Calcium controls the assembly of the photosynthetic water-oxidizing complex: a cadmium(II) inorganic mutant of the Mn₄Ca core

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Perturbation of the catalytic inorganic core (Mn₄CaO₄Cl₂) of the photosystem II-water-oxidizing complex (PSII-WOC) isolated from spinach is examined by substitution of Ca²⁺ with cadmium(II) during core assembly. Cd²⁺ inhibits the yield of reconstitution of O₂-evolution activity, called photoactivation, starting from the free inorganic cofactors and the cofactor-depleted apo-WOC-PSII complex. Ca²⁺ affinity increases following photooxidation of the first Mn²⁺ to Mn³⁺ bound to the ‘high-affinity’ site. Ca²⁺ binding occurs in the dark and is the slowest overall step of photoactivation (IM₁→IM₄ step). Cd²⁺ competitively blocks the binding of Ca²⁺ to its functional site with 10- to 30-fold higher affinity, but does not influence the binding of Mn²⁺ to its high-affinity site. By contrast, even 10-fold higher concentrations of Cd²⁺ have no effect on O₂-evolution activity in intact PSII-WOC. Paradoxically, Cd²⁺ both inhibits photoactivation yield, while accelerating the rate of photoassembly of active centres 10-fold relative to Ca²⁺. Cd²⁺ increases the kinetic stability of the photooxidized Mn¹⁺ assembly intermediate(s) by twofold (mean lifetime for dark decay). The rate data provide evidence that Cd²⁺ binding following photooxidation of the first Mn³⁺, IM₁→IM₄, causes three outcomes: (i) a longer intermediate lifetime that slows IM₄ decay to IM₀ 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 Mn¹⁺ that form the functional cluster, and (iii) increased lability of Cd²⁺ following Mn₁ cluster assembly results in (re)exchange of Cd²⁺ by Ca²⁺ which restores active O₂-evolving centres. Prior EPR spectroscopic data provide evidence for an oxo-bridged assembly intermediate, Mn³⁺(µ-O²⁻)Ca²⁺, for IM₄. We postulate an analogous inhibited intermediate with Cd²⁺ replacing Ca²⁺.

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

Abbreviations: Chl, chlorophyll a; FeCN, potassium ferricyanide K₃[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 O₂ 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, Mn₄CaO₄Cl₂, 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 (Mn²⁺, Ca²⁺, Cl⁻, HCO₃⁻), an electron acceptor and light. Reconstitution of O₂-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 O₂-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). Ca²⁺ is an essential cofactor for proper photoassembly of the Mn₄ core and for O₂-evolution activity. Its selective removal from the holoenzyme reversibly abolishes activity (Vander Meulen et al. 2002). If Ca²⁺ is left out of the photoactivation medium, the apo-WOC-PSII protein binds and photooxidizes an excess number of Mn²⁺ ions (as many as 20) and no O₂-evolution activity is observable (Chen et al. 1995; Ananyev & Dismukes 1996a).

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The kinetically resolved steps of \textit{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 \cite{Ananyev1996a, Ananyev1999a, Baranov2004}. Mn\(^{2+}\) that binds to the high-affinity site has been shown to speciate among different chemical forms depending upon the solution pH, presence/absence of Ca\(^{2+}\) and bicarbonate \cite{Ananyev1999a, Baranov2004, Tyryshkin2006, Dasgupta}. This speciation greatly influences the 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 \cite{Zaltsman1997}. 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 \cite{Ananyev2001}. This intermediate, designated IM\(_1^*\), has been proposed to be an oxo- or bishydroxo-bridged species, either [Mn\(^{3+}\) (\(\mu\)-O)\(\mu\)-Ca\(^{2+}\)] or [Mn\(^{3+}\) (\(\mu\)-OH)\(\mu\)-Ca\(^{2+}\)], on the basis of several electron paramagnetic resonance (EPR) spectroscopic properties \cite{Tyryshkin2006}. 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\(_2\) step occurs in the dark and is the slowest step in the overall photoactivation process \cite{Ananyev2001}. 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 \cite{Cheniae1971, Tamura1987, Hwang2005}. 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 \cite{Zaltsman1997} if Cl\(^{-}\) is present in the medium \cite{Ananyev1998, Ananyev2001}. 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 \cite{Peloquin2001, Carrell2002}, and subsequently shown also by structural data derived from X-ray diffraction of a PSII core complex \cite{Zouni2001, Ferreira2004}.

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 \cite{Ghanotakis1984}. 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 \cite{Baranov2004}. All samples

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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 homebuilt 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 $Y_{SS}$, 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:

$$Y(t) = Y_{SS} + A_1 e^{-2t/K_1} - A_2 e^{-2t/K_2}.$$  (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 ($Y_{SS}$) is reached increases (is accelerated) by Cd²⁺. This is seen most easily as the number of flashes needed to reach 50% saturation of $Y_{SS}$. On the other hand, Cd²⁺ was found to exhibit only weak inhibition of O₂ evolution when given to intact isolated PSII membranes (figure 1b); in this case, 1 mM Cd²⁺ inhibits O₂ evolution by less than 3%.

Figure 1c shows that this inhibition of $Y_{SS}$ by Cd²⁺ is competitive with Ca²⁺ between 10 and 50 mM. This range of concentrations of Ca²⁺ is saturating for recovery of O₂-evolution activity by photoactivation (Ananyev & Dismukes 1996). On the other hand, increasing the amount of Mn²⁺ by threefold to 30 μM did not produce any change in the cadmium inhibition

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Figure 2. Representative kinetic data for O2 recovery during photoactivation and fitting to two models: (a) comparison of the kinetics of O2 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 µM MnCl2, 10 mM CaCl2 and 100 µM CdCl2. 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.

profile. The latter concentration of Mn2+ is sufficient to appreciably change the fraction of PsII centres with bound Mn2+, as it is nearly equal to the dissociation constant for Mn2+ at the high-affinity manganese site (Kd = 0.45 mM; Tyryshkin et al. 2006). The data in figures 1 and 2 indicate that Cd2+ binds to the calcium effector site required for photoactivation and not to the high-affinity Mn2+ site.

Next we fitted the kinetic profile of photoactivation using the previously described two-step kinetic model. Figure 2 shows that addition of Cd2+ 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 kinetic 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 A2 and λ2, respectively:

\[ Y(t) = Y_{SS} + A_1 e^{-\lambda_1 t} - A_2 e^{-\lambda_2 t} + A_2 e^{-\lambda_2 t} \] (3.2)

Excellent fits were obtained using the three-exponent model at all concentrations of Cd2+. As shown in figure 3a, by systematically titrating the concentration of Cd2+ we observed that the amplitude of this new

Figure 3. Effect of Cd2+ concentration on (a) the relative amplitudes A2 and A2' and (b) the corresponding rate constants λ2 and λ2', 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 µM Mn2+ and 10 mM Ca2+, and open and filled squares represent data at 10 µM Mn2+ and 10 mM Ca2+. Error bars represent one standard deviation of the mean (σ), except at the lowest [Cd2+] (for A2) and highest [Cd2+] (for A2') 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+σ). (c) Effect of Cd2+ and Ca2+ concentrations on the relative amplitude A2, 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 µM Mn2+ and 50 mM Ca2+, filled circles represent data at 10 µM Mn2+ and 30 mM Ca2+, filled squares represent data at 10 µM Mn2+ and 10 mM Ca2+. Each dataset was normalized to the A2 value at zero CdCl2. All experiments were performed using samples and conditions that are the same as given in figure 1.
Mn$^{2+}$ 10 mM Ca$^{2+}$ represent one standard deviation of the mean (using samples and conditions as in figure 1. Error bars in figure 3. Filled circles represent data at 30 mmol Mn$^{2+}$ and 10 mM Ca$^{2+}$, and filled squares represent data at 10 μM 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 (σ).

Kinetic phase increases to a maximum, while the amplitude of the dark slow step ($A_2$) associated with the uptake of Ca$^{2+}$ decreases in parallel. An identical trend was observed at both 30 and 10 μM Mn$^{2+}$ as shown, except the amplitudes were smaller at the lower Mn$^{2+}$ concentrations. Figure 3b plots the dependence of the corresponding rate constants, $\lambda_2$ and $\lambda_3$, as a function of the Cd$^{2+}$ concentration. The rate constant for the Cd$^{2+}$-induced slow phase $\lambda_3$ is 10-fold or more faster than $\lambda_2$, and both of these rate constants get slower as the Cd$^{2+}$ concentration increases. Both trends hold true at 30 and 10 μM Mn$^{2+}$ as shown, and both rate constants are slower at the lower Mn$^{2+}$ concentration. These data show that Cd$^{2+}$ introduces a 10-fold faster kinetic phase which replaces the slow Ca$^{2+}$ phase. However, Cd$^{2+}$ inhibits recovery of O$_2$ evolution and both of these rate constants are appreciably slowed by further addition of Cd$^{2+}$.

Figure 3c shows that addition of Ca$^{2+}$, in the concentration range 10–50 mM, suppresses the inhibition caused by Cd$^{2+}$ of the amplitude of the slow step of photoactivation ($A_2$), effectively competing with Cd$^{2+}$. By contrast, figure 4 shows that addition of Ca$^{2+}$ does not change the rate constant $\lambda_1$ for the first step in photoactivation involving binding and photodestruction of Mn$^{2+}$ at the high-affinity site. This is shown to be true at two Mn$^{2+}$ concentrations of 10 and 30 μM. Hence, Cd$^{2+}$ does not compete with Mn$^{2+}$ at the high-affinity Mn$^{2+}$ site.

The previous data were acquired at a fixed dark time of 3 s between the 30 ms light pulses. By varying the dark time between flashes, one can probe the mean lifetime of the unstable light-induced intermediates as they decay back to earlier intermediates or the dark state. In figure 5, the yield of O$_2$ produced at the end of the photoactivation process ($Y_{SS}$) is plotted as function of the dark time between pulses at different Cd$^{2+}$ concentrations. This reveals that Cd$^{2+}$ increases the most probable (peak) lifetime of intermediates formed during photoactivation approximately from 3 to 5 s compared with Ca$^{2+}$ alone. The shape of the distribution is also shifted towards longer lifetime for the whole population, yielding approximately a twofold increase in the mean lifetime of the whole distribution with Cd$^{2+}$.

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 ($A_2$), which increases the yield of conversion ($\lambda_2$) of intermediate IM$_1$→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 dominant 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 $A_l'$, $\lambda_l'$) 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 $\rightarrow$ IM1$'$ and produces the observed 10-fold acceleration of $\lambda_l$ (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 photoactivation. 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 $\mu$M for $Y_{SS}$ and 50 $\mu$M 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 $\rightarrow$ 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 Ca2+ binding in the native system, e.g. IM1 $\rightarrow$ 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 Mn,Cd, the Cd2+ is more easily replaced by Ca2+ which produces a normal Mn,Ca 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 UO2+ 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 Ca-depleted holoenzyme (Vrettos et al. 2001). Our IC50 value for Cd2+ inactivation of $Y_{SS}$ (120 $\mu$M) during photoactivation compares well with its IC50 reported for O2 evolution from intact functional PSII membrane complexes ($K_D$ = 144 $\mu$M; Vrettos et al. 2001). These authors report a higher binding affinity for Ca2+ to the intact functional PSII complex ($K_D$ = 69 $\mu$M) 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 $\mu$M Cd2+ in the presence of 10 mM Ca2+ versus 125–140 $\mu$M 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, Q$. This positive mutual cooperativity provides a more efficient photoassembly process. It is counter-intuitive that adding a divalent cation like Ca2+ (or Cd2+) close to Mn3+ would slow electron recombination, unless this binding were to create a thermodynamically more stable Mn3+ having a less positive electrochemical reduction potential. Such a potential decrease would be expected if proton ionization of a ligand coordinated directly to Mn3+ in IM1 were to occur, as depicted in scheme 1. The case illustrated is for proton ionization of Mn3+ (OH$^{-}$) to form a bridged oxide Mn3+(O2)2Cd2+. Independent evidence in support of this model comes from studies of
flash-induced pH changes showing that a proton is released at IM₁', either upon Ca²⁺ binding or Mn²⁺ photooxidation (Ananyev et al. 2001). This same structural model for conversion of IM₁ → IM₂ was inferred based upon EPR spectroscopic data describing the ligand field at Mn³⁺ and its environment (Tyryshkin et al. 2006).

It is chemically reasonable to assume that Cd²⁺ binds in exactly the same way as Ca²⁺ to the calcium effector site to form a bridged structure: [Mn²⁺ (μ-O²⁻−)Cd²⁺]. Slightly stronger ionic binding by Cd²⁺ versus Ca²⁺ to the μ-O²⁻− bridge might be expected based on its slightly greater charge density (ionic radius = 0.97 versus 0.99 Å for Ca²⁺). Although this is borne out by the pKa 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(s) versus CaO(s). The latter is measured by the large difference in their heats of formation ΔHf = −258 versus 635 kJ mol⁻¹, respectively (Frenkel 2005). This undoubtedly arises from stronger interligand repulsions and from antibonding repulsion with the filled 4d¹⁰ shell in Cd²⁺. Consequently, Cd²⁺ is expected to release electron density from μ-O²⁻− making it a stronger field ligand and more available for binding to Mn²⁺ ions in the WOC cluster compared with Ca²⁺.

The foregoing analysis provides evidence that Cd²⁺ binding during the step IM₁ → IM₂ causes three outcomes: (i) a longer intermediate lifetime that slows IM₁ reductive decay to IM₀, (ii) ten times higher probability of the cofactor (or protein?) degrees of freedom needed to bind and photooxidize the remaining 3 Mn²⁺ that form the functional cluster, and (iii) exchange of Cd²⁺ by Ca²⁺ following the latter steps restores active O₂-evolving centres. The second outcome may involve forming additional bridges to the second (or multiple) Mn²⁺ ions which is the one-electron precursor to intermediate IM₂ 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 Mn²⁺ comprising the active cluster may also contribute. In this regard, it was previously shown by EPR spectroscopy that at higher cofactor concentrations, Ca²⁺ templates binding of the initial 2 Mn²⁺ 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 Ca²⁺ (or Sr²⁺) excess Mn²⁺ gets photooxidized during photoassembly, with as many as 20 Mn²⁺ oxidations and no functional O₂ evolution observable (Chen et al. 1995; Ananyev & Dismukes 1996b; Ananyev et al. 2001). Collectively, these studies strongly suggest that Ca²⁺ directs the final steps of photoassembly of the WOC by creating sites for the binding and photooxidation of the final 3 Mn²⁺, coincident with the formation of the oxo bridge at IM₁', e.g. [Mn²⁺ (μ-O²⁻−)Cd²⁺]. At intracellular cofactor concentrations found in vitro, it does so ‘on the fly’, after photochemical formation of IM₁', by forming solvent-derived hydroxide/oxide bridge(s) which enable formation of the functional Mn₃O₄·Ca core. An oxo-bridged structure for IM₁' 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).

Cd²⁺ does not contribute to the greater photooxidation yield of the first Mn²⁺ (high-affinity Mn²⁺), as is clear from the independence of the rate constant λ₁ on Cd²⁺ concentration. In this regard, the function of Cd²⁺ in photoassembly differs completely from the role of bicarbonate which accelerates λ₁ by direct thermodynamic stabilization of the photooxidized high-affinity Mn³⁺ (Baranov et al. 2004; Kozlov et al. 2004).

Sr²⁺ is the only other functionally active metal known to support water oxidation/O₂ production in the Ca²⁺ effector site of PSII. Sr²⁺ substituted PSII has been prepared by biochemical exchange using isolated functional PSII centres, by biosynthetic growth on Sr²⁺ and by complete cofactor reassembly upon photoactivation (Westphal et al. 2000; Ananyev et al. 2001; Boussac et al. 2004). In all cases, similar O₂ evolution rates were found (30–35% of the Ca²⁺ 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: S₁ → S₃ + O₂. Previous photoactivation studies found that Sr²⁺ replacement for Ca²⁺ accelerates the forward dark step (λ₁) by twofold and slows the rate of decay of the high-affinity Mn³⁺ by fivefold (λ₋₁ step; Ananyev et al. 2001). The latter process is believed to involve Mn³⁺ₐQₐ charge recombination to form IM₀ at the high-affinity site coupled to displacement of the resulting Mn²⁺(OH⁻) by Me²⁺(OH⁻), equation (4.1) (Ananyev et al. 2001):

\[ IM₁[Mn³⁺(OH⁻)₂] + (Me²⁺ = Ca²⁺, Sr²⁺, Cd²⁺) \rightarrow Mn²⁺(OH⁻) + IM₀[Me²⁺(OH⁻)]. \]

By this mechanism, the capacity for Sr²⁺ and Cd²⁺ to slow this process relative to Ca²⁺ should correlate with the binding affinity for Me²⁺(OH⁻) at the high-affinity Mn³⁺ site. By contrast, a considerably faster rate acceleration of λ₁ occurs in the presence of Cd²⁺ (10×) versus Sr²⁺ (2×) relative to Ca²⁺. We have interpreted this to indicate that Cd²⁺ and Sr²⁺ more rapidly find and assemble the proposed bridged intermediate IM₁' (scheme 1). In future work it would be worthwhile to investigate the Cd²⁺ substituted IM₁' intermediate by EPR spectroscopy, as it should be possible to distinguish the ligand field parameters of the Mn³⁺ to which it is bridged in the proposed model of scheme 1, much as has been achieved with the native Ca²⁺ intermediate IM₁' (Tyryshkin et al. 2006).

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