Investigation of the Functional Role of Ca^{2+} in the Oxygen-Evolving Complex of Photosystem II: A pH-Dependence Study of the Substitution of Ca^{2+} by Sr^{2+}

Cheng-I Lee and Gary W. Brudvig*
Department of Chemistry, Yale University, P. O. Box 208107, New Haven, CT 06520-8107, USA

The oxygen-evolving complex (OEC) of photosystem II (PSII) is the catalytic site for the oxidation of water to O_2 in photosynthesis. The OEC consists of a tetranuclear manganese (Mn_4) cluster with calcium and chloride ions functioning as essential cofactors. Previous studies have shown that substitution of Ca^{2+} in the OEC by various divalent and trivalent metal ions results in the loss of oxygen-evolution activity of PSII. Sr^{2+} is the only ion that has been found to restore the oxygen-evolving activity in PSII. Although several models have been proposed for the mechanism of water oxidation in PSII, the functional role of Ca^{2+} remains unclear. In order to test the proposal that Ca^{2+} functions as a Lewis acid in water oxidation chemistry, the pH dependence of the oxygen-evolution activity of intact Ca^{2+}-containing PSII and Sr^{2+}-substituted PSII has been investigated. In both samples, the pH dependence exhibits a bell-shaped curve. Sr^{2+} substitution shifts the peak of the curve to higher pH in comparison to Ca^{2+}-containing PSII. The pH-dependent O_2-evolving rates of these two samples are fit to a diprotic model in which protonation of an essential basic group at acidic pH inhibits activity (pKa_1) and deprotonation of an essential acidic group at higher pH inhibits activity (pKa_2). Sr^{2+} substitution has no effect on pKa_2. This result suggests that the high pH inhibition may be on the electron-acceptor side of PSII. On the other hand, Sr^{2+} substitution causes pKa_1 to be shifted to higher pH by 1.0 pH unit. This pH-shift indicates a direct role of Ca^{2+}/Sr^{2+} in the OEC. The functional role of Ca^{2+} in oxygen evolution in PSII is discussed based on these results.

Keywords: Photosystem II; Calcium; Strontium; Oxygen-evolving complex; Water oxidation; pH-Dependence.

INTRODUCTION

Photosystem II (PS II) is an integral membrane protein complex that utilizes light energy to oxidize water to O_2. Photosynthetic oxygen evolution is carried out at the oxygen-evolving complex (OEC) of PSII, which includes a tetranuclear manganese cluster (Mn_4), a redox-active tyrosine residue (Y_Z), and Cl^- and Ca^{2+} cofactors.[1] When one of the chlorophylls or carotenoids in PSII absorbs a photon of visible light, the excited state is transferred to a special chlorophyll called P_{680}, forming the excited singlet state P_{680}*. P_{680}* donates an electron to a pheophytin (Pheo) cofactor, resulting in the charge-separated $P_{680}^+Pheo^-$ state. This charge separation is stabilized by the rapid transfer of the electron to a membrane bound quinone, Q_A, forming the $P_{680}^+Q_A^-$ state. Then, the electron on Q_A^- is transferred to an exchangeable quinone, Q_B, which dissociates from PSII upon its two-electron/two-proton reduction. P_{680}^+ is reduced by the Y_Z residue, which subsequently gets re-reduced by the Mn_4 cluster. In turn, the Mn_4 cluster is successively oxidized through five 'S_n' states (where n = 0-4), in which S_0 is the most reduced state, S_1 is the most dark-stable state and S_4 is an unstable,

Dedicated to Professor Sunney I. Chan on the occasion of his 67th birthday and his retirement from professional life.

* Corresponding author. Tel: (203) 432-5202; fax: (203) 432-6144; e-mail: gary.brudvig@yale.edu

Abbreviations: DCBQ, 2,5-dichloro-*p*-benzoquinone; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; FTIR, Fourier Transfer Infrared; MES, 2-(N-morpholino)ethanesulfonic acid; Mn_4, tetranuclear manganese cluster; OEC, oxygen-evolving complex; Pheo, pheophytin; PSII, photosystem II; PIPES, 1,4-bis(4-sulphobutyl)piperazin; Q_A, tightly bound plastoquinone in PSII; Q_B, exchangeable plastoquinone in PSII; Y_D, tyrosine 160 of the D2 protein; Y_Z, tyrosine 161 of the D1 protein.

transient state. Upon formation of the S_4 state, the Mn_4 cluster is rapidly reduced by four electrons by oxidizing H_2O, and one dioxygen molecule is released during the $S_3 \rightarrow [S_4] \rightarrow S_0$ state transition. The PSII core complex contains a number of transmembrane polypeptides that bind the photoactive pigment molecules and redox cofactors.[2] In addition, several extrinsic polypeptides are bound to the lumenal surface of the PSII protein complex. In higher plants, these extrinsic polypeptides include the 17, 23 and 33 kDa proteins. The 17 and 23 kDa polypeptides are barriers for diffusion of Ca^{2+} and Cl^-, to prevent dissociation of these ions into the solvent. The 33 kDa polypeptide stabilizes the Mn_4 cluster.

The X-ray crystal structure of PSII has been emerging, but remains at low resolution in comparison to soluble proteins whose structures are typically solved at 1~2 Å, as is the case of most membrane proteins because of their complexity and the difficulties in sample preparations. Three crystal structures of PSII have been solved: the first by electron crystallography at 8 Å resolution,[3] and two recent X-ray crystal structures at 3.8 Å[4] and 3.7 Å[5] resolution, respectively. The crystal structures identify the location of the Mn_4 cluster at a position 15 Å from the center of P_{680}, but the structure of the Mn_4 cluster and the location of the Ca^{2+} and Cl^- cofactors in the OEC cannot be discerned. The exact structure of the OEC is still under debate, although several spectroscopic methods have been applied. Extended X-ray absorption fine structure (EXAFS) studies have reported a tetramer of μ-oxo-bridged units with Mn-Mn separations of 2.7 Å and 3.3 Å.[6-8] EXAFS data of the native and Sr^{2+}-substituted OEC suggest that Ca^{2+} is very close to the Mn_4 cluster; Mn-Ca distances of 3.3/3.4 Å[9,10] and 4.2 Å[11] have been reported.

The S_2 state is an electron paramagnetic resonance (EPR) active state and the oxidation state of the Mn_4 cluster in the S_2 state is generally considered to be $Mn(III)Mn(IV)_3$.[6-7] The Mn_4 cluster has a pronounced multiline EPR signal at $g \sim 2$ in the S_2 state. In addition to the multiline signal, the S_2 state also exhibits an EPR signal at $g = 4.1$ from a high-spin conformation. These signals have been very useful to characterize the S_2 state and the S_1 to S_2 transition.

The number of Ca^{2+} in the OEC has been determined by several methods, including steady-state activity measurement,[12] atomic absorption,[13] scintillation counting with the use of ^{45}Ca,[14] and equilibrium measurements by using a Ca^{2+}-sensitive electrode.[15] Overall, there is general agreement on the presence of one high-affinity Ca^{2+} site in the OEC that affects O_2-evolution rates ($K_m \sim$ μM). In addition, ~4 other Ca^{2+} ions bind with high affinity to the light-harvesting proteins in spinach PSII membrane preparations ($K_d \sim 2$ μM).[15]

It has been demonstrated that Ca^{2+} is required for oxygen evolution and that the depletion of Ca^{2+} from PSII causes loss of activity.[16] Ca^{2+} has been proposed and is generally believed to have its functional role beyond the S_2 state, because removal of Ca^{2+} from PSII blocks the S_2 to S_3 state transition and results in the formation of the $S_2Y_Z^{\bullet}$ "split" EPR signal.[17,18] Many research groups have been working to understand the role of Ca^{2+} in the OEC. Because Ca^{2+} ions lack unpaired electrons for magnetic resonance studies and do not have accessible electronic transitions for optical detection, many studies have been made on samples in which Ca^{2+} has been replaced with other cations. Therefore, procedures for depletion of Ca^{2+} from PSII and for substitution of the site by another cation are necessary. Several methods for Ca^{2+} depletion have been reported. These include a high salt (1-2 M NaCl) wash followed by the addition of a chelating agent (EDTA or EGTA) at neutral pH, and incubation with citrate at pH 3.0 followed by adjustment of the pH back to neutral pH. Conducting the treatment in the presence or absence of light has been reported to affect the rate of Ca^{2+} depletion. Various divalent cations or lanthanide ions have been substituted into the vacant Ca^{2+} site and the properties of the substituted samples have been investigated. Interpretation of these results requires that any changes are from the specific substitution of Ca^{2+} in the OEC. However, the previous reported treatments used to deplete PSII of Ca^{2+} could cause effects unrelated to Ca^{2+} depletion. To avoid possible nonspecific effects, a moderate treatment for the Ca^{2+} site is required. Vrettos et al. have successfully replaced Ca^{2+} with various divalent and trivalent cations by using a 4-h cation-exchange incubation.[19] This reversible method for substitution of Ca^{2+} without prior Ca^{2+} depletion is used in this work to study the role of Ca^{2+} in the OEC.

A Fourier Transfer Infrared (FTIR) study has shown the similarity of S_2/S_1 difference spectra between untreated PSII and Ca^{2+}-depleted PSII, but the addition of chelating agents (EDTA or EGTA) caused the disappearance of carboxylate bands.[20] An EPR power-saturation study indicated that EGTA can significantly decrease the S_2-multiline signal amplitude, and this effect is unrelated to Ca^{2+} binding.[17] Therefore, we cannot neglect the alteration of the spectroscopic signal contributed by the chelating agents and need to examine carefully those results in which chelating agents were used. In the present study, the cation-exchange method used for substitution of Ca^{2+} avoids the use of chelating agents and, thus, potential complications from the effects of chelating agents on the properties of the OEC are circumvented.

In many Ca^{2+}-containing proteins, a variety of multivalent cations are capable of replacing Ca^{2+} based on their size and charge similarity.[21] The Ca^{2+} site in the OEC has been found to be size-selective;[19] only divalent cations and lanthanide ions with an ionic radius similar to that of Ca^{2+} are capable of binding with high affinity to the Ca^{2+} site. Substitution of Ca^{2+} in the OEC by various divalent cations and lanthanide ions results in the loss of oxygen-evolution activity of PSII. However, substitution of Sr^{2+} ions into the Ca^{2+}-binding site restores ~40% of the O_2-evolution activity.[22]

The S_2 state exhibits an altered and more stable multiline EPR signal after the removal of Ca^{2+} from PSII. Addition of Sr^{2+} into the vacant Ca^{2+} site yields a different altered multiline EPR signal and also gives a higher yield of the S_2 state g = 4.1 EPR signal. The S_2-state multiline EPR signal has not been observed in any other cation-substituted (La^{3+}, Dy^{3+}, Cd^{2+}) PSII.

Many models of oxygen evolution have been proposed,[23,24] but the functional role of Ca^{2+} was not incorporated until the models of Limburg et al.,[25] Pecoraro et al.[26] and Siegbahn and Crabtree[27] were reported. Vrettos et al. proposed a mechanism based on proton-coupled electron transfer (PCET) during the advancement of the S states, in which consecutive PCET and concerted PCET proceeds before and after S_2 state, respectively.[28] In this Brudvig model, the Ca^{2+} ion plays the role of a Lewis acid to bind and activate a substrate water molecule. This Ca^{2+}-bound water molecule is deprotonated by a nearby protein residue and then nucleophilically attacks a Mn^{V}=O species to form the O-O bond, which is subsequently released from the OEC as O_2 (Fig. 1). Therefore, cations with a Lewis acidity similar to that of Ca^{2+}, such as Sr^{2+}, are capable of functionally replacing Ca^{2+} in the OEC.[19,28]

In this work, we have investigated the pH dependence of O_2 evolution in intact Ca^{2+}-containing PSII and in Sr^{2+}-substituted PSII, as Sr^{2+} is the only cation other than Ca^{2+} that has been found to yield O_2-evolving activity. We observed a bell-shaped pH dependence in both samples and a significant shift of the pH dependence in Sr^{2+}-substituted PSII in comparison to Ca^{2+}-containing PSII. The functional role of Ca^{2+} in the OEC of PSII is discussed based on these results.

EXPERIMENTAL SECTION

Protein Preparation

BBY-type PSII-enriched membrane fragments were isolated from market spinach according to the procedure of Berthold et al.[29] with the modification of Beck et al.[30] The PSII samples were stored until use in a liquid nitrogen freezer after freezing in a buffer containing 20 mM MES (pH 6.0), 15 mM NaCl, and 30% (w/v) ethyl glycol.

Preparation of Sr^{2+}-Substituted Samples

In order to substitute Sr^{2+} into the OEC of PSII, a large excess of $SrCl_2$ instead of $CaCl_2$ was added to PSII samples. For studies at different pH's, the buffers contained 20 mM MES, 20 mM PIPBS (pKa_1 = 4.6 and pKa_2 = 8.6), 15 mM NaCl, and either 20 mM $CaCl_2$ (for Ca^{2+}-containing PSII) or 40 mM $SrCl_2$ (Sr^{2+}-substituted PSII). The pH of each sample was adjusted to the desired value between 4 and 8. PSII samples were incubated in Sr^{2+}-containing buffers on ice for 4 h. No incubation was required for 20 mM $CaCl_2$ containing samples.

Oxygen-evolution Activity Measurement

O_2-evolving activities were measured with a Clark-type electrode at 25 °C. An Oriel 1000 W tungsten lamp, fitted

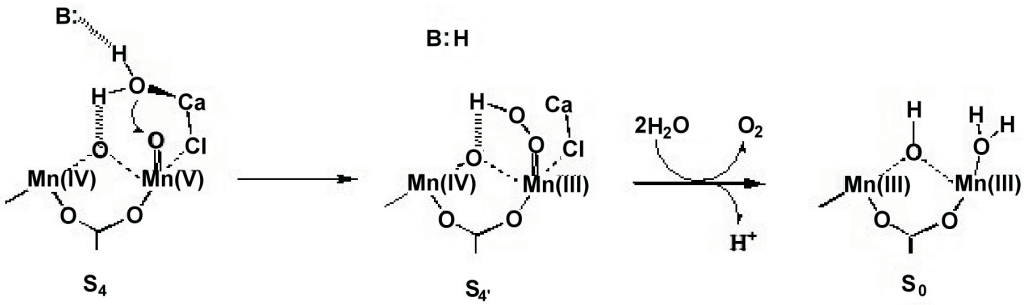

Fig. 1. Proposed mechanism of O-O bond formation by the OEC showing the role of Ca^{2+} as a Lewis acid to activate one substrate water as a nucleophile (adapted from Vrettos et al.[28]). Only one di-manganese unit within the tetranuclear Mn cluster in the OEC is shown. An unidentified residue acts as a base, B, for deprotonation of a Ca^{2+}-bound water in formation of the O-O bond, which is subsequently released as O_2.

with a liquid filter (filled with distilled water), a 610 nm cut-off filter (LP 610) and a heat filter, was used to initiate the photochemistry. All the samples were allowed to equilibrate at room temperature for 15 min prior to conducting the O_2-evolution assay. 250 μM DCBQ and 1 mM $K_3Fe(CN)_6$ were added to all samples. Typical O_2-evolution rates were 400-600 μmoles O_2 (mg Chl)$^{-1}$ hr^{-1} for PSII membranes in buffer containing 20 mM MES (pH 6.0), 15 mM NaCl and 20 mM $CaCl_2$.

RESULTS

Substitution of Sr^{2+} and Reversibility

The substitution of Sr^{2+} reaches equilibrium in 4 h, at which point the activity does not change with time (data not shown). After the 4 h incubation, 43% of the activity remains in the Sr^{2+}-substituted PSII (Table 1), as has been reported in a previous study on the substitution of Sr^{2+} into a vacant Ca^{2+} site.[22] The reconstitution was done by using one wash followed by the addition of Ca^{2+}, and 76% of the activity was recovered. The reversibility of acidic (pH 4.0) or basic (pH 8.0) treatments was also examined through suspension of the pellet of an acid- or base-treated sample into a buffer at pH = 6.0. Significant inhibition (only 7% of activity remains) is caused by either acidic or basic treatment and most of the activity is recovered by adjustment of the pH back to 6.0 (Table 2). After re-adjustment with neutral buffer (pH 6.0), the activity of PSII is restored to 79% (acidic treated) or 88% (basic treated) of the activity of an untreated PSII sample.

The pH-Dependence of Oxygen-evolution Activity

The oxygen evolution profiles as a function of pH in the presence of $CaCl_2$ and $SrCl_2$ are shown (Fig. 2). The bell-shaped curves are generated by individual measurements from the same batch of samples that were adjusted to the indicated pH prior to conducting the assay. These curves show that both deprotonation and protonation steps affect the rate of oxygen evolution and can be simulated by a diprotic model. The diprotic system can be expressed by the following equations:

$$\mathrm{PSII \cdot BH \cdot A} \equiv \text{Active} \quad (1)$$

$$\mathrm{PSII \cdot BH \cdot A + H^+} \xleftrightarrow{Ka_1} \mathrm{PSII \cdot BH \cdot AH^+} \quad (2)$$

$$\mathrm{PSII \cdot BH \cdot A} \xleftrightarrow{Ka_2} \mathrm{PSII \cdot B^- \cdot A + H^+} \quad (3)$$

In such a diprotic system, the reaction rate can be furthermore expressed as following mathematical equation.[31]

Table 1. The substitution of metal ions into the Ca^{2+} site in PSII

	O_2-evolution activity (μmol O_2/mg Chl h) at pH 6.0		
	Untreated PSII	Treated PSII	Reconstitution with Ca^{2+}
This work			
Sr^{2+} (40 mM)	430 ± 10 (100)[a]	190 ± 10 (43)	330 ± 10 (76)
Dy^{3+} (40 mM)	700 ± 30 (100)	44 ± 9 (6)	68 ± 7 (10)
Previous work			
Sr^{2+} (15 mM)[b]	780 (100)	330 (42)	
Dy^{3+} (10 mM)[c]	720 (100)	0	0

[a] The numbers in parentheses represent the percentage of activity relative to that of untreated PSII.
[b] From Ghanotakis et al.[22]
[c] From Bakou et al.[39]

Table 2. Reversibility of acidic and basic treatments of PSII

O_2-evolution activity (μmol O_2/mg Chl h) measured at the indicated pH			
Untreated PSII at pH 6	Treated at pH 4	Treated at pH 8	Treated PSII returned to pH 6
400 ± 10 (100)[a]	26 ± 6 (7)		320 ± 20 (79)
400 ± 10 (100)		26 ± 4 (7)	350 ± 40 (88)

[a] The numbers in the parentheses are the percentages of activity relative to that of untreated PSII.

$$V\max_{app} = \frac{V\max}{1+\frac{[H^+]}{Ka_1}+\frac{Ka_2}{[H^+]}} \quad (4)$$

where $V\max_{app}$ is the observed rate and $V\max$ is the theoretical maximum. Only the *PSII·BH·A* species is active, and neither the protonated (*PSII·BH·AH*$^+$) nor the deprotonated (*PSII·B*$^-$*·A*) form is active. The constants, Ka_1 and Ka_2, represent the equilibrium constants of the protonation and deprotonation reactions, respectively.

Simulated protonation and deprotonation reactions and their superposition by using this diprotic model eq (4) are shown (Fig. 3).

After a 4 h-incubation, we still can not expect a complete substitution of Ca^{2+} with Sr^{2+}. Therefore, for Sr^{2+}-substituted PSII, we consider the residual Ca^{2+}-bound PSII that contributes some of the O_2-evolution activity and modify eq (4) as

$$V\max_{app} = F\times\left(\frac{V\max}{1+\frac{[H^+]}{Ka_1}+\frac{Ka_2}{[H^+]}}\right) + (1-F)\times\left(\frac{V\max}{1+\frac{[H^+]}{Ka_1(Ca)}+\frac{Ka_2(Ca)}{[H^+]}}\right) \quad (5)$$

where the substitution factor, F, is the fraction of Sr^{2+}-substituted PSII, and the factor (1-F) represents the residual Ca^{2+}-containing PSII that is fully functional. The values of Ka_1 and Ka_2 obtained from the curve fitting of Ca^{2+}-containing PSII (Ka_1 (Ca) = 5.2 ± 0.04 and Ka_2 (Ca) = 6.8 ± 0.05) are substituted as fixed constants into the fitting for Sr^{2+}-substituted PSII. The fits of pKa_1, pKa_2 and Vmax (normalized to the activity of Ca^{2+}-containing PSII at pH 6.0) of Ca^{2+}-containing PSII and Sr^{2+}-substituted PSII based on the diprotic model (eq (4) and eq (5), respectively) are listed (Table 3). Additionally the fraction of Sr^{2+}-substituted PSII was calculated to be 0.90 ± 0.07. The same experiment was repeated and the reported values are the average of fits from two independent sets of data in which different batches of PSII were used.

DISCUSSION

In this investigation, we have used a cation-exchange method to substitute Sr^{2+} into the Ca^{2+} site in the OEC. This method avoids the use of high-salt or low-pH conditions, which may cause irreversible effects on the OEC or perturb the PSII sample in ways not related to the exchange of metal

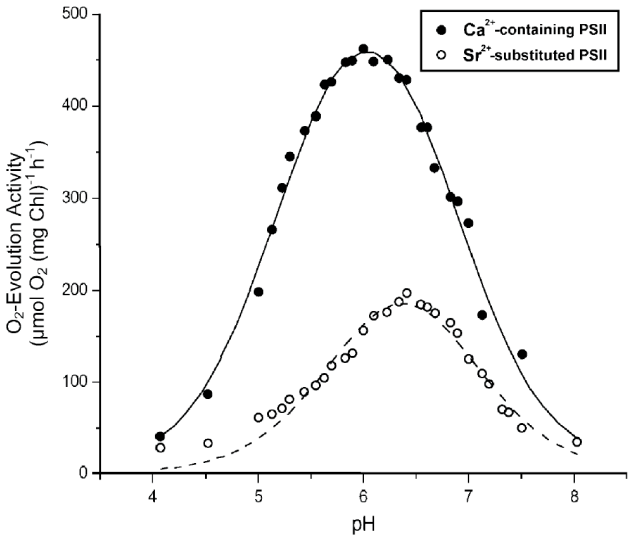

Fig. 2. pH-dependence of O_2-evolution activity of Ca^{2+}-containing PSII (filled circles), Sr^{2+}-substituted PSII (open circles). The lines through the data points show the fits using eq (4) for Ca^{2+}-containing PSII and eq (5) for Sr^{2+}-substituted PSII. The O_2-evolution activities were measured at various pH values independently. See the text for the assay conditions and the curve-fitting models.

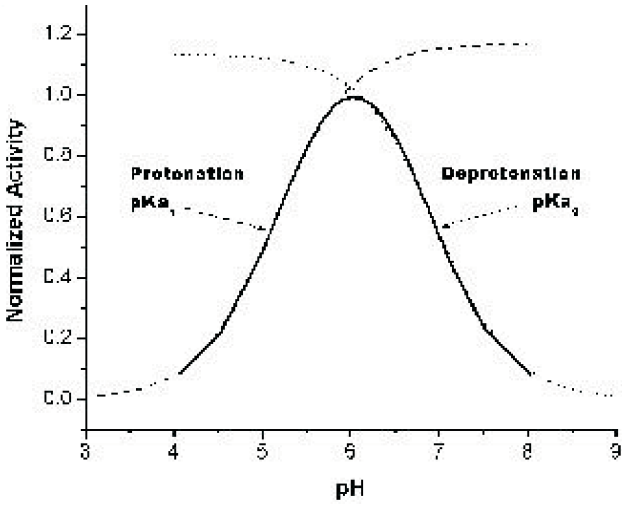

Fig. 3. Simulation of the pH-dependence of O_2-evolution activity using a diprotic model that includes a protonation reaction (dashed line), a deprotonation reaction (dotted line) and their superposition (solid line).

Table 3. Fits of pK_1 and pK_2 based on the diprotic model from two independent sets of data

Sample	pKa_1	pKa_2	Vmax	F[a]
PSII	5.20 ± 0.04	6.83 ± 0.05	1.30 ± 0.03	n/a
Sr^{2+}-PSII	6.2 ± 0.2	6.7 ± 0.2	0.7 ± 0.2	0.90 ± 0.07

[a] F is the fraction of Sr^{2+}-substituted PSII. The remaining fraction is assumed to consist of native, Ca^{2+}-containing PSII.

ions in the Ca^{2+} site in the OEC. It also avoids the use of chelators, such as EDTA or EGTA, which have been found to alter the OEC in ways that are unrelated to Ca^{2+} binding. Equilibrium of cation exchange in the Ca^{2+} site is reached after 4 h. By exchanging the endogenous Ca^{2+} in PSII with a solution containing 40 mM $SrCl_2$, we find that 90% of the Ca^{2+} in the OEC is replaced by Sr^{2+}. Furthermore, the exchange is reversible. By pelleting and resuspending the Sr^{2+}-substituted sample a single time in a solution containing 20 mM $CaCl_2$, we find that ~80% of the activity is restored; a more complete cation exchange can be obtained by using a 4 h-incubation and/or multiple washes. Therefore, the cation-exchange method we have used to prepare Sr^{2+}-substituted PSII is a good method to study the role of Ca^{2+} in the OEC.

pH Dependence and the Diprotic Model

Both Ca^{2+}-containing and Sr^{2+}-substituted PSII exhibit a bell-shaped pH dependence as shown in Fig. 2. Before analyzing these data, it is important to establish that the effects of pH are reversible. Acidic and basic treatments are common methods for protein denaturation. In the moderate range of pH 4 to 8 that we have used, PSII is less likely to denature. Nevertheless, the reversibility of acidic/basic treatments was tested. As shown in Table 2, a large fraction of the activity was recovered following adjustment of the pH to either 4 or 8 and then back to 6.

We find that the maximum O_2-evolving activity is at pH 6.00 and pH 6.35 in the presence of Ca^{2+} and Sr^{2+}, respectively. Our results on the pH dependence of O_2-evolution activity for untreated, Ca^{2+}-containing PSII membranes agree with previous studies by Berthold et al.[29] and Bernat et al.,[32] who found that the pH of maximum activity was at approximately pH 6 within a bell-shaped curve. However, Damoder and Dismukes found the pH of maximum activity at pH 7 within an asymmetric bell-shaped curve.[33] In our work, pH steps of 0.1 were used so that we could define the pH profile more precisely.

The pH-dependence data were fit to a diprotic model in which protonation of an essential basic group at acidic pH inhibits activity (pKa_1) and deprotonation of an essential acidic group at higher pH inhibits activity (pKa_2). The diprotic model (eq (4) and eq (5)) fits the data very well ($R^2 > 0.963$).

The pKa_2 values of Ca^{2+}-containing PSII (6.8 ± 0.05) and Sr^{2+}-substituted PSII (6.7 ± 0.2) are about the same. The observation that Sr^{2+} substitution has no effect on pKa_2 suggests that the high pH inhibition may be on the electron-acceptor side of PSII. The acceptor-side reaction of PSII involves the two-electron/two-proton reduction of plastoquinone. It is known in the analogous bacterial reaction center (bRC) that proton transport pathways involving a series of His, Asp and Glu residues are involved in the quinone reduction reaction.[34] The rate of reduction of Q_B in the bRC is greatly slowed with increasing pH due to ionization of Glu-L212 (pKa ~ 8.5). The value of pKa_2 identified for PSII may be associated with an analogous proton transport pathway on the electron-acceptor side of PSII.

Sr^{2+} substitution caused pKa_1 to be shifted to higher pH by 1.0 pH unit (5.2 ± 0.04 for Ca^{2+}-containing PSII, 6.2 ± 0.2 for Sr^{2+}-substituted PSII). The significant effect of Sr^{2+} on pKa_1 provides further evidence for a direct role of Ca^{2+}/Sr^{2+} in the OEC and indicates that Ca^{2+} affects the rate of O_2 evolution via a step involving a protonation reaction (discussed in the next section). It is notable that the shift of pKa_1 to higher pH upon substitution of Sr^{2+} for Ca^{2+} correlates with the different Lewis acidities of Ca^{2+} and Sr^{2+}. Ca^{2+} is a somewhat stronger Lewis acid than Sr^{2+}, as reflected by the difference in their pKa values in water (12.80 for Ca^{2+} and 13.18 for Sr^{2+}).[35] Our group has proposed that Ca^{2+} functions in the OEC as a Lewis acid to activate one substrate water molecule as a nucleophile (Fig. 1).[28] We also proposed that Sr^{2+} is able to functionally replace Ca^{2+} because it is the only metal cation that has a similar Lewis acidic strength to Ca^{2+} among the divalent and trivalent cations with a similar size to Ca^{2+} so that they bind to the OEC with high affinity.[19] According to this proposal, Sr^{2+}-substituted PSII should be most active when the pH of the sample is adjusted to compensate for its slightly weaker Lewis acidity compared to Ca^{2+}. The finding that Sr^{2+} substitution caused pKa_1 to be shifted to higher pH by 1.0 pH unit provides strong support for these proposals.

Role of Ca^{2+} in the OEC

At low pH, protonation of an essential basic group inhibits O_2-evolving activity (pKa_1). Owing to the large effect of Sr^{2+}-substitution on pKa_1, we conclude that this essential basic group is closely associated with Ca^{2+} in the OEC. One

possibility is that the essential basic group is the base (B, Fig. 1) that has been proposed to be required to accept a proton from the Ca^{2+}-bound water during its nucleophilic attack on the second substrate water. This assignment is consistent with studies of other metalloenzymes in which a metal ion functions as a Lewis acid to activate water as a nucleophile. The pH dependences of Zn^{2+} enzymes that catalyze hydrolytic reactions, such as carbonic anhydrase and carboxypeptidase A, have been studied in detail.[36] For carboxypeptidase A, k_{cat}/K_m pH profiles are bell-shaped and characterized by an inhibition at acidic pH with a p*K*a of about 6. This p*K*a has been assigned to protonation of the Glu-270-coordinated H_2O species (Fig. 4A). In this case, the enzymatic reaction is inhibited upon protonation of the base that is required to accept a proton from water during its nucleophilic attack on the substrate. Similarly, inhibition of the O_2-evolution reaction in PSII at low pH could be due to protonation of an essential base that is required to deprotonate a substrate water molecule bound to Ca^{2+}.

The shift of pKa_1 to higher pH upon substitution of Sr^{2+} for Ca^{2+} provides additional information about the role of Ca^{2+}. It is expected that this shift should correlate with the different chemical properties of Ca^{2+} and Sr^{2+}. Ca^{2+} is a slightly stronger Lewis acid and, therefore, the p*K*a of a Ca^{2+}-bound ligand should be lower than that of the homologous Sr^{2+}-bound ligand. On the other hand, the p*K*a of a basic residue that is hydrogen bonded to a Ca^{2+}-bound ligand is expected to be less affected by Sr^{2+} substitution. Therefore, we consider the possibility that B is directly coordinated to Ca^{2+}/Sr^{2+} (Fig. 4B). In this case, the p*K*a of the coordinated base should shift to higher pH upon substitution of Sr^{2+} for Ca^{2+} and be strongly affected by the metal ion, both of which are observed.

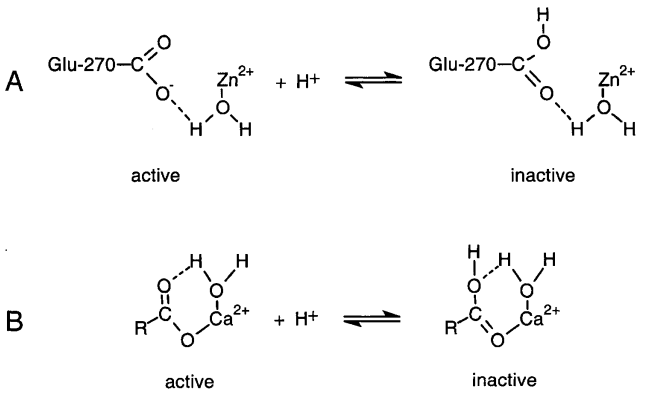

Fig. 4. Models of the protonation reactions in: (A) carboxypeptidase A and (B) the OEC.

CONCLUSIONS

The observed effects on the pH dependence of O_2 evolution upon substitution of Sr^{2+} for Ca^{2+} provide strong support for the proposal that Ca^{2+} plays a role as a Lewis acid in the OEC. The data indicate that Ca^{2+} is specifically involved in a protonation step that is crucial to the O_2-evolution reaction. The observed inhibition of O_2 evolution at low pH can be accounted for by protonation of an essential basic residue that is required to accept a proton from a Ca^{2+}-bound water during its nucleophilic attack on the substrate. A consideration of the shift in the pH dependence upon substitution of Sr^{2+} for Ca^{2+}, in light of the different chemical properties of Ca^{2+} and Sr^{2+}, suggests that this basic residue could be directly coordinated to Ca^{2+}. Potential candidates for this coordinated base are monodentate carboxylic acid side chains from Glu or Asp residues,[2] the carboxy terminus of the D1 protein,[37] or bicarbonate, which has been suggested to be a cofactor in the OEC.[38]

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REFERENCES

1. Debus, R. J. *Biochim. Biophys. Acta* **1992**, *1102*, 269.
2. Debus, R. J. *Metal Ions Biol. Syst.* **2000**, *37*, 657.
3. Rhee, K. H.; Morriss, E. P.; Barber, J.; Kühlbrandt, W. *Nature (London)* **1998**, *396*, 283.
4. Zouni, A.; Witt, H. T.; Kern, J.; Fromme, P.; Krauss, N.; Saenger, W.; Orth, P. *Nature (London)* **2001**, *409*, 739.
5. Kamiya, N.; Shen, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 98.
6. Yachandra, V. K.; Sauer, K.; Klein, M. P. *Chem. Rev.* **1996**, *96*, 2927.
7. Penner-Hahn, J. E. *Struct. Bonding* **1998**, *90*, 1.
8. Maclachlan, D. J.; Hallahan, B. J.; Ruffle, S. V.; Nugent, J. H. A.; Evans, M. C. W.; Strange, R. W.; Hasnain, S. S. *Biochem. J.* **1992**, *285*, 569.
9. Latimer, M. J.; DeRose, V. J.; Yachandra, V. K.; Sauer, K.; Klein, M. P. *J. Phys. Chem. B* **1998**, *102*, 8257.

10. Cinco, R. M.; Holman, K. L. M.; Robblee, J. H.; Yano, J.; Pizarro, S. A.; Bellacchio, E.; Sauer, K.; Yachandra, V. K. *Biochemistry* **2002**, *41*, 12928.
11. Penner-Hahn, J. E.; Fronko, R. M.; Pecoraro, V. L.; Yocum, C. F.; Betts, S. D.; Bowlby, N. R. *J. Am. Chem. Soc.* **1990**, *112*, 2549.
12. Han, K. C.; Katoh, S. *Biochim. Biophys. Acta* **1995**, *1232*, 230.
13. Kalosaka, K.; Beck, W. F.; Brudvig, G. W.; Cheniae, G. In *Current Research in Photosynthesis*; M. Baltsheffsky, Ed.; Kluwer: Dordrecht, The Netherlands, 1990; p 721.
14. Ädelroth, P.; Lindberg, K.; Andréasson, L. E. *Biochemistry* **1995**, *34*, 9021.
15. Grove, G. N.; Brudvig, G. W. *Biochemistry* **1998**, *37*, 1532.
16. Yocum, C. F. *Biochim. Biophys. Acta* **1991**, *1059*, 1.
17. Boussac, A.; Zimmermann, J. L.; Rutherford, A. W. *Biochemistry* **1989**, *28*, 8984.
18. Lakshmi, K. V.; Eaton, S. S.; Eaton, G. R.; Frank, H. A.; Brudvig, G. W. *J. Phys. Chem. B* **1998**, *102*, 8327.
19. Vrettos, J. S.; Stone, D. A.; Brudvig, G. W. *Biochemistry* **2001**, *40*, 7937.
20. Kimura, Y.; Ono, T. A. *Biochemistry* **2001**, *40*, 14061.
21. Falke, J. J.; Drake, S. K.; Hazard, A. L.; Peersen, O. B. *Quart. Rev. Biophys.* **1994**, *27*, 219.
22. Ghanotakis, D. F.; Babcock, G. T.; Yocum, C. F. *FEBS Lett.* **1984**, *167*, 127.
23. Hoganson, C. W.; Babcock, G. T. *Science* **1997**, *277*, 1953.
24. Chu, H. A.; Hillier, W.; Law, N. A.; Babcock, G. T. *Biochim. Biophys. Acta* **2001**, *1503*, 69.
25. Limburg, J.; Szalai, V. A.; Brudvig, G. W. *J. Chem. Soc., Dalton Trans.* **1999**, 1353.
26. Pecoraro, V. L.; Baldwin, M. J.; Caudle, M. T.; Hsieh, W.-Y.; Law, N. A. *Pure & Appl. Chem.* **1998**, *70*, 925.
27. Siegbahn, P. E. M.; Crabtree, R. H. *J. Am. Chem. Soc.* **1999**, *121*, 117.
28. Vrettos, J. S.; Limburg, J.; Brudvig, G. W. *Biochim. Biophys. Acta* **2001**, *1503*, 229.
29. Berthold, D. A.; Babcock, G. T.; Yocum, C. F. *FEBS Lett.* **1981**, *134*, 231.
30. Beck, W. F.; De Paula, J. C.; Brudvig, G. W. *Biochemistry* **1985**, *24*, 3035.
31. Segel, I. H. *Enzyme Kinetics*; John Wiley & Sons: New York, NY, 1975.
32. Bernat, G.; Morvaridi, F.; Feyziyev, Y.; Styring, S. *Biochemistry* **2002**, *41*, 5830.
33. Damoder, R.; Dismukes, G. C. *FEBS Lett.* **1984**, *174*, 157.
34. Okamura, M. Y.; Paddock, M. L.; Graige, M. S.; Feher, G. *Biochim. Biophys. Acta* **2000**, *1458*, 148.
35. Weast, R. C. In *CRC Handbook of Chemistry and Physics*; Weast, R. C., Ed.; CRC Press, Inc.: West Palm Beach, FL, 1978.
36. Bertini, I.; Luchinat, C. In *Bioinorganic Chemistry*; I. Bertini; H. B. Gray; S. J. Lippard; J. S. Valentine, Ed.; University Science Books: Sausalito, CA, 1994; p 37.
37. Nixon, P. J.; Trost, J. T.; Diner, B. A. *Biochemistry* **1992**, *31*, 10859.
38. Klimov, V. V.; Baranov, S. V. *Biochim. Biophys. Acta* **2001**, *1503*, 187.
39. Bakou, A.; Buser, C.; Dandulakis, G.; Brudvig, G.; Ghanotakis, D. F. *Biochim. Biophys. Acta* **1992**, *1099*, 131.