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Identification of a Na\textsuperscript{+}-Binding Site Near the Oxygen-Evolving Complex of Spinach Photosystem II

Jimin Wang\textsuperscript{1,*}, Joshua M. Perez-Cruet\textsuperscript{2,*}, Hao-Li Huang\textsuperscript{2,*}, Krystle Reiss\textsuperscript{2}, Christopher J. Gisriel\textsuperscript{2}, Gourab Banerjee\textsuperscript{2}, Divya Kaur\textsuperscript{3,4}, Ipsita Ghosh\textsuperscript{2}, Alisha Dziarski\textsuperscript{2}, M. R. Gunner\textsuperscript{3,4}, Victor S. Batista\textsuperscript{2}, Gary W. Brudvig\textsuperscript{1,2,*}

\textsuperscript{1}Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114, USA. \textsuperscript{2}Department of Chemistry, Yale University, New Haven, CT 06520-8107, USA. \textsuperscript{3}Department of Physics, City College of New York (CCNY), New York, NY 10031, USA. \textsuperscript{4}Department of Chemistry, The Graduate Center of the City University of New York, New York, NY 10016, USA.

\textsuperscript{*}Equal contributions

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ABSTRACT: The oxygen-evolving complex (OEC) of photosystem II (PSII) is an oxomanganese cluster composed of four redox-active Mn ions, and one redox-inactive Ca\textsuperscript{2+} ion, with two nearby bound Cl\textsuperscript{−} ions. Sodium is a common counter-ion of both chloride and hydroxide anions, and a sodium-specific binding site has not been identified near the OEC. Here, we find that the oxygen-evolution activity of spinach PSII increases with [Na\textsuperscript{+}], particularly at high pH. A Na\textsuperscript{+}-specific binding site next to the OEC, upon protonation of the D1-H337 amino-acid residue, is suggested by the analysis of two recently published PSII cryo-EM maps in combination with quantum mechanical calculations and molecular dynamics simulations.

INTRODUCTION

The oxygen-evolving complex (OEC) of photosystem II (PSII) catalyzes the oxidation of water, generating molecular dioxygen (O\textsubscript{2}), protons (H\textsuperscript{+}), and electrons (e\textsuperscript{−}), during the light period of photosynthesis:\textsuperscript{1-4}

\[ 2 \text{H}_2\text{O} \rightarrow \text{O}_2 + 4e^- + 4H^+ \]

Many simple inorganic salts such as MnCl\textsubscript{2}, CaCl\textsubscript{2}, and NaCl modulate the oxygen-evolution ability of PSII, influence the OEC structure, and regulate its activity. Earlier studies of the oxygen-evolution activity of spinach PSII implicitly suggested a possible functional role for sodium in OEC function.\textsuperscript{4} For example, oxygen evolution of spinach PSII membranes in MES [i.e., 2-(N-morpholino)ethanesulfonic acid] buffer with Ca(OH\textsubscript{2}) produced 100 units [which is µmol O\textsubscript{2} (mg Chl)\textsuperscript{−1} hr\textsuperscript{−1}] in the presence of 2 mM Na\textsubscript{+}, while O\textsubscript{2} evolution was completely suppressed when Na\textsubscript{+} was removed.\textsuperscript{1} However, in NaOH-MES buffer, oxygen evolution increased to 450 units with 2 mM Na\textsubscript{+} while remained at 250 units without Na\textsubscript{+}.\textsuperscript{3} The upshift in the kinetic profile may have been caused by the estimated 8 mM Na\textsuperscript{+} present in the NaOH-containing buffer, suggesting that oxygen evolution is activated at low Na\textsuperscript{+} concentration. Oxygen evolution is inhibited at high Na\textsuperscript{+} concentration because Na\textsuperscript{+} becomes competitive with Ca\textsuperscript{2+}; thus Na\textsuperscript{+} likely binds in place of Ca\textsuperscript{2+} in the OEC under these conditions.\textsuperscript{3} In contrast, Na\textsuperscript{+} also becomes an uncompetitive inhibitor at high [Ca\textsuperscript{2+}], which suggests the presence of another yet-to-be-identified Na\textsuperscript{+}-specific binding site that may be distal to the OEC.\textsuperscript{5} Uncompetitive inhibition is not observed with other monovalent ions such as K\textsuperscript{+}, Cs\textsuperscript{+}, and [N(CH\textsubscript{3})\textsubscript{3}]\textsuperscript{+}.

The effects of Na\textsuperscript{+} on the PSII activity are complicated and dependent on many conditions, including Ca\textsuperscript{2+} concentration and pH. Na\textsuperscript{+} is almost always present as a counterion, and no pH-dependent kinetic studies have yet been carried out to explicitly determine the potential functional role of Na\textsuperscript{+} in oxygen evolution. The crystal structure of Thermosynechococcus vulcanus PSII determined at 1.9-Å resolution at pH 6.5 has established the Mn\textsubscript{5}CaO\textsubscript{5} OEC structure, with Mn\textsuperscript{3+/4+} and Ca\textsuperscript{2+} as integral components, and two nearby bound Cl\textsuperscript{−} ions, although Na\textsuperscript{+}-binding near the OEC has not been identified.\textsuperscript{6} The presence of bound Na\textsuperscript{+}, however, cannot be ruled out because the electron density peaks for Na\textsuperscript{+} and H\textsubscript{2}O are very similar since both species have the same number of electrons. In contrast, the electrostatic potential (ESP) maps of cryo-electron microscopy (cryo-EM)\textsuperscript{7,8} can differentiate Na\textsuperscript{+} from H\textsubscript{2}O since the scattering factors of cations are much higher than those of neutral species.\textsuperscript{10} Thus, we have carefully examined the recently published cryo-EM data to explore whether we can identify a Na\textsuperscript{+}-binding site. Indeed, the recently published ESP maps of PSII from Spinacea oleracea (spinach) and Pisum sativum (pea) at pH 7.5,\textsuperscript{7,8} allow us to identify a Na\textsuperscript{+}-specific binding site adjacent to the OEC near D1-H337. Computational simulations show that sodium binding occurs at high pH when the nearby D1-H337 is deprotonated, with Na\textsuperscript{+} supplementing the missing positive charge of D1-H337. Consistent with the nature of the proposed binding site, our measurements of oxygen-evolution activity of purified spinach PSII membranes at pH 6.0 and 8.0 show that the addition of Na\textsuperscript{+} increases oxygen evolution quite significantly at pH 8.0, but not at pH 6.0.

METHODS AND MATERIALS

Preparation of spinach PSII membranes and Na\textsuperscript{+} depletion

PSII-enriched thylakoid membrane fragments were prepared from spinach as previously described and suspended in buffer containing 15 mM NaCl, 20 mM MES, and 30% (v/v) ethylene glycol.\textsuperscript{11,13} The pH was adjusted to 6.0 with NaOH. Suspended and purified PSII membranes were stored in liquid nitrogen. Sodium was removed from PSII membranes by washing with a Na\textsuperscript{+}-depleted buffer. The PSII sample was homogenized, centrifuged and resuspended three times with the Na\textsuperscript{+}-depleted buffer to dilute [Na\textsuperscript{+}] by a factor of more than 50-fold. The Na\textsuperscript{+}-depleted buffer contained 20 mM CaCl\textsubscript{2}, 20 mM MES, and 20
Oxygen assay of Na+-depleted PSII membranes

Oxygen-evolution activity of the Na+-depleted PSII membranes was measured using a Clark-type O₂ electrode as previously described.₁¹⁻¹⁴ The assay buffers were prepared at 0 mM, 10 mM, 20 mM, and 40 mM NaCl and contained 20 mM CaCl₂ (i.e., [Cl⁻] ≥ 40 mM), 20 mM MES, and 20 mM HEPES. The pH was adjusted to 6.0 and 8.0 using N(CH₃)₂OH. Both 1 mM K₃[Fe(CN)₆] and 0.25 mM PBQ (phenyl-p-benzoquinone) were used as electron acceptors. The chlorophyll concentration for the assay was 3.8 µg/mL. The oxygen-evolution activity assay was also performed at pH 8 in the presence of either 40 mM N(CH₃)₂Cl/40 mM NaCl or 0 mM N(CH₃)₂Cl/40 mM NaCl with the fixed total concentration of [Cl⁻] of 80 mM to rule out Cl⁻ activation.

Density-functional theory (DFT) geometry optimization for ESP calculations

DFT geometry optimization for ESP calculations

ESP maps were calculated for a 250-atom model taken from the 3ARC/V2C PDB structure of PSII,⁶,¹³ including D1-[M329-N338], D2-[R348-L352], and two water molecules (D1-370 and D2-381), both of which were free to move during DFT geometry optimization. A Na⁺ complex ion was inserted into the binding pocket with as many H₂O ligands as possible, which were also free to move during optimization. In these Na+-containing models, D1-H337 was deprotonated. In Na+-free models, D1-H337 was treated separately as both deprotonated and protonated at its N61 position. DFT geometry optimization was performed using a B3LYP functional of the Gaussian 2016 software package with the 6-31G basis set for C, N, and H and the 6-31G(d) basis set for O, S, and Na⁺.¹⁵,¹⁶ ESP maps were generated using the Merz-Singh-Kollman scheme.¹⁷,¹⁸

Corresponding atomic charges were derived from ESP maps, and charge-only Coulomb’s ESP maps were recalculated using the DelPhi program.¹⁹,²⁰ Inside the protein boundary, the macroscopic dielectric constant was set to 4 or 1, whereas that of solvent continuum was always set to 80. These ESP maps were Fourier inverted using the Phenix software suite to obtain the corresponding structure factors.²¹ The maps were recalculated using structure factors with appropriate Wilson B-factors on spacing grids corresponding to a fixed 0.18 Å resolution with varying reciprocal resolution using CCP4.²² This calculation was done in two sets of grid spacing after adding Wilson B-factors of 41.3 Å² and 64.6 Å² to the structure factors. The first set of spacing is fixed at the effective resolution of 0.18 Å, and the second set varies with the effective resolutions of 2.5 Å and 3.2 Å, respectively. The effective resolution of the van der Waal (vdW) component of cryo-EM maps was estimated using an empirical relationship obtained for X-ray crystallographic data sets.²³

$$ln[B] = 2.0548 - 1.8169ln[S_{max}]$$

where B is the Wilson B-factor of the diffraction data, and S_{max} is the maximum reciprocal resolution. The accuracy of both electron density and ESP peaks is dependent on the real-space grid resolution that is used to sample corresponding functions for exactly the same structure factors.

**RESULTS AND DISCUSSION**

**pH-Dependent Na⁺ activation of the oxygen-evolution activity of spinach PSII membranes**

Oxygen evolution was fast at pH 6.0, but was considerably slower at pH 8.0 under the same Na⁺ concentration (Fig. 1A), which is consistent with a previous study. In the presence of 40 mM Na⁺ at pH 8, oxygen evolution is 2- to 3-fold faster compared to Na+-depleted PSII without any Na⁺ ion (Fig. 1). At the optimal pH of 6.0 for spinach PSII activity, there is only a slight increase in the activity with increasing Na⁺ (Fig. 1). At pH 8.0, Cl⁻ activation is ruled out because the rate of oxygen evolution remains low when NaCl is substituted with an equal concentration of N(CH₃)₂Cl (Fig. 1B). Earlier measurements always included 15 mM NaCl to maintain a sufficiently high chloride concentration for optimal oxygen evolution so that the Na⁺ dependence was hidden. At low pH, [N(CH₃)₂]⁺ was shown to have no effect on the oxygen-evolution activity of NaCl-washed thylakoid membranes where PSII lacked extrinsic subunits. In this study, extrinsic proteins are preserved; therefore, it is unlikely that [N(CH₃)₂]⁺ could bind near the active site to impact oxygen evolution. Thus, we ascribe the observed difference in activity at pH 8 to the Na⁺ concentration. Based on our current pH-dependent oxygen-evolution activity results, we anticipated that ligands of this Na⁺-binding site might include an ionizable group with a pKₐ within the experimental range of pH 6 and 8, such as a His residue.

Our study shows that Na⁺ is required for optimal oxygen-evolution activity of spinach PSII, and that this activation is most pronounced at higher pH. These effects are
difficult to observe when either the pH or $[\text{Na}^+]$ is not varied individually. This study does not directly distinguish between active and passive modes of activation. In a passive mode, $\text{Na}^+$ would simply play an essential role for maintaining the structural integrity of PSII at higher pH where it is especially fragile. In an active mode, $\text{Na}^+$ would accelerate one of the slow steps of oxygen evolution. At high pH, some extrinsic subunits may be partially dissociated. This dissociation should not affect the $\text{Na}^+$-dependent activation of oxygen evolution observed here. We have not examined additional roles of $\text{Na}^+$ at higher concentrations in this study, which could be more complicated because it can bind many more sites.

Figure 1. $\text{Na}^+$ dependence of PSII. The oxygen-evolution activity of $\text{Na}^+$-depleted PSII membranes is shown as a function of varying NaCl concentration and pH. (A). Red: at pH 8 with N(CH$_3$)$_4$Cl, but no NaCl, very low oxygen evolution is observed. With the addition of NaCl, oxygen evolution is increased to >100 µmol O$_2$/mg Chl/hr. Blue: at pH 6 the high OEC activity shows a smaller NaCl dependence. (B). Activities in the presence of either 40 mM N(CH$_3$)$_4$Cl or 40 mM NaCl showing that Cl$^-$ does not activate oxygen evolution at pH 8.0. Averaged activities [µmol O$_2$ (mg Chl)$^{-1}$ hr$^{-1}$] with standard deviations (in error bars) from three or more assays are shown.

Identification of a $\text{Na}^+$-specific binding site from recent cryo-EM maps

Two cryo-EM maps recently reported at ~ 3.2 Å resolution for plant PSII super-complexes (with light-harvesting complex II) at pH 7.5 one from spinach (EMD-6617/PDB-3JCU) (Fig. 2), and the other from pea (EMD-6742/PDB-5XNM). These maps were examined for a $\text{Na}^+$-specific binding site near the OEC in order to provide a structural basis for the pH-dependent $\text{Na}^+$-specific activation of spinach PSII observed here. We are aware that the actual pH of the cryo-EM samples may differ from the cited values due to the freezing process.

An examination of the two cryo-EM maps revealed an unexplained positive peak of approximately spherical symmetry inside a pocket (Fig. 2). Map-sharpening routinely carried out for cryo-EM maps can produce artifacts, especially in an extreme case when dynamic experimental electric potential (EP) maps are converted to ESP maps by setting the underlying B-factors to zero and by making the amplitudes of the corresponding structure factors independent of resolution. We have verified that the peak we observed in the cryo-EM map is not an artifact of map-sharpening. A similar positive peak is present after a Laplacian operation on the original, unsharpened cryo-EM maps, which produces locally distributed charge-density (CD) maps without introducing any artifacts (Fig. 3A, 3B).
In both of the cryo-EM maps, the ESP value of this peak is higher than that of its ligands, including D1-H337 and other surrounding protein residues, suggesting that a positively charged species occupies this site (Fig. 2, 3). We assign this positive peak to a Na\(^+\) ion, which is the only monovalent cation present in the buffer of the protein samples imaged.\(^7\) This putative Na\(^+\)-binding pocket is ~ 7.6 Å and 9.5 Å away from the two Cl\(^-\) ions near the OEC and ~ 7.6 Å away from Mn1 of the OEC.\(^6\) This pocket is located at the C-terminus of a D1 α-helix and corresponds to the pocket occupied by two ordered water molecules (water-370 and water-381) in the 1.9-Å resolution crystal structure of *T. vulcanus* PSII determined at pH 6.5 (Fig. 4).\(^6\)

There are two possible interpretations for an ESP peak with such a large peak maximum. One is a Na\(^+\) ion with +1 formal charge. The other is that two water molecules may be merged together to exhibit a single positive ESP peak. However, an analysis of a DFT-derived ESP map excludes the second interpretation while supporting the first interpretation (see below, Fig. 5-7). We have verified that the two water molecules in the *T. vulcanus* PSII crystal structure (pH 6.5) are not a mis-identified Na\(^+\) ion because when water-370 was replaced with Na\(^+\) in a DFT-calculated ESP map, its atomic B-factor value assigned in model refinement was larger than those of atoms it interacts with by ~ 7 Å\(^2\). With a water molecule at this position, there was no difference in atomic B-factors among all interacting atoms in this area, implying that water molecule is correctly assigned.

Two additional cryo-EM structures were reported at lower pH values (5.7 and 6.5) and higher resolution (~ 2.7 Å),\(^6,9\) which provide direct evidence for pH-dependent Na\(^+\) binding to D1-H337. These structures of PSII purified at these two lower pHs differ from the one at pH 7.5. At pH 7.5, Na\(^+\) is seen to bind to D1-H337 (EMD-6741/PDB-5XNM) in the aforementioned pea PSII super-complexes, which are arranged in an unstacked configuration. However, at pH 5.7 in a stacked configuration (EMD-7424/PDB-5XNL), D1-H337 is likely to be protonated, and two water molecules were observed to bind in this pocket in an arrangement that is almost identical to the high-resolution cyanobacterial PSII crystal structure at pH 6.5.\(^6,9\) At pH 6.5, an additional cryo-EM structure (EMD-9955/PDB-6KAC) at 2.7-Å resolution was computationally extracted from the supercomplex PSII structure from the green algae, Chlamydomonas reinhardtii.\(^9\) In that structure, there is a single resolved ESP peak in the pocket, which differs from the cryo-EM PSII crystal structure at the same pH.\(^9\) The maximum for this lone peak is much smaller than the one found at pH 7.5 relative to ESP peaks for D1-H337. It is likely that under these conditions, D1-H337 is partially deprotonated, and that the observed single ESP peak corresponds to a mixture of both Na\(^+\) and water-occupied binding pockets.

![Figure 3](image)

**Figure 3.** A single, well-isolated, approximately spherically symmetric peak observed in the cryo-EM map (EMD-6617) recently reported for spinach PSII (3JCU) at the putative Na\(^+\)-binding site in a close-up view. (A) Two contouring levels (left: +4.0σ; right: +5.0σ) of the EMD-6617 map. (B) Two contouring levels (left: +1.5σ; right: +2.0σ) of the unsharpened CD map.

**Evidence for pH-dependent Na\(^+\) binding from recent cryo-EM maps**

Utilizing charge properties in experimental cryo-EM maps

Cryo-EM maps display the spatial variation of ESP, which is the sum of the Coulombic and van der Waals (vdW) potentials at each point.\(^31-33\) The ESP peak for a cation is more easily visualized than that of a neutral atom because the net positive partial charge generates an extra positive Coulomb ESP that augments the underlying vdw EP peak.\(^34\) The ESP peak of an anion is less easily visualized than that of a neutral atom because the net negative partial charge generates an extra negative Coulomb ESP function that diminishes the underlying vdw EP peaks.\(^33\) These charge effects make the ESP peak of Na\(^+\) more conspicuous than those of Cl\(^-\) and H\(_2\)O (i.e., Na\(^+\) > H\(_2\)O > Cl\(^-\)). This simple principle is useful for an initial
identification of ions in cryo-EM maps. This expectation is met in that both cryo-EM maps published for spinach PSII exhibit no isolated positive peak corresponding to the two known Cl− ions. The authors of those studies probably retained the Cl− ions in their starting model from an extrapolation of the previously-determined X-ray structures of cyanobacterial PSII.5 The X-ray crystallographic electron density peak of Cl− is more easily visualized than that of Na+ because Cl− has nearly twice the number of electrons of Na+, whereas the electron density peaks of Na+ and H2O are difficult to distinguish because these species have the same number of electrons, i.e., Cl− ≫ H2O ~ Na+. This highlights the importance of considering charges for proper interpretation of cryo-EM maps in this range of resolution.

Cryo-EM ESP maps can be viewed as the convolution products of static ESP maps with dynamic motions (i.e., B-factors), which include a time average of large numbers of macromolecules frozen at different time points of trajectory, as well as measurement errors. DFT calculations were used to generate theoretical static ESP maps from the accurate atomic coordinates obtained using X-ray crystallography, which can be compared with experimental data only after properly adding the atomic motion described by the Wilson B-factor. However, when ESP maps are calculated using atomic scattering factors, the Coulomb and the vdW ESP components are separated. The charges used for calculation of the Coulomb maps are derived from the DFT-derived total ESP map. The charge-only Coulomb ESP map can then be recalculated using DelPhi, which provides a finite difference solution to the Poisson-Boltzmann equation, with appropriate Wilson B-factors added (Fig. 5G-5I).19,20

![Figure 5](image)

**Figure 5.** The DFT-derived theoretical EP and DelPhi-calculated EP functions of the first atomic model. (A-F) DFT-derived EP maps. (G-I) DelPhi-calculated EP maps. Right two panels are in stereodiagram (C, F, and I). First row (A-C) with increasing atomic motions (ΔB = 0, first panel at 0.18-Å resolution; second panel with ΔB = 41.3 Å2 at ~ 2.50-Å resolution; third panel with ΔB = 64.6 Å2 at ~ 3.20-Å resolution) but with the fixed real-space super-sampling at 0.18-Å resolution. Map is contoured at +2.5σ. The second row is the same as the first row in reciprocal spacing but with reduced real-space grid sampling (which is approximately corresponding to their reciprocal space resolution). The third row is the Delphi-calculated EP function contoured at +2.5σ (green) and -2.5σ (red) with the same reciprocal resolutions as the first row, but with fixed grid spacing corresponding at 0.50 Å resolution.

The charge-only ESP map shows the following: (1) Na+ has a large positive ESP at its nuclear position, (2) the O atom of H2O has a negative ESP, and (3) H atoms of H2O have only very small effects on the ESP at the O position. The sum of these contributions makes the total ESP value, which corresponds to the experimental ESP value at the nuclear position of Na+, much larger than that of neutral H2O species, assuming the same small atomic motions of the two species (Fig. 5C, 5D). The differences between one Na+ and two water molecules become smaller with increasing atomic motions. However, the summed ESP values of H2O molecules disappear below the noise level before that of Na+ at any given contouring level. Given the typical H2O-H2O distance of ~ 2.8 Å, ESP peaks of two H2O molecules will never merge into a single elongated peak with increasing atomic motions before their peaks disappear below the noise level. Thus, the single ESP peak inside the putative Na+-binding pocket observed in the two cryo-EM maps reported for spinach PSII cannot be assigned as two merged H2O molecules, but only as a Na+ cation or a partially hydrated Na+ complex ion since Na+ is only the monovalent cation present in the buffer of these protein samples.7,8

The contribution of a Coulomb component to the cryo-EM map, which we did not include in an estimation of the local resolution, can greatly alter the appearance of ESP peaks for both cations and anions.23,34 This makes it unreliable to use the appearance (i.e., the apparent spatial resolution) of electron density maps for a comparison with the appearance of cryo-EM maps to calibrate their effective resolution (i.e., the underlying B-factors), as suggested recently.15 At ~ 3.2 Å, one can definitely identify the binding of Cl− in electron density maps but not in cryo-EM maps. One can definitely identify Na+ in cryo-EM maps, but it is nearly impossible in electron density maps at medium resolution. These contrasting features of electron density maps and cryo-EM maps can be reproduced using both DFT calculation as done in this study and electron scattering factors published for a small set of ions with integral charge numbers.36

**Ligand assignment in the hydrated Na+ complex cation using DFT**

While we can qualitatively identify the isolated positive peak observed in the two cryo-EM maps at pH 7.5 as a bound Na+ cation on the basis of its net +1 charge, the map at ~ 3.2 Å resolution does not provide sufficient information to fully determine its specific H2O ligands and coordination geometry. In such a case, the ligands and coordination geometry may be proposed upon combining the experimental information from the cryo-EM maps with physicochemical principles.

We calculated DFT-ESP maps for three atomic models directly derived from the X-ray crystallography structure of cyanobacterial PSII and chose the one that is the most consistent with the cryo-EM maps. In the first model, we inserted a hydrated Na+ complex into several initial binding sites with as many H2O ligands as possible, and let DFT optimize the coordination geometry, assuming that D1-H337 is deprotonated (Fig. 5). The second and third atomic models feature protonated and deprotonated D1-H337, respectively, and include two water molecules (W370 and W381 as in 3WU2/5V2C) but without Na+(Figs. 6, 7).6,13 The DFT models were built by using the OEC optimized in the S1 state in...
cyanobacterial PSII. All H atoms of the water ligands are explicitly modeled with no rotational disorder of torsional angles of any kind. Spinach PSII, analyzed in this work, and cyanobacterial PSII are highly conserved in sequence. Although they have different pH kinetic profiles, these differences can be assigned to a replacement of D1-A87 in spinach PSII with D1-N87 in cyanobacterial PSII near the OEC. This residue is ~15 Å away from the putative Na\(^{+}\)-binding site, and there is no difference in the protein sequence near the Na\(^{+}\)-binding site, so that we assume that this should not affect our DFT calculations of Na\(^{+}\) binding.

After DFT optimization, the first Na\(^{+}\)-bound atomic model matched the cryo-EM maps better than either of the two Na\(^{+}\)-free atomic models (Fig. 5-7). The Na\(^{+}\)-binding pocket, buried inside the protein, appears only large enough to accommodate a maximum of three water ligands to the bound Na\(^{+}\). Two protein ligands of the Na\(^{+}\) ion are the deprotonated D1-H337 side chain and the D2-N350 backbone carbonyl (Fig. 8A, 8B). Effectively, a partially hydrated [Na(H\(_2\)O)\(_3\)]\(^{+}\) complex ion replaces the two water molecules (W370 and W381) found inside the binding pocket of the crystal structure of \(T.\: vulcanus\) PSII.\(^{6,13}\) The mean Na\(^{+}\) coordination bond length in our DFT model is 2.40 ± 0.13 Å, varying from 2.24 Å to 2.61 Å. The small size of this binding pocket may prevent larger K\(^{+}\) and Cs\(^{+}\) monovalent cations from binding. Neither K\(^{+}\) nor Cs\(^{+}\) ion exhibits non-competitive inhibition as Na\(^{+}\) does.\(^{5}\) Nor have they been tested to see if they enhance oxygen-evolution activity at high pH. The pentavalent coordination geometry also rules out binding of NH\(_4^{+}\). These stipulations support the presence of a highly specific Na\(^{+}\)-binding pocket.

![Figure 6](image1.png)

**Figure 6.** The DFT-QM/MM model of the second atomic model. (A) The same as (A-C) of Figure 5 but contoured at +3σ. (B) DFT-derived EP function for two H\(_2\)O molecules bound as in the \(T.\: vulcanus\) PSII crystal structure with protonated D1-H337, contoured at +2σ (middle row) and +3σ (bottom row) with atomic motions included as indicated.

In our DFT-optimized structure, one water ligand is almost precisely superimposed onto the water designated as W370 in the cyanobacterial structure 5V2C.\(^{6,13}\) It retains the same pattern of H bonding interactions with the D1 protein as found in the crystal structure. The two other new water molecules functioning as Na\(^{+}\) ligands (Z1 and Z2, i.e., hydrated Na\(^{+}\) ion) also have three and two H bonds with the protein, respectively (Fig. 8A, 8B, green dashes). Both Z1 and Z2 form H bonds with D1-protein residues, and Z1 also interacts with D2-N350. With the three H bonds, the Z1 O atom has perfect tetrahedral interaction geometry (Fig. 8B).

Deprotonation of D1-H337 and pH-dependent binding of Na\(^{+}\)Cryo-EM maps correspond to the ensemble of configurations of microstates in the frozen samples, and their corresponding thermal fluctuations, are sampled during the cryo-EM imaging. We have analyzed an ensemble of protein microstates with and without Na\(^{+}\) using GCMC as implemented in MCCE.\(^{24,25}\) MCCE will bring the protonation states of all amino acids into equilibrium with the cofactor redox states at the defined pH and relative Na\(^{+}\) chemical potential with a background 150 mM ionic strength. The full PSII complex can be analyzed in MCCE.\(^{23}\) Here, 137 residues near the OEC in the S\(_1\) state were simulated.\(^{27}\)
Our MCCE simulations of the protonation states of amino-acid residues in the cyanobacterial PSII structure find that there is a single high-affinity Na⁺-binding site near D1-H337. In the absence of Na⁺, D1-H337 is protonated between pH 6.5 and 9.0, while it always remains neutral in the presence of Na⁺. Thus, the protonation state of D1-H337 is dependent on the pH and on [Na⁺]. At a given pH, increasing [Na⁺] leads to both Na⁺ binding and deprotonation of D1-H337. Previous experimental and computational data have shown that D1-337 is protonated at low pH for the OEC to function properly. At higher pH it is easier to deprotonate the His, so the Na⁺ affinity increases. Because of the competition between H⁺ and Na⁺, the calculated Na⁺ affinity increases 10-fold/pH unit as the pH is raised from 6 to 9.

The high pKₐ for D1-H337 and the ability of Na⁺ to replace the His cation at higher pH reflect the affinity for a cation in this region of PSII. The backbone dipoles as well as the nearby bound Cl⁻ and D1-Asp61 contribute a negative Coulomb potential that favors the positive charge. The summed contribution of the OEC and its primary protein ligands affect the cation affinity by less than 1 kcal/mol. In displacing water ligands of the hydrated [Na(H₂O)]⁺ complex ion, we see that the protein is able to provide higher affinity ligands, making it a specific Na⁺-binding site. Although either a deprotonated His side chain or a carboxylate could serve as a ligand for Na⁺, our calculation shows that neither alone provides sufficient interaction energy for binding of Na⁺. In PSII, the Na⁺-specific binding pocket is located at the C-terminus of a long α-helix, and this helix has a cumulative negative ESP that originates from the aligned dipoles of the backbone carboxyls. At low pH, this negative ESP stabilizes protonated D1-H337, whereas at high pH, it stabilizes the bound Na⁺ that binds when D1-H337 is deprotonated.

Figure 8. DFT-optimized Na⁺-bound structure of spinach PSII and molecular mechanical basis for EP disparity between Na⁺ and HOH in the Na⁺-specific binding pocket. (A) Na⁺ coordination geometry in stereodiagram. Its ligands include three water molecules (W370 plus two new water molecules named Z1 and Z2), D1-H337 side chain, and D2-N350 backbone carbonyl group. D1-H337 is H bonded to O3 of the OEC. (B) Zoomed-out stereodiagram view shows how three water molecule ligands are held in place in the Na⁺ binding pocket. (C) DFT-derived EP functions plotted along the Na⁺/Water-370 axis on the absolute voltage scale as a function of B-factor (i.e., effective resolution, see text). (D) DelPhi-calculated charge-only Coulomb potentials along the Na⁺/Water-370 axis. (E) DFT-derived EP functions plotted along the Water-381/Water-370 axis on the absolute voltage scale as a function of B-factor. (F) A close-up view of (E).

Environmental factors for modeling of the Na⁺-binding site

Two notable long-range environmental factors observed in PSII, which have been omitted in this study, could affect the absolute EP value of Na⁺ and its H₂O ligands as found in additional DFT calculations. These factors include long-range ESP contributions from the aligned dipole moments of backbone carboxyls of the long D1 α-helix (Fig. 4A). The Na⁺-binding pocket is located at the C-terminus of the D1 helix and is subject to the cumulative effects of aligned dipole moments. In addition, partial occupancy of the OEC in the PSII samples imaged, which may be due to partial loss during electron scattering, will affect the ESP nearby. However, it is likely that these long-range ESP effects are similar regardless of whatever species occupies the Na⁺-binding pocket. Therefore, these factors do not alter the overall conclusion about the relative ESP magnitude of Na⁺ and H₂O.

CONCLUSION

In this study, we have identified a possible Na⁺-specific binding site immediately adjacent to the OEC at high pH, next to deprotonated D1-H337. Na⁺ binding is consistent with recently reported cryo-EM maps, our DFT-QM/MM and MCCE calculations, and our kinetic measurements of oxygen-evolution activity of spinach PSII as a function of both [Na⁺] and pH. We suggest that Na⁺ binding upon deprotonation of D1-H337 allows for the conservation of charge near the OEC and helps to fine-tune a wide pH range for oxygen-evolution activity.

AUTHOR INFORMATION

Corresponding Author

To whom correspondence should be addressed: J.W. (jimin.wang@yale.edu), or G.W.B (gary.brudvig@yale.edu).

Author Contributions

J.W., G.W.B., V.S.B., and M.R.G conceived the concept and designed experiments for this study. J.M.P.-C., H.-L.H., C.J.G., G.B., and I.G. executed biochemical studies, K.R. did DFT calculations, D.K. carried out MCCE calculations, and J.W. analyzed cryo-EM maps. All coauthors contributed to writing the manuscript.

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ABBREVIATIONS

ACS Paragon Plus Environment
PSII, photosystem II; OEC, oxygen-evolving complex; cryo-EM, cryo-electron microscopy; ESP, electrostatic potential; EP, electric potential; vdW, van der Waals; MES, 2-(N-morpholino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-(piperazine)ethanesulfonic acid; PPBQ, phenyl-p-benzoquinone.

Uniprot accession numbers for Spinacia oleracea PSII complex:

- PSII protein D1 (PSII-D1, PSII-A), psbA: P69560.
- PSII CP-47 (PSII-CP47, PSII-B), psbB: P04600.
- PSII protein D2 (PSII-D2, PSII-D), P60065.
- PSII cytochrome b553 α (reaction center V, PSII-E): P69383.
- PSII cytochrome b553 β (reaction center VI, PSII-F): P60128.
- PSII reaction center I (PSII-I): P62103.
- PSII reaction center J (PSII-J): Q9M3L2.
- PSII reaction center K (PSII-K): P21163.
- PSII reaction center M (PSII-M): P62112.
- PSII reaction center L (PSII-L): P60150.
- PSII Mn-stabilizing protein O (PSII-O): P12359.
- PSII reaction center L (PSII-L): P60150.
- PSII reaction center YCF12 (PSII-Y): P80470.
- PSII reaction center W (PSII-W): Q41387.

REFERENCES


SYNOPSIS TOC: Binding of Na\(^+\) often escapes attention because it is always present in biological systems and is difficult to distinguish from water using X-ray crystallography. Its charge property is enhanced and can be seen in cryo-electron microscopic maps.

Topics of Content (TOC). DFT-calculated ESP map filtered at 3.2 Å resolution shows that a large symmetric ESP peak in a binding pocket next to the OEC is consistent with a monovalent Na\(^+\) ion.