Calcium controls the assembly of the photosynthetic water-oxidizing complex: a cadmium(II) inorganic mutant of the Mn$_4$Ca core

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Perturbation of the catalytic inorganic core (Mn$_4$Ca$_1$O$_x$Cl$_y$) of the photosystem II-water-oxidizing complex (PSII-WOC) isolated from spinach is examined by substitution of Ca$^{2+}$ with cadmium(II) during core assembly. Cd$^{2+}$ inhibits the yield of reconstitution of O$_2$-evolution activity, called photoactivation, starting from the free inorganic cofactors and the cofactor-depleted apo-WOC-PSII complex. Ca$^{2+}$ affinity increases following photooxidation of the first Mn$^{3+}$ to Mn$^{1+}$ bound to the ‘high-affinity’ site. Ca$^{2+}$ binding occurs in the dark and is the slowest overall step of photoactivation (IM$_1$→IM$_3^*$ step). Cd$^{2+}$ competitively blocks the binding of Ca$^{2+}$ to its functional site with 10- to 30-fold higher affinity, but does not influence the binding of Mn$^{2+}$ to its high-affinity site. By contrast, even 10-fold higher concentrations of Cd$^{2+}$ have no effect on O$_2$-evolution activity in intact PSII-WOC. Paradoxically, Cd$^{2+}$ both inhibits photoactivation yield, while accelerating the rate of photoassembly of active centres 10-fold relative to Ca$^{2+}$. Cd$^{2+}$ increases the kinetic stability of the photooxidized Mn$^1$ assembly intermediate(s) by twofold (mean lifetime for dark decay). The rate data provide evidence that Cd$^{2+}$ binding following photooxidation of the first Mn$^{3+}$, IM$_1$→IM$_3^*$, causes three outcomes: (i) a longer intermediate lifetime that slows IM$_1$ decay to IM$_0$, by charge recombination, (ii) 10-fold higher probability of attaining the degrees of freedom (either or both cofactor and protein d.f.) needed to bind and photooxidize the remaining 3 Mn$^{3+}$ that form the functional cluster, and (iii) increased lability of Cd$^{2+}$ following Mn$_1$ cluster assembly results in (re)exchange of Cd$^{2+}$ by Ca$^{2+}$ which restores active O$_2$-evolving centres. Prior EPR spectroscopic data provide evidence for an oxo-bridged assembly intermediate, Mn$^{3+}$($\mu$-O$^{2-}$)Ca$^{2+}$, for IM$_1^*$. We postulate an analogous inhibited intermediate with Cd$^{2+}$ replacing Ca$^{2+}$.

**Keywords:** calcium; manganese; oxygen evolution; photosystem II; photosynthesis; water oxidation

**Abbreviations:** Chl, chlorophyll $a$; FeCN, potassium ferricyanide K$_3$[Fe(CN)$_6$]; IM, intermediate; PSII, photosystem II; WOC, water-oxidizing complex

1. **INTRODUCTION**

The process of solar energy conversion that occurs in photosynthetic organisms ultimately stores energy via oxidizing water to produce O$_2$ gas, higher energy electrons and protons (ΔpH gradient). This is a profoundly important natural process having global biogeochemical impact and significance for bio-inspired catalyst design. The catalytic site of water oxidation occurs within a subdomain of the photosystem II pigment–protein complex (PSII) called the water oxidation centre (WOC) comprising an inorganic core, Mn$_4$Ca$_1$O$_x$Cl$_y$, found in all oxygenic phototrophs examined thus far. This core is assembled in a process called photoactivation during biogenesis and repairs of the enzyme, starting with the apo-WOC-PSII protein complex, free cofactors (Mn$^{3+}$, Ca$^{2+}$, Cl$^-$, HCO$_3^-$), an electron acceptor and light. Reconstitution of O$_2$-evolution capacity by photoactivation has been extensively studied in vitro using isolated PSII complexes (Miller & Brudvig 1989; Ananyev et al. 2001; Ono 2001; Dismukes et al. 2005; Fuller et al. 2005) and in vivo using intact cells (Nixon & Diner 1992; Burnap et al. 1996; Boussac et al. 2004; Burnap 2004; Dasgupta et al. in press). Previous O$_2$-evolution measurements by Cheniae and co-workers have shown that a two-step kinetic sequence occurs during assembly of the inorganic core comprising the PSII-WOC (Radmer & Cheniae 1971; Tamura & Cheniae 1987; Frasch & Sayre 2001). Ca$^{2+}$ is an essential cofactor for proper photoassembly of the Mn$_4$ core and for O$_2$-evolution activity. Its selective removal from the holoenzyme reversibly abolishes activity (Vander Meulen et al. 2002). If Ca$^{2+}$ is left out of the photoactivation medium, the apo-WOC-PSII protein binds and photooxides an excess number of Mn$^{2+}$ ions (as many as 20) and no O$_2$-evolution activity is observable (Chen et al. 1995; Ananyev & Dismukes 1996a).

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rstb.2007.2222 or via http://journals.royalsociety.org.

One contribution of 20 to a Discussion Meeting Issue ‘Revealing how nature uses sunlight to split water’.

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The kinetically resolved steps of in vitro photoactivation of spinach PSII membranes are shown in scheme 1. The process starts with the photooxidation of Mn$^{2+}$ to Mn$^{3+}$ bound to the ‘high-affinity’ site within PSII, symbolically denoted by square brackets (Ananyev & Dismukes 1996a; Ono 2001; Burnap 2004). Mn$^{2+}$ that binds to the high-affinity site has been shown to be spectrally different among different chemical forms depending upon the solution pH, presence/absence of Ca$^{2+}$ and bicarbonate (Ananyev et al. 1999; Baranov et al. 2004; Tyryshkin et al. 2006; Dasgupta et al. in press, unpublished work). This speciation greatly influences the copper quantum efficiency of photooxidation to Mn$^{3+}$ which yields the first intermediate IM$_1$. The Ca$^{2+}$ binding affinity increases following photooxidation of the first Mn$^{2+}$ and leads to the binding of 1 Ca$^{2+}$ ion in the dark during the second (rate-limiting) step of the two-step photoactivation kinetic sequence (Zaltsman et al. 1997). This photooxidation step, or the subsequent dark step, in which Ca$^{2+}$ binds to its effector site, is coupled to the release of a second H$^+$ in solution (Ananyev et al. 2001). This intermediate, designated IM$_1^*$, has been proposed to be an oxo- or bishydroxo-bridged species, either [Mn$^{3+}$-(µ-O)Ca$_{2+}$]$^-$ or [Mn$^{3+}$-(µ-OH)$_2$Ca$_{2+}$]$^-$, on the basis of several electron paramagnetic resonance (EPR) spectroscopic properties (Tyryshkin et al. 2006). The presence of Ca$^{2+}$ bound to its effector site, eliminates a strong pH dependence of the EPR properties of this Mn$^{3+}$ caused by locking of its ligand coordination environment.

The IM$_1$–IM$_1^*$ step occurs in the dark and is the slowest step in the overall photoactivation process (Ananyev et al. 2001). It has been postulated to be associated with a protein conformational change, principally because it is a slow process, but direct evidence for protein degrees of freedom is still lacking. The infrequency of this dark process in which Ca$^{2+}$ binding occurs to its effector site following the newly photooxidized Mn$^{3+}$ formation is responsible for the low quantum efficiency of photoactivation (Cheniae & Martin 1971; Tamura & Cheniae 1987; Hwang & Burnap 2005). The formation of this intermediate templates the system for the subsequent rapid cooperative binding and photooxidation of the final 3 Mn$^{2+}$ ions in a kinetically unresolved process that creates a functional O$_2$-evolving complex (Zaltsman et al. 1997) if Cl$^-$ is present in the medium (Ananyev et al. 1998, 2001). This 1+3 Mn stoichiometry of the overall photoassembly sequence provided early evidence for the non-equivalent partitioning of the Mn ions in the apo-WOC-PSII protein complex, as seen also by $^{55}$Mn spin densities in the S$_2$ state measured by EPR spectroscopy of the intact WOC from detergent-isolated PSII (Peloquin & Britt 2001; Carrell et al. 2002), and subsequently shown also by structural data derived from X-ray diffraction of a PSII core complex (Zouni et al. 2001; Ferreira et al. 2004).

Herein we examine the consequences of replacing Ca$^{2+}$ by the smaller more acidic Cd$^{2+}$ ion on the rate of each of the resolved steps of the photoactivation process and the yield of reconstituted O$_2$ evolution. We provide evidence that Cd$^{2+}$ acts specifically by replacing Ca$^{2+}$ and provide a critical analysis of the related literature. We provide a structural model for its site of action based on prior EPR spectroscopic and X-ray diffraction data of isolated PSII complexes.

2. MATERIAL AND METHODS

Spinach PSII enriched membranes, prepared by the Berthold–Babcock–Yocum (BBY) method were used for photoactivation samples (Ghanotakis et al. 1984). The preparation of PSII samples lacking Mn and Ca (apo-WOC-PSII membranes) was performed by alkaline washing at pH 9.0 as described previously (Baranov et al. 2004). All samples
contain 2 mM FeCN (electron acceptor) and 0.3 M sucrose 2-(N-morpholino)-ethanesulfonic acid (MES)/NaOH buffer at pH 6.0. Photoactivation was conducted at 25°C using previously described homemade instrumentation using red LED illumination with pulse duration of 30 ms and interpulse dark time of 3 s, unless otherwise stated. Complete removal of Mn was confirmed by EPR spectroscopy and by the absence of residual O₂ activity upon photoactivation using complete medium lacking added Mn²⁺. Unless otherwise stated, complete medium comprised Mn²⁺, Ca²⁺, Cl⁻ and FeCN at concentrations as indicated in the figure legends. All error bars not specifically explained represent one standard deviation of the mean (σ). Fits of the kinetic data to one-, two- and three-exponential models were performed using graphical software (OriginLab Corp., USA), as previously described (Baranov et al. 2004). To obtain the maximum value of the O₂ yield expected at the end of a complete photoactivation flash period, called Yₜₚ, the experimental time course of the photoactivated O₂ yield was fitted to a single site binding curve and the extrapolated endpoint. The actual raw data recovered is 90–95% of O₂ yield.

3. RESULTS

Figure 1a shows the kinetics of recovery of O₂ evolution during pulsed light photoactivation over a period of 400 flashes applied to apo-WOC-PSII membranes measured in the presence of the usual cofactors (Mn²⁺, Ca²⁺ and FeCN). A short initial lag period in the first approximately 10 flashes is present where there is no O₂ recovery, and this was previously identified as due to the pre-steady-state build-up of the first Mn³⁺ photo intermediate. In previous reports we have established that the time course of photoactivation follows a bi-exponential kinetic law given by equation (3.1), and this has been shown to hold true over a wide range of concentrations of both Mn²⁺ and Ca²⁺ (250-fold) when there are no competing metal ions or inhibitors present. The maximum deviation from this two-step model over this 250-fold range of cofactor concentrations was previously shown to be less than 5% based on rigorous least-square curve fits:

\[ Y(t) = Y_{ss} + A_1 e^{-kt_1} - A_2 e^{-kt_2} \]

(3.1)

Figure 1a also shows that the addition of Cd²⁺ inhibits the recovery of O₂ evolution during photoactivation in proportion to the amount of Cd²⁺ added. If calcium is replaced entirely with cadmium no recovery of O₂ evolution occurred (not shown). It can also be seen that while the final O₂ yield is lowered, the number of flashes (and hence the rate) at which this final yield (Yₜₚ) is reached increases (is accelerated) by Cd²⁺. This is seen most easily as the number of flashes needed to reach 50% saturation of Yₜₚ on the standard sucrose buffer at pH 6.0 and FeCN electron acceptor (see text). (c) Effect of Cd²⁺ concentration on the maximum oxygen yield recovered at the end of photoactivation (Yₜₚ) using apo-WOC-PSII, as a function of the indicated concentrations of MnCl₂ (µM), CaCl₂ (mM) and CdCl₂ (µM). Conditions are identical to (a). All traces are normalized to unit amplitude at 0 mM Cd²⁺. All error bars represent one standard deviation of the mean (σ). Yₜₚ was obtained from fits to the model by extrapolation of the data to infinite time. The absolute values of Yₜₚ (mmol O₂/Chl/flash) at 0 [Cd] are as follows: 10 µM Mn/10 mM Ca: Yₜₚ = 2.72 ± 0.01; 10 µM Mn/30 mM Ca: Yₜₚ = 2.97 ± 0.01; 10 µM Mn/50 mM Ca: Yₜₚ = 1.90 ± 0.01; 30 µM Mn/10 mM Ca: Yₜₚ = 3.88 ± 0.01.
The latter concentration of Mn\(^{2+}\) is sufficient to appreciably change the fraction of PSII centres with bound Mn\(^{2+}\), as it is nearly equal to the dissociation constant for Mn\(^{2+}\) at the high-affinity manganese site (\(K_d = 0.45 \text{ mM; Tyryshkin et al.} 2006\)). The data in figures 1 and 2 indicate that Cd\(^{2+}\) binds to the calcium effector site required for photoactivation and not to the high-affinity Mn\(^{2+}\) site.

Next we fitted the kinetic profile of photoactivation using the previously described two-step kinetic model. Figure 2 shows that addition of Cd\(^{2+}\) to the photoactivation components causes the bi-exponential kinetic model to fail severely, with peak deviations as large as 30\% for the representative data shown. However, as shown in this figure, addition of a third exponential component to the model recovers excellent fits of the experimental data with r.m.s. deviations less than 1\% and peak deviation less than 3\% in the steady-state region. This new kinetic phase is identified by its amplitude and rate constant, \(A_2'\) and \(\lambda_2'\), respectively:

\[
Y(t) = Y_{SS} + A_1 e^{-\lambda_1 t} - A_2 e^{-\lambda_2 t} + A_2' e^{-\lambda_2' t}.
\]  

Excellent fits were obtained using the three-exponential model at all concentrations of Cd\(^{2+}\). As shown in figure 3a, by systematically titrating the concentration of Cd\(^{2+}\) we observed that the amplitude of this new kinetic phase is identified by its amplitude and rate constant, \(A_2'\) and \(\lambda_2'\), obtained from least-squares fits of the experimental data to the three-exponential model, as shown in figure 2. Open and filled circles represent data at 30 \(\mu\)M Mn\(^{2+}\) and 10 mM Ca\(^{2+}\), and open and filled squares represent data at 10 \(\mu\)M Mn\(^{2+}\) and 10 mM Ca\(^{2+}\). Error bars represent one standard deviation of the mean (\(\sigma\)), except at the lowest [Cd\(^{2+}\)] (for \(A_2\)) and highest [Cd\(^{2+}\)] (for \(A_2'\)) where these parameters contribute very little to the fits and correspondingly exhibit very large errors. For these two points the error shown is given as log(1+\(\sigma\)). (c) Effect of Cd\(^{2+}\) and Ca\(^{2+}\) concentrations on the relative amplitude \(A_2\), obtained from least-squares fits to the experimental data using the three-exponential model, as described in figure 2. Filled triangles represent data at 10 \(\mu\)M Mn\(^{2+}\) and 50 mM Ca\(^{2+}\), filled circles represent data at 10 \(\mu\)M Mn\(^{2+}\) and 30 mM Ca\(^{2+}\), filled squares represent data at 10 \(\mu\)M Mn\(^{2+}\) and 10 mM Ca\(^{2+}\). Each dataset was normalized to the \(A_2\) value at zero CdCl\(_2\). All experiments were performed using samples and conditions that are the same as given in figure 1.

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**Figure 2.** Representative kinetic data for O\(_2\) recovery during photoactivation and fitting to two models: (a) comparison of the kinetics of O\(_2\) recovery to bi- and tri-exponential models obtained by least-squares fits of each model to the data and (b) residuals = deviation between the data and the two models. The experiment was performed using apo-WOC-PSII, 30 \(\mu\)M MnCl\(_2\), 10 mM CaCl\(_2\) and 100 \(\mu\)M CdCl\(_2\). Other conditions are the same as given in figure 1. The equations used for the two- and three-exponent models are described in the text.

**Figure 3.** Effect of Cd\(^{2+}\) concentration on (a) the relative amplitudes \(A_2\) and \(A_2'\) and (b) the corresponding rate constants \(\lambda_2\) and \(\lambda_2'\), obtained from least-squares fits of the experimental data to the three-exponential model, as shown in figure 2. Open and filled circles represent data at 30 \(\mu\)M Mn\(^{2+}\) and 10 mM Ca\(^{2+}\), and open and filled squares represent data at 10 \(\mu\)M Mn\(^{2+}\) and 10 mM Ca\(^{2+}\). Error bars represent one standard deviation of the mean (\(\sigma\)), except at the lowest [Cd\(^{2+}\)] (for \(A_2\)) and highest [Cd\(^{2+}\)] (for \(A_2'\)) where these parameters contribute very little to the fits and correspondingly exhibit very large errors. For these two points the error shown is given as log(1+\(\sigma\)). (c) Effect of Cd\(^{2+}\) and Ca\(^{2+}\) concentrations on the relative amplitude \(A_2\), obtained from least-squares fits to the experimental data using the three-exponential model, as described in figure 2. Filled triangles represent data at 10 \(\mu\)M Mn\(^{2+}\) and 50 mM Ca\(^{2+}\), filled circles represent data at 10 \(\mu\)M Mn\(^{2+}\) and 30 mM Ca\(^{2+}\), filled squares represent data at 10 \(\mu\)M Mn\(^{2+}\) and 10 mM Ca\(^{2+}\). Each dataset was normalized to the \(A_2\) value at zero CdCl\(_2\). All experiments were performed using samples and conditions that are the same as given in figure 1.
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Figure 4. Dependence on Cd$^{2+}$ concentration of the rate constant $\lambda_1$, obtained from least-squares fits to the experimental data using the three-exponential model, as described in figure 3. Filled circles represent data at 30 $\mu$M Mn$^{2+}$ and 10 mM Ca$^{2+}$, and filled squares represent data at 10 $\mu$M Mn$^{2+}$ and 10 mM Ca$^{2+}$. All experiments were performed using samples and conditions as in figure 1. Error bars represent one standard deviation of the mean ($\sigma$).

Figure 5. Dependence on Cd$^{2+}$ concentration of the steady-state oxygen yield recovered during photoactivation ($Y_{SS}$), measured as a function of the dark times between light pulses. Each dataset was normalized to the yield at 3 s. One micromolar apo-WOC-PSII samples contain 10 $\mu$M MnCl$_2$, 10 mM CaCl$_2$, and the indicated concentrations of CdCl$_2$ ($\mu$M). Experiments were performed using samples and conditions as in figure 1.

4. DISCUSSION

The data in this paper shows that Cd$^{2+}$ does not support photoactivation if used without Ca$^{2+}$ and binds selectively to the Ca$^{2+}$ effector site, in competition with Ca$^{2+}$. The Cd$^{2+}$ binding causes a large (10-fold) acceleration of the rate-limiting step of photoassembly ($\lambda_2$), which increases the yield of conversion ($\langle \lambda_2 \rangle$) of intermediate IM$_1$ $\rightarrow$ IM$_1^*$ by the same amount. Cd$^{2+}$ blocks expression of O$_2$-evolution activity during photoassembly, while producing minimal loss of O$_2$-evolution activity in intact PSII. Cd$^{2+}$ does not compete with Mn$^{2+}$ for binding to the high-affinity Mn$^{2+}$ site during photoassembly. Inhibition by Cd$^{2+}$ of later photoassembly steps following formation of IM$_1^*$, involving the remaining 3 Mn$^{2+}$, cannot be ruled out or in, as these steps are not resolved kinetically, and thus remains a possible primary cause for inhibition of O$_2$-evolution activity, but not for acceleration of the rate-limiting step.

Using paired flash experiments, Burnap and co-workers have revealed a third kinetic phase during in vivo photoactivation studies of Synechocystis that is a minor component in terms of amplitude and is 5–10 times faster than the rate-limiting dark rearrangement step that is the dominate phase (Hwang & Burnap 2005). This step presumably represents an alternate pathway to the intermediate, IM$_1^*$. Assuming that the dark rearrangement step is a protein conformational change, they propose the possibility that a fraction of the centres may already exist in the rearranged state during or shortly after the first light-induced step. These centres are thus able to process the second
quantum without the prior delay required by the conformational change. Burnap’s results share two important similarities with those of our cadmium experiments: (i) the appearance of two populations of centres which proceed to IM1 at different rates and (ii) a 5- to 10-fold acceleration of the normally unseen pathway with respect to the previously observed dark rearrangement step. It thus seems plausible that Cd²⁺ promotes a shift between two populations, so that the faster pathway (our $A'_2$, $\bar{A}'_2$) plays a significant role in photoassembly.

Our data suggest two possible models that could account for the opposing influence of Cd²⁺ on the rate and yield of photoactivation. In both of these models, Cd²⁺ binds to the calcium effector site during the dark step IM1→IM1 and produces the observed 10-fold acceleration of $\lambda_2$ (scheme 1). Model 1: the remaining photoassembly steps, involving binding and photooxidation of 3 Mn²⁺, occur as usual, Cd²⁺ binds only to the functional calcium effector site and its binding to this site blocks expression of O₂ activity. In this case, Cd²⁺ replacement by Ca²⁺ in subsequent assembly steps would be required to account for the observed acceleration of recovery of O₂-evolution activity. Model 2: this claims that Cd²⁺ binding to the calcium effector site actually supports some lower level of O₂-evolution activity, but that inhibition of O₂ yield occurs at higher concentrations of Cd²⁺ because it blocks one or more of the remaining 3 Mn²⁺ in later photoassembly steps. Since we did not observe any O₂ recovery during photoactivation in the absence of Ca²⁺, model 1 offers the best description of how Cd²⁺ blocks photoassembly. Model 1 predicts that the binding of Cd²⁺ must be weaker in the steps following IM1 formation.

We compared the IC₅₀ values corresponding to Cd²⁺ concentrations that cause 50% reduction of: (i) the O₂ yield produced by photoactivation ($Y_{SS}$) obtained from figure 1c, and (ii) the amplitude of the slow photoassembly intermediate ($A_2$) obtained from figure 3c, as a function of the Ca²⁺ concentration (figures S1 and S2 in the electronic supplementary material). These IC₅₀ values were extrapolated to zero Ca²⁺ concentration to obtain the apparent dissociation constants for Cd²⁺. These values are approximately 120 μM for $Y_{SS}$ and 50 μM for $A_2$. We previously reported the affinities for Ca²⁺ activation of $Y_{SS}$ and $\lambda_2$ to be exactly the same at 1.4 mM (Ananyev & Dismukes 1996b; Zaltsman et al. 1997). Thus, Cd²⁺ exhibits a 10- to 30-fold higher affinity than Ca²⁺ for binding on the IM1→IM1 step when measured under identical conditions. From this we see that the Cd²⁺ binding affinity increases by 2.5-fold when measured using the specific reaction yield ($A_2$) that is coupled to Cd³⁺ binding in the native system, e.g. IM1→IM1, compared to using the overall reconstituted O₂ yield ($Y_{SS}$) which reflects the average affinity after the remaining 3 Mn²⁺ are photoassembled. This lower affinity for Cd²⁺ following the uptake and photooxidation of the remaining 3 Mn²⁺ fits well with the exchange model described above, whereby in fully reconstituted centres containing Mn₃Cd, the Cd³⁺ is more easily replaced by Ca²⁺, which produces a normal Mn₃Ca WOC that has assembled ten times faster. Thus, binding of Cd²⁺ to a single site, the Ca²⁺ effector site, can account for the full range of observations on both the rate of acceleration and yield inhibition of photoactivation. This proposal does not exclude a mixed mode of inhibition involving Cd²⁺ additionally blocking Mn²⁺ uptake in later steps after IM1, which produce inactive centres that do not progress to active ones.

The relative binding affinities of various divalent metals (Mg²⁺, Ca²⁺, Mn²⁺, Sr²⁺) and the oxo-cation UO₂²⁺ have been previously measured for the Ca²⁺ effector site during in vitro photoactivation of apo-WOC-PSII (Ananyev et al. 1999, 2001) and for the Cd-depleted holoenzyme (Vrettos et al. 2001). Our IC₅₀ value for Cd²⁺ inactivation of $Y_{SS}$ (120 μM) during photoactivation compares well with its IC₅₀ reported for O₂ evolution from intact functional PSII membrane complexes ($K_D=144$ μM; Vrettos et al. 2001). These authors report a higher binding affinity for Ca²⁺ to the intact functional PSII complex ($K_D=69$ μM) compared with other monovalent (Na, K) and divalent (Mn, Ni, Cu, Co, Cd, Sr, Ba) ions. Our results also agree with a report by Faller et al. (2005) who provide a measurement of the IC₅₀ for Cd²⁺ inhibition of O₂ yield during photoactivation with continuous illumination (50% inhibition of photoactivation at 200 μM Cd²⁺ in the presence of 10 mM Ca²⁺ versus 125–140 μM Cd²⁺ for our result, figure 1c). These authors also concluded that Cd²⁺ binds at the calcium effector site and not at the high-affinity Mn²⁺ site based on competition studies. These authors did not explore the kinetic dimensions of the Cd²⁺ effect on photoactivation. However, in agreement with our finding, although not discussed in the article, their in vivo fluorescence measurements of the green alga Chlamydomonas reinhardtii appear to show an acceleration of reconstitution of PSII activity with the addition of Cd²⁺, even though maximum activity is decreased. Other published studies have reported IC₅₀ values in the low micromolar range for Cd²⁺ inhibition of O₂ evolution in a few plants, algae and cyanobacteria (Stobart et al. 1985; Ata et al. 1991; Schafer et al. 1994; Nagel & Voigt 1995; Okamoto et al. 1996). Although these measurements did not distinguish between PSII inhibition during photoactivation or functional PSII, it is likely that Cd²⁺ binding during photoactivation played a more important role than Cd²⁺ binding to functional PSII.

The data in figure 5, showing that Cd²⁺ binding increases by twofold the mean lifetime for decay of photointermediates, indicate that Cd²⁺ interacts with the high-affinity Mn³⁺ to cause greater kinetic stability by slowing charge recombination with the reduced primary electron acceptor, Q₅. This positive mutual cooperativity provides a more efficient photoassembly process. It is counter-intuitive that adding a divalent cation like Ca²⁺ (or Cd²⁺) close to Mn³⁺ would slow electron recombination, unless this binding were to create a thermodynamically more stable Mn³⁺ having a less positive electrochemical reduction potential. Such a potential decrease would be expected if proton ionization of a ligand coordinated directly to Mn³⁺ in IM1 were to occur, as depicted in scheme 1. The case illustrated is for proton ionization of Mn³⁺(OH⁻)⁺ to form a bridged oxide Mn³⁺(O⁻²⁻)Ca²⁺⁺. Independent evidence in support of this model comes from studies of
flash-induced pH changes showing that a proton is released at IM1\textsubscript{t}, either upon Ca\textsuperscript{2+} binding or Mn\textsuperscript{2+} photooxidation (Ananyev et al. 2001). This same structural model for conversion of IM\textsubscript{t}→IM\textsubscript{1} was inferred based upon EPR spectroscopic data describing the ligand field at Mn\textsuperscript{3+} and its environment (Tyrshkin et al. 2006).

It is chemically reasonable to assume that Cd\textsuperscript{2+} binds in exactly the same way as Ca\textsuperscript{2+} to the calcium effector site to form a bridged structure: [Mn\textsuperscript{3+} (μ-O\textsuperscript{2−})Cd\textsuperscript{2+}]. Slightly stronger ionic binding by Cd\textsuperscript{2+} versus Ca\textsuperscript{2+} to the μ-O\textsuperscript{2−} bridge might be expected based on its slightly greater charge density (ionic radius = 0.97 versus 0.99 Å for Ca\textsuperscript{2+}). Although this is borne out by the pK\textsubscript{a} values of the corresponding aquo ions (9.00 versus 12.80, respectively), reflecting their affinity for hydroxide versus water, considerably weaker binding of oxide anions exists in solid CdO\textsubscript{(s)} versus CaO\textsubscript{(s)}. The latter is measured by the large difference in their heats of formation ΔH\textsubscript{f} = −258 versus 635 kJ mol\textsuperscript{−1}, respectively (Frenkel 2005). This undoubtedly arises from stronger interligand repulsions and from antibonding repulsion with the filled 4d\textsuperscript{10} shell in Cd\textsuperscript{2+}. Consequently, Cd\textsuperscript{2+} is expected to release electron density from μ-O\textsuperscript{2−} making it a stronger field ligand and more available for binding to Mn\textsuperscript{2+} ions in the WOC cluster compared with Ca\textsuperscript{2+}.

The foregoing analysis provides evidence that Cd\textsuperscript{2+} binding during the step IM\textsubscript{t}→IM\textsubscript{1} causes three outcomes: (i) a longer intermediate lifetime that slows IM\textsubscript{1} reductive decay to IM\textsubscript{0}, (ii) ten times higher probability of the cofactor (or protein?) degrees of freedom needed to bind and photooxidize the remaining 3 Mn\textsuperscript{2+} that form the functional cluster, and (iii) (re)exchange of Cd\textsuperscript{2+} by Ca\textsuperscript{2+} following the latter steps restores active O\textsubscript{2}-evolving centres. The second outcome may involve forming additional bridges to the second (or multiple) Mn\textsuperscript{2+} ions which is the one-electron precursor to intermediate IM\textsubscript{2} in scheme 1. Other degrees of freedom, such as the folding of the D1 protein into a productive conformation that permits faster assembly and photooxidation of the second and remaining Mn\textsuperscript{2+} comprising the active cluster may also contribute. In this regard, it was previously shown by EPR spectroscopy that at higher cofactor concentrations, Ca\textsuperscript{2+} templates binding of the initial 2 Mn\textsuperscript{2+} to apo-WOC-PSII even in the dark, yielded a spin-coupled dimanganese (II,II) pair (separation <4.25 Å) that can go on to productive photoactivation (Ananyev & Dismukes 1997). Additionally, without Ca\textsuperscript{2+} (or Sr\textsuperscript{2+}) excess Mn\textsuperscript{2+} gets photooxidized during photoassembly, with as many as 20 Mn\textsuperscript{2+} oxidations and no functional O\textsubscript{2} evolution observable (Chen et al. 1995; Ananyev & Dismukes 1996b; Ananyev et al. 2001). Collectively, these studies strongly suggest that Ca\textsuperscript{2+} directs the final steps of photoassembly of the WOC by creating sites for the binding and photooxidation of the final 3 Mn\textsuperscript{2+}, coincident with the formation of the oxo bridge at IM\textsubscript{1}, e.g. [Mn\textsuperscript{3+} (μ-O\textsuperscript{2−})Cd\textsuperscript{2+}]. At intracellular cofactor concentrations found \textit{in vivo}, it does so ‘on the fly’, after photochemical formation of IM\textsubscript{1}, by forming solvent-derived hydroxide/oxide bridge(s) which enable formation of the functional Mn\textsubscript{3}O\textsubscript{4}Ca core. An oxo-bridged structure for IM\textsubscript{1} is consistent with the X-ray diffraction and EXAFS-derived models of the intact PSII-WOC isolated from a cyanobacterial source (Loll et al. 2005; Yano et al. 2006; Barber & Murray in press).

Cd\textsuperscript{2+} does not contribute to the greater photooxidation yield of the first Mn\textsuperscript{2+} (high-affinity Mn\textsuperscript{2+}), as is clear from the independence of the rate constant λ\textsubscript{1} on Cd\textsuperscript{2+} concentration. In this regard, the function of Cd\textsuperscript{2+} in photoassembly differs completely from the role of bicarbonate which accelerates λ\textsubscript{1} by direct thermodynamic stabilization of the photooxidized high-affinity Mn\textsuperscript{3+} (Baranov et al. 2004; Kozlov et al. 2004). Sr\textsuperscript{2+} is the only other functionally active metal known to support water oxidation/O\textsubscript{2} production in the Ca\textsuperscript{2+} effector site of PSII. Sr\textsuperscript{2+} substituted PSII has been prepared by biochemical exchange using isolated functional PSII centres, by biosynthetic growth on Sr\textsuperscript{2+} and by complete cofactor reassembly upon photoactivation (Westphal et al. 2000; Ananyev et al. 2001; Boussac et al. 2004). In all cases, similar O\textsubscript{2} evolution rates were found (30–35% of the Ca\textsuperscript{2+} level), indicating the effect arises from a native-like replacement and not an artefact of the method. This reduced rate arises primarily from a four- to sevenfold slower overall rate of the final photochemical step: S\textsubscript{1}→S\textsubscript{0}. Previous photoactivation studies found that Sr\textsuperscript{2+} replacement for Ca\textsuperscript{2+} accelerates the forward dark step (λ\textsubscript{2}) by twofold and slows the rate of decay of the high-affinity Mn\textsuperscript{3+} by fivefold (S→S\textsubscript{1} step; Ananyev et al. 2001). The latter process is believed to involve Mn\textsuperscript{3+}OX charge recombination to form IM\textsubscript{0} at the high-affinity site coupled to displacement of the resulting Mn\textsuperscript{3+} (OH\textsuperscript{−}) by Me\textsuperscript{2+} (OH\textsuperscript{−}), equation (4.1) (Ananyev et al. 2001):

\[
\text{IM}_1 [\text{Mn}^{3+} (\text{OH})_2] + (\text{Me}^{2+} = \text{Ca}^{2+}, \text{Sr}^{2+}, \text{Cd}^{2+}) \\
\rightarrow \text{Mn}^{2+} (\text{OH}^-) + \text{IM}_0 [\text{Me}^{2+} (\text{OH}^-)].
\] (4.1)

By this mechanism, the capacity for Sr\textsuperscript{2+} and Cd\textsuperscript{2+} to slow this process relative to Ca\textsuperscript{2+} should correlate with the binding affinity for Me\textsuperscript{2+} (OH\textsuperscript{−}) at the high-affinity Mn\textsuperscript{3+} site. By contrast, a considerably faster rate acceleration of λ\textsubscript{2} occurs in the presence of Cd\textsuperscript{2+} (10×) versus Sr\textsuperscript{2+} (2×) relative to Ca\textsuperscript{2+}. We have interpreted this to indicate that Cd\textsuperscript{2+} and Sr\textsuperscript{2+} more rapidly find and assemble the proposed bridged intermediate IM\textsubscript{1} (scheme 1). In future work it would be worthwhile to investigate the Cd\textsuperscript{2+} substituted IM\textsubscript{1} intermediate by EPR spectroscopy, as it should be possible to distinguish the ligand field parameters of the Mn\textsuperscript{3+} to which it is bridged in the proposed model of scheme 1, much as has been achieved with the native Ca\textsuperscript{2+} intermediate IM\textsubscript{1} (Tyrshkin et al. 2006).

This work was supported by the National Institutes of Health (GM 39932).

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