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

John E. Bartlett^{1,2}, Sergei V. Baranov^{1,2}, Gennady M. Ananyev^{1,2}
and G. Charles Dismukes^{1,2,*}

¹Department of Chemistry, and ²Environmental Institute, Princeton University, Princeton, NJ 08544, USA

Perturbation of the catalytic inorganic core (Mn₄Ca₁O_xCl_y) 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 → IM₁^{*} 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 Mn³⁺ 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 → IM₁^{*}, causes three outcomes: (i) a longer intermediate lifetime that slows IM₁ 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 Mn²⁺ that form the functional cluster, and (iii) increased lability of Cd²⁺ following Mn₄ 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 IM₁^{*}. 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, Mn₄Ca₁O_xCl_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 (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; Faller *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).

* Author for correspondence (dismukes@princeton.edu).

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Scheme 1. (a) The sequence of kinetic intermediates formed during assembly of the inorganic core of the WOC by photoactivation (A, B, C). (b) Proposed chemical formulation of intermediates (Dasgupta *et al.* in press). The microscopic rate constants (k_1 , k_{-1} and k_2) can be related to the fitted rate constants at commonly encountered conditions of the photoactivation experiments ($k_1 \gg k_2$) as follows (Baranov *et al.* 2004): $\lambda_1 = (k_1 + k_{-1})$ and $\lambda_2 = k_2 k_1 / (k_1 + k_{-1})$.

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 speciate among different chemical forms depending upon the solution pH, presence/absence of Ca^{2+} and bicarbonate (Ananyev *et al.* 1999; Baranov *et al.* 2004; Tyrshkin *et al.* 2006; Dasgupta *et al.* in press, unpublished work). 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 (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 $[\text{Mn}^{3+}(\mu\text{-O})\text{Ca}^{2+}]$ or $[\text{Mn}^{3+}(\mu\text{-OH})_2\text{Ca}^{2+}]$, on the basis of several electron paramagnetic resonance (EPR) spectroscopic properties (Tyrshkin *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 $\text{IM}_1 \rightarrow \text{IM}_1^*$ 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 (Chenae & Martin 1971; Tamura & Chenae 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 Bertold-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 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 1*a* 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:

$$Y(t) = Y_{SS} + A_1 e^{-\lambda_1 t} - A_2 e^{-\lambda_2 t}. \quad (3.1)$$

Figure 1*a* 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 1*b*); in this case, 1 mM Cd²⁺ inhibits O₂ evolution by less than 3%.

Figure 1*c* 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*b*). On the other hand, increasing the amount of Mn²⁺ by threefold to 30 μ M did not produce any change in the cadmium inhibition

Figure 1. Dependence on Cd²⁺ concentration of oxygen evolution yield per flash: photoactivation using (a) 1 μ M apo-WOC-PSII and 10 μ M MnCl₂ and 10 mM CaCl₂ and (b) 1 μ M intact PSII with no added Mn²⁺ or Ca²⁺. All samples contain the indicated concentrations of CdCl₂ (μ M) in 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 (Y_{SS}) using apo-WOC-PSII, as a function of the indicated concentrations of MnCl₂ (μ M), CaCl₂ (mM) and CdCl₂ (μ M). Conditions are identical to (a). All traces are normalized to unit amplitude at 0 μ M Cd²⁺. All error bars represent one standard deviation of the mean (σ). Y_{SS} was obtained from fits to the model by extrapolation of the data to infinite time. The absolute values of Y_{SS} (mmol O₂/Chl/flash) at 0 [Cd] are as follows: 10 μ M Mn/10 mM Ca: $Y_{SS}=2.72 \pm 0.01$; 10 μ M Mn/30 mM Ca: $Y_{SS}=2.97 \pm 0.01$; 10 μ M Mn/50 mM Ca: $Y_{SS}=1.90 \pm 0.01$; 30 μ M Mn/10 mM Ca: $Y_{SS}=3.88 \pm 0.01$.

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 μM $MnCl_2$, 10 mM $CaCl_2$ and 100 μM $CdCl_2$. 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.

profile. 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 ($K_d = 0.45$ 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 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, A_2' and λ_2' , respectively:

$$Y(t) = Y_{ss} + A_1 e^{-\lambda_1 t} - A_2 e^{-\lambda_2 t} + A_2' e^{-\lambda_2' t}. \quad (3.2)$$

Excellent fits were obtained using the three-exponent 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

Figure 3. Effect of Cd^{2+} concentration on (a) the relative amplitudes A_2 and A_2' and (b) the corresponding rate constants λ_2 and λ_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 μM Mn^{2+} and 10 mM Ca^{2+} , and open and filled squares represent data at 10 μM Mn^{2+} and 10 mM Ca^{2+} . Error bars represent one standard deviation of the mean (σ), 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 μM Mn^{2+} and 50 mM Ca^{2+} , filled circles represent data at 10 μM Mn^{2+} and 30 mM Ca^{2+} , filled squares represent data at 10 μM 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.

Figure 4. Dependence on Cd^{2+} concentration of the rate constant λ_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 μM 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 3*b* plots the dependence of the corresponding rate constants, λ'_2 and λ_2 , as a function of the Cd^{2+} concentration. The rate constant for the Cd^{2+} -induced slow phase λ'_2 is 10-fold or more faster than λ_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 3*c* 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 Cd^{2+} does not change the rate constant λ_1 for the first step in photoactivation involving binding and photo-oxidation 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

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 μM MnCl_2 , 10 mM CaCl_2 , and the indicated concentrations of CdCl_2 (μM). Experiments were performed using samples and conditions as in figure 1.

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 (λ_2), which increases the yield of conversion (λ'_2) of intermediate $\text{IM}_1 \rightarrow \text{IM}_1^*$ 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 IM_1^* 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 Cd^{2+} promotes a shift between two populations, so that the faster pathway (our A_2' , λ_2') plays a significant role in photoassembly.

Our data suggest two possible models that could account for the opposing influence of Cd^{2+} on the rate and yield of photoactivation. In both of these models, Cd^{2+} binds to the calcium effector site during the dark step $\text{IM}_1 \rightarrow \text{IM}_1^*$ and produces the observed 10-fold acceleration of λ_2 (scheme 1). Model 1: the remaining photoassembly steps, involving binding and photooxidation of 3 Mn^{2+} , occur as usual, Cd^{2+} binds only to the functional calcium effector site and its binding to this site blocks expression of O_2 activity. In this case, Cd^{2+} replacement by Ca^{2+} in subsequent assembly steps would be required to account for the observed acceleration of recovery of O_2 -evolution activity. Model 2: this claims that Cd^{2+} binding to the calcium effector site actually supports some lower level of O_2 -evolution activity, but that inhibition of O_2 yield occurs at higher concentrations of Cd^{2+} because it blocks one or more of the remaining 3 Mn^{2+} in later photoassembly steps. Since we did not observe any O_2 recovery during photoactivation in the absence of Ca^{2+} , model 1 offers the best description of how Cd^{2+} blocks photoactivation. Model 1 predicts that the binding of Cd^{2+} must be weaker in the steps following IM_1^* formation.

We compared the IC_{50} values corresponding to Cd^{2+} concentrations that cause 50% reduction of: (i) the O_2 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 Ca^{2+} concentration (figures S1 and S2 in the electronic supplementary material). These IC_{50} values were extrapolated to zero Ca^{2+} concentration to obtain the apparent dissociation constants for Cd^{2+} . These values are approximately 120 μM for Y_{SS} and 50 μM for A_2 . We previously reported the affinities for Ca^{2+} activation of Y_{SS} and k_2 to be exactly the same at 1.4 mM (Ananyev & Dismukes 1996b; Zaltsman *et al.* 1997). Thus, Cd^{2+} exhibits a 10- to 30-fold higher affinity than Ca^{2+} for binding on the $\text{IM}_1 \rightarrow \text{IM}_1^*$ step when measured under identical conditions. From this we see that the Cd^{2+} binding affinity increases by 2.5-fold when measured using the specific reaction yield (A_2) that is coupled to Ca^{2+} binding in the native system, e.g. $\text{IM}_1 \rightarrow \text{IM}_1^*$, compared to using the overall reconstituted O_2 yield (Y_{SS}) which reflects the average affinity after the remaining 3 Mn^{2+} are photoassembled. This lower affinity for Cd^{2+} following the uptake and photooxidation of the remaining 3 Mn^{2+} fits well with the exchange model described above, whereby in fully reconstituted centres containing Mn_4Cd , the Cd^{2+} is more easily replaced by Ca^{2+} which produces a normal Mn_4Ca WOC that has assembled ten times faster. Thus, binding of Cd^{2+} to a single site, the Ca^{2+} 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 Cd^{2+} additionally blocking Mn^{2+} uptake in later steps after IM_1^* , which produce inactive centres that do not progress to active ones.

The relative binding affinities of various divalent metals (Mg^{2+} , Ca^{2+} , Mn^{2+} , Sr^{2+}) and the oxo-cation UO_2^{2+} have been previously measured for the Ca^{2+} 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 IC_{50} value for Cd^{2+} inactivation of Y_{SS} (120 μM) during photoactivation compares well with its IC_{50} reported for O_2 evolution from intact functional PSII membrane complexes ($K_{\text{D}} = 144 \mu\text{M}$; Vrettos *et al.* 2001). These authors report a higher binding affinity for Ca^{2+} to the intact functional PSII complex ($K_{\text{D}} = 69 \mu\text{M}$) 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 IC_{50} for Cd^{2+} inhibition of O_2 yield during photoactivation with continuous illumination (50% inhibition of photoactivation at 200 μM Cd^{2+} in the presence of 10 mM Ca^{2+} versus 125–140 μM Cd^{2+} for our result, figure 1c). These authors also concluded that Cd^{2+} binds at the calcium effector site and not at the high-affinity Mn^{2+} site based on competition studies. These authors did not explore the kinetic dimensions of the Cd^{2+} 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 Cd^{2+} , even though maximum activity is decreased. Other published studies have reported IC_{50} values in the low micromolar range for Cd^{2+} inhibition of O_2 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 Cd^{2+} binding during photoactivation played a more important role than Cd^{2+} binding to functional PSII.

The data in figure 5, showing that Cd^{2+} binding increases by twofold the mean lifetime for decay of photointermediates, indicate that Cd^{2+} interacts with the high-affinity Mn^{3+} to cause greater kinetic stability by slowing charge recombination with the reduced primary electron acceptor, Q_A^- . This positive mutual cooperativity provides a more efficient photoassembly process. It is counter-intuitive that adding a divalent cation like Ca^{2+} (or Cd^{2+}) close to Mn^{3+} would slow electron recombination, unless this binding were to create a thermodynamically more stable Mn^{3+} having a less positive electrochemical reduction potential. Such a potential decrease would be expected if proton ionization of a ligand coordinated directly to Mn^{3+} in IM_1 were to occur, as depicted in scheme 1. The case illustrated is for proton ionization of $\text{Mn}^{3+}(\text{OH}^-)$ to form a bridged oxide $\text{Mn}^{3+}(\text{O}^{2-})\text{Ca}^{2+}$. Independent evidence in support of this model comes from studies of

flash-induced pH changes showing that a proton is released at IM_1^* , either upon Ca^{2+} binding or Mn^{2+} photooxidation (Ananyev *et al.* 2001). This same structural model for conversion of $IM_1 \rightarrow IM_1^*$ was inferred based upon EPR spectroscopic data describing the ligand field at Mn^{3+} and its environment (Tyryshkin *et al.* 2006).

It is chemically reasonable to assume that Cd^{2+} binds in exactly the same way as Ca^{2+} to the calcium effector site to form a bridged structure: $[Mn^{3+}(\mu-O^{2-})Cd^{2+}]$. Slightly stronger ionic binding by Cd^{2+} versus Ca^{2+} to the $\mu-O^{2-}$ bridge might be expected based on its slightly greater charge density (ionic radius = 0.97 versus 0.99 Å for Ca^{2+}). Although this is borne out by the pK_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 $CdO_{(s)}$ versus $CaO_{(s)}$. The latter is measured by the large difference in their heats of formation $\Delta H_f^0 = -258$ versus 635 kJ mol^{-1} , respectively (Frenkel 2005). This undoubtedly arises from stronger interligand repulsions and from antibonding repulsion with the filled $4d^{10}$ shell in Cd^{2+} . Consequently, Cd^{2+} is expected to release electron density from $\mu-O^{2-}$ making it a stronger field ligand and more available for binding to Mn^{n+} ions in the WOC cluster compared with Ca^{2+} .

The foregoing analysis provides evidence that Cd^{2+} binding during the step $IM_1 \rightarrow IM_1^*$ causes three outcomes: (i) a longer intermediate lifetime that slows IM_1 reductive decay to IM_0 , (ii) ten times higher probability of the cofactor (or protein?) degrees of freedom needed to bind and photooxidize the remaining 3 Mn^{2+} that form the functional cluster, and (iii) (re)exchange of Cd^{2+} by Ca^{2+} following the latter steps restores active O_2 -evolving centres. The second outcome may involve forming additional bridges to the second (or multiple) Mn^{2+} ions which is the one-electron precursor to intermediate IM_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^{2+} comprising the active cluster may also contribute. In this regard, it was previously shown by EPR spectroscopy that at higher cofactor concentrations, Ca^{2+} templates binding of the initial 2 Mn^{2+} to apo-WOC-PSII even in the dark, yielded a spin-coupled dimanganese (II,II) pair (separation $< 4.25 \text{ Å}$) that can go on to productive photoactivation (Ananyev & Dismukes 1997). Additionally, without Ca^{2+} (or Sr^{2+}) excess Mn^{2+} gets photooxidized during photoassembly, with as many as 20 Mn^{2+} oxidations and no functional O_2 evolution observable (Chen *et al.* 1995; Ananyev & Dismukes 1996b; Ananyev *et al.* 2001). Collectively, these studies strongly suggest that Ca^{2+} directs the final steps of photoassembly of the WOC by creating sites for the binding and photooxidation of the final 3 Mn^{2+} , coincident with the formation of the oxo bridge at IM_1^* , e.g. $[Mn^{2+}(\mu-O^{2-})Cd^{2+}]$. At intracellular cofactor concentrations found *in vivo*, it does so 'on the fly', after photochemical formation of IM_1^* , by forming solvent-derived hydroxide/oxide bridge(s) which enable formation of the functional Mn_4O_xCa core. An oxo-bridged structure for IM_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^{2+} does not contribute to the greater photooxidation yield of the first Mn^{2+} (high-affinity Mn^{2+}), as is clear from the independence of the rate constant λ_1 on Cd^{2+} concentration. In this regard, the function of Cd^{2+} in photoassembly differs completely from the role of bicarbonate which accelerates λ_1 by direct thermodynamic stabilization of the photooxidized high-affinity Mn^{3+} (Baranov *et al.* 2004; Kozlov *et al.* 2004).

Sr^{2+} is the only other functionally active metal known to support water oxidation/ O_2 production in the Ca^{2+} effector site of PSII. Sr^{2+} substituted PSII has been prepared by biochemical exchange using isolated functional PSII centres, by biosynthetic growth on Sr^{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_2 evolution rates were found (30–35% of the Ca^{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: $S_3 \rightarrow S_4 \rightarrow S_0 + O_2$. Previous photoactivation studies found that Sr^{2+} replacement for Ca^{2+} accelerates the forward dark step (λ_2) by twofold and slows the rate of decay of the high-affinity Mn^{3+} by fivefold (k_{-1} step; Ananyev *et al.* 2001). The latter process is believed to involve $Mn^{3+}Q_A^-$ charge recombination to form IM_0 at the high-affinity site coupled to displacement of the resulting $Mn^{2+}(OH^-)$ by $Me^{2+}(OH^-)$, equation (4.1) (Ananyev *et al.* 2001):

By this mechanism, the capacity for Sr^{2+} and Cd^{2+} to slow this process relative to Ca^{2+} should correlate with the binding affinity for $Me^{2+}(OH^-)$ at the high-affinity Mn^{2+} site. By contrast, a considerably faster rate acceleration of λ_2 occurs in the presence of Cd^{2+} ($10\times$) versus Sr^{2+} ($2\times$) relative to Ca^{2+} . We have interpreted this to indicate that Cd^{2+} and Sr^{2+} more rapidly find and assemble the proposed bridged intermediate IM_1^* (scheme 1). In future work it would be worthwhile to investigate the Cd^{2+} substituted IM_1^* intermediate by EPR spectroscopy, as it should be possible to distinguish the ligand field parameters of the Mn^{3+} to which it is bridged in the proposed model of scheme 1, much as has been achieved with the native Ca^{2+} intermediate IM_1^* (Tyryshkin *et al.* 2006).

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