medium and the Mn cluster. This shift in the structure of the OEC is reversible, in that rebinding of Ca\(^{2+}\) partially occludes the pathway by which \(\text{NH}_2\text{OH}\) and its N-methyl derivative access the Mn cluster.

Calcium depletion of PSII under conditions where the extrinsic polypeptides are retained affects the Cl\(^-\) Km of the enzyme (Table 1); there is little difference in the Cl\(^-\) Km values for salt-washed and for Ca\(^{2+}\)-extracted samples. The Cl\(^-\) affinity of citrate-treated PSII samples was examined after the reconstitution of Ca\(^{2+}\) in two ways: first, Ca\(^{2+}\)-reconstituted samples were dark incubated with Cl\(^-\) for 60 min. and assayed in Ca\(^{2+}\)/Cl\(^-\) free medium to measure stable reincorporation of Cl\(^-\). The second method used Cl\(^-\) addition to the assay medium to assess Cl\(^-\) reincorporation in the light, as was done with the samples shown in Table 1. The results are presented in Table 2. Comparison of these data with those of Table 1 reveal that reincorporation of Ca\(^{2+}\) into PSII has decreased the Cl\(^-\) Km (the lower V\(_{MAX}\) values are a result of incomplete reconstitution of high affinity Cl\(^-\) binding). These samples therefore exhibit the “one site/two affinity” Cl\(^-\) binding reported by Lindberg, et al. (1996). It is significant that we cannot observe high affinity Cl\(^-\) binding in the dark in Ca\(^{2+}\)-depleted samples; the Cl\(^-\) Km appears to be about 300 mM (not shown.

**Discussion**

Citrate-induced Ca\(^{2+}\) extraction from PSII is not an innocent process; at least 20% of centers are exposed to large reductants (data not shown) and exhibit rapidly exchangeable Ca\(^{2+}\) binding, a characteristic of salt-washed PSII preparations lacking the 23 and 17 kDa polypeptides (Table 1). Nevertheless, the remaining 80% of centers in this preparation remain intact, and as we show here, exhibit a poor affinity (high Km) for Ca\(^{2+}\). The reductant probing approach offers a facile method for assessing both the integrity of the OEC, as well as for examining the steric properties of access channels leading from the external medium to the Mn cluster. We have exploited this methodology to probe the consequences of Ca\(^{2+}\) extraction, using the citrate method to produce a sample that is minimally perturbed with respect to binding of the extrinsic polypeptides.

The data of Fig. 1 show that after Ca\(^{2+}\) removal, a pathway for \(\text{NH}_2\text{OH}\) and its N-methyl derivative is present in PSII that permits these reductants to access and destroy the Mn cluster. This pathway imposes steric restrictions on the reductants; N,N-dimethyl hydroxylamine is unable to inhibit H\(_2\)O oxidation under the incubation conditions employed for these experiments. This pathway is likely to be identical with that by which small inhibitory ligands like NH\(_3\) as well as substrate H\(_2\)O gain access to the Mn cluster. The results

<table>
<thead>
<tr>
<th>Sample Treatment</th>
<th>(K_m) (µM)</th>
<th>(V_{MAX}) (µmol O(_2)/hr/mg Chl)</th>
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<tbody>
<tr>
<td>Dark incubation with Cl(^-)</td>
<td>350</td>
<td>80</td>
</tr>
<tr>
<td>(no Cl(^-) in the assay buffer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl(^-) added to assay buffer,</td>
<td>620</td>
<td>120</td>
</tr>
<tr>
<td>(no preincubation with Cl(^-))</td>
<td></td>
<td></td>
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summarized in Fig. 2 and Table 2 show that the “open” conformation of PSII induced by Ca\textsuperscript{2+} extraction can be made to revert partially to a “closed” conformation by the simple expedient of restoring Ca\textsuperscript{2+} to its binding site.

Beyond its role in regulating the structure of the OEC (Mei and Yocum, 1991), Ca\textsuperscript{2+} has been proposed to act as a ligation site for Cl\textsuperscript{−} (Tommos and Babcock, 1998), for H\textsubscript{2}O (Rutherford, 1989; Riggs-Gelasco, et al. 1996; Pecoraro, et al. 1998) or for –OH (Vrettos, et al., 2001a). In the absence of Ca\textsuperscript{2+}, Cl\textsuperscript{−} is unable to restore H\textsubscript{2}O oxidation, even at very high concentrations (data not shown). This would not be observed if Ca\textsuperscript{2+} were necessary only to enhance Cl\textsuperscript{−} binding near the OEC. The alternate proposals view Ca\textsuperscript{2+} as a key component of H\textsubscript{2}O oxidation chemistry, i.e., as a site for binding a substrate molecule that attacks a Mn=O species in the S\textsubscript{4} state (Vrettos, et al., 2001a). Our data are consistent with these proposals. As shown in Fig. 3, the topology of Ca\textsuperscript{2+} binding, as revealed by reductant probing, places the metal in a position where it is capable of binding substrate H\textsubscript{2}O destined for oxidation by the PSII Mn cluster.

**Fig 3.** Schematic diagram of an OEC structure illustrating the topological/structural role of Ca\textsuperscript{2+} in blocking rapid access of small reductants to the PSII Mn cluster.

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**References**