Ammonia Binding in the Second Coordination Sphere of the Oxygen-Evolving Complex of Photosystem II

David J. Vinyard
_Yale University_

Mikhail Askerka
_Yale University_

Richard J. Debus
_University of California, Riverside_

Victor S. Batista
_Yale University_

Gary W. Brudvig
_Yale University_

Follow this and additional works at: https://digitalcommons.lsu.edu/biosci_pubs

**Recommended Citation**

This Article is brought to you for free and open access by the Department of Biological Sciences at LSU Digital Commons. It has been accepted for inclusion in Faculty Publications by an authorized administrator of LSU Digital Commons. For more information, please contact ir@lsu.edu.
Ammonia Binding in the Second Coordination Sphere of the Oxygen-Evolving Complex of Photosystem II

David J. Vinyard,† Mikhail Askerka,† Richard J. Debus,‡ Victor S. Batista,† and Gary W. Brudvig*†

†Department of Chemistry, Yale University, New Haven, Connecticut 06520-8107, United States
‡Department of Biochemistry, University of California, Riverside, California 92521, United States

Supporting Information

ABSTRACT: Ammonia binds to two sites in the oxygen-evolving complex (OEC) of Photosystem II (PSII). The first is as a terminal ligand to Mn in the S2 state, and the second is at a site outside the OEC that is competitive with chloride. Binding of ammonia in this latter secondary site results in the S2 state $S = 5/2$ spin isomer being favored over the $S = 1/2$ spin isomer. Using electron paramagnetic resonance spectroscopy, we find that ammonia binds to the secondary site in wild-type Synechocystis sp. PCC 6803 PSII, but not in D2-K317A mutated PSII that does not bind chloride. By combining these results with quantum mechanics/molecular mechanics calculations, we propose that ammonia binds in the secondary site in competition with D1-D61 as a hydrogen bond acceptor to the OEC terminal water ligand, W1. Implications for the mechanism of ammonia binding via its primary site directly to Mn4 in the OEC are discussed.

Oxygenic photosynthesis in cyanobacteria, algae, and plants converts solar energy to chemical energy by oxidizing water to molecular oxygen (O₂) in the Photosystem II (PSII) reaction center. The protons stripped from water contribute to the proton motive force across the thylakoid membrane, and the electrons are used to reduce plastoquinone to plastoquinol.1 The active site of water oxidation is the oxygen-evolving complex [OEC (Figure 1)], which is a Mn₄CaO₅ inorganic cluster ligated by amino acid residues and water molecules.2 The OEC cycles through four metastable redox intermediates ($S_n$ states, where $n = 0−3$) during its catalytic cycle as described by Kok and co-workers.3 The $S_3$ state is oxidized to a proposed transient $S_4$ intermediate that spontaneously releases O₂, binds H₂O, and reforms $S_0$. X-ray crystallography, X-ray absorption and emission spectroscopies, EPR spectroscopy, and computational modeling have revealed key features of the OEC structure in states $S_0−S_3$ (reviewed in ref 4). However, the nature of $S_4$ and, therefore, the chemical mechanism of O−O bond formation have not been experimentally characterized.

The precise binding sites of the two substrate waters that give rise to O₂ are not known.4 An oxo–oxyl radical coupling mechanism for O−O bond formation has been proposed with the substrate waters bound as a terminal oxyl ligand to a Mn⁴⁺ and a $\mu$-oxo bridge in the $S_4$ state.5,6 Alternatively, a water nucleophile attack mechanism has been proposed for which the substrate waters are bound as a terminal oxo ligand to a Mn⁵⁺ and a terminal water ligand to Ca²⁺.⁷−⁹ Therefore, identifying the sites of substrate water binding is a strategy for understanding the chemical mechanism of photosynthetic water oxidation.

Ammonia is an electronic and structural analogue of water and binds in two distinct sites in PSII.¹¹ Upon formation of the $S_1$ state, ammonia was recently proposed to bind as an additional¹⁰ terminal ligand to Mn⁴⁺ (in the $S = 5/2$ spin isomer with Mn oxidation states IV, IV, IV, and III for Mn1−Mn4, respectively), thus completing the octahedral coordination sphere and

Figure 1. QM/MM-optimized structure of the $S_2$ state $S = 5/2$ spin isomer of the PSII OEC.¹⁰ Ammonia binds outside the OEC at a site competitive with chloride in an area hypothesized to be within the dashed square.
stabilizing the complex in the \( S = 1/2 \) spin isomer (with Mn oxidation states III, IV, IV, and IV for Mn1–Mn4, respectively). We previously showed that ammonia binding in the \( S_2 \) state is analogous to water binding during the \( S_2 \) to \( S_1 \) transition. Quantitative mechanical studies of ammonia binding to the \( S_2 \) state suggest that previously bound terminal waters (W1 and W2) move in a "carousel" around Mn4. This mechanism, a similar version of which was later proposed by Pandit and coworkers, helps to explain how water is directed from hydrogen bonding networks within PSII to the OEC and further activated for \( O_2 \) production.

Ammonia also binds outside the OEC at a previously unresolved site that is competitive with chloride. A high-affinity chloride is bound approximately 7 Å from the dmapn Mn in the OEC as shown in Figure 1. When dark-adapted PSII membranes from spinach are illuminated at 200 K to advance the OEC from the S1 state to the S2 state in the presence of ammonia, the free base and a halide would occupy the same site in the protein matrix. While this secondary ammonia-binding site and chloride are clearly competitive, it is not obvious that an amine free base and a halide would occupy the same site in the protein matrix.

Previously, we showed that chloride binding requires the presence of D2-K317 in Synecocystis sp. PCC 6803. In wild-type (WT) PSII, chloride prevents D2-K317 from forming a salt bridge with D1-D61 and likely regulates the hydrogen bonding network within the broad channel to promote efficient proton transfer away from the OEC. Notably, neither WT cyanobacterial PSII nor PSII from the D2-K317A mutant exhibits a \( g = 4.1 \) EPR signal in the \( S_2 \) state.

Herein, we show that ammonia binding in the secondary binding site is inhibited in D2-K317A PSII. The combination of this observation and QM/MM modeling suggests that ammonia replaces D1-D61 as a hydrogen bond acceptor to W1 in the second coordination sphere of the OEC. This proposed binding site is distinct from, but intimately connected to, the chloride-binding site.

**MATERIALS AND METHODS**

PSII core complexes were purified from His-tagged CP43 and CP47 wild-type and the D2-K317A mutant of Synecocystis sp. PCC 6803 as previously described. Briefly, cells were grown mixotrophically with 5 mM glucose and warm white lighting (80 \( \mu \)E m\(^{-2}\) s\(^{-1}\)) and bubbled with 5% CO2 in air. Cells were filtered on a gravity driven clarifier, and thylakoid membranes were isolated by lysis using silica/zirconia beads in a chilled centrifuge. Beadbeater, and thylakoid membranes were isolated by lysis using silica/zirconia beads in a chilled centrifuge. Membranes from spinach are illuminated at 200 K to advance the OEC.

MgCl\(_2\), 10% (v/v) glycerol, 1.2 M betaine, and 0.03% DM. Additional experiments were performed in which 1 M sucrose was replaced with 1.2 M betaine or 25% (v/v) glycerol. For buffer exchange, PSII core complexes were diluted by approximately 10-fold and concentrated using centrifugal filters. This process was repeated 10–14 times. EPR samples were prepared by adding 100 mM NH\(_4\)Cl (from a 2 M stock) to PSII core complexes at approximately 1.5 mg of Chl mL\(^{-1}\) (∼48 \( \mu \)M PSII and 1.8 mM NH\(_3\)). The S2 state was generated by illuminating dark-adapted PSII samples at 200 K. Annealed samples were warmed to 273 K in an ice water bath for approximately 30 s and then frozen in liquid nitrogen.

EPR spectra were recorded using a Bruker ELEXYS E500 spectrometer equipped with a SHQ cavity and an Oxford ESR-900 helium flow cryostat at 6–7 K. Instrument parameters were as follows: microwave frequency, 9.39 GHz; microwave power, 2–5 mW; modulation frequency, 100 kHz; modulation amplitude, 19.5 G; sweep time, 84 s; conversion time, 41 ms; time constant, 82 ms. Four scans were averaged for each experiment.

QM/MM calculations were performed as previously described with the B3LYP functional, with the LANL2DZ pseudopotential for Ca and Mn and the 6-31G basis set for all other atoms. The AMBER force field was used for all MM layer atoms. The 2011.9 Å structure was used as an initial approximation for the S state models. The models included all amino acid residues within 15 Å of the OEC, as well as the water molecules and chloride cofactors. The capping ACE/NME groups were added to the C/N termini at the chain breaks. Two sodium atoms were added to the model to compensate for the excess negative charge of the overall 15 Å cut. A neutral NH\(_3\) molecule was added to investigate the secondary ammonia-binding motif. The total model included 2492 atoms. The QM layer contained 131 atoms, including D1-A344, D1-E189, CP43-E354, D1-H332, D1-D170, D1-E333, D1-D342, D1-H337, CP43-R357, D1-D61, D2-K317, chloride (except in the secondary ammonia structure), and all waters in the vicinity of these residues.

**RESULTS AND DISCUSSION**

In spinach PSII membranes, the relative populations of the \( S_2 \) state \( S = 1/2 \) and \( S = 5/2 \) spin isomers are sensitive to the choice of cryoprotectant. The typical elution buffer for Synecocystis PSII core complexes purified by Ni-affinity chromatography includes both 10% (v/v) glycerol and 1.2 M betaine, which would favor formation of the \( S = 1/2 \) spin isomer based on results with spinach PSII core complexes. To test the effect of cryoprotectant on the equilibrium between the \( S_2 \) state spin isomers in cyanobacteria, Synecocystis PSII core complexes were rigorously buffer exchanged into 1 M sucrose, 45 mM HEPES (pH 7.5), 11 mM Ca(OH)\(_2\), 0.5 mM EDTA, and 0.03% DM (see Materials and Methods). As shown in Figure 2, only the \( S_2 \) state multiline (g = 2) EPR signal is observed in WT and K317A PSII in all cases.

Then, 100 mM NH\(_4\)Cl was added to Samples containing sucrose as a cryoprotectant, and the samples were illuminated at 200 K. As shown in Figure 3 (spectra A), a broad g = 4.1 EPR signal is observed in WT PSII corresponding to the \( S_2 \) state \( S = 5/2 \) spin isomer.
The sample is annealed at 273 K, an altered S2 state observed upon 200 K illumination (Figure 3, spectrum C). When in the primary site (directly to the OEC) causes an alteration of spectra are shown in Figure S1 of the Supporting Information. When illuminated at 200 K (C) and then annealed to 273 K (D). Spectra were recorded at 6.2–6.4 K using a microwave power of 2 mW. Unsubtracted spectra are shown in Figure S1 of the Supporting Information.

Figure 2. $S_1$ state light-minus-dark EPR spectra of (A) WT PSII core complexes in elution buffer (1.2 M betaine and 10% (v/v) glycerol (pH 6.0)), (B) WT PSII core complexes in 1 M sucrose (pH 7.5), and (C) K317A PSII core complexes in 1 M sucrose (pH 7.5). Spectra were recorded at 6.2–6.4 K using a microwave power of 2 mW. Unsubtracted spectra are shown in Figures S1 of the Supporting Information.

Figure 3. $S_2$ state light-minus-dark EPR spectra in the presence of 100 mM NH$_4$Cl (pH 7.5). WT PSII core complexes illuminated at 200 K (A) and then annealed to 273 K (B). K317A PSII core complexes illuminated at 200 K (C) and then annealed to 273 K (D). Spectra were recorded at 6.2–6.4 K using a microwave power of 2 mW. Unsubtracted spectra are shown in Figures S3 and S4 of the Supporting Information.

A $5/2$ spin isomer. The appearance of this signal is independent of cryoprotectant choice (see Figure S2 of the Supporting Information). When the sample is annealed to 273 K, the $g = 4.1$ signal is lost and the $g = 2$ multiline signal is altered (Figure 3, spectrum B). This behavior is analogous to that found in previous studies of spinach PSII membranes: ammonia binding in the secondary site (outside the OEC) induces a $g = 4.1$ EPR signal in the $S_2$ state formed by 200 K illumination, and ammonia binding in the primary site (directly to the OEC) causes an alteration of the $S_2$ state $g = 2$ EPR signal upon annealing.

For K317A PSII, only a very weak $g = 4.1$ EPR signal is observed upon 200 K illumination (Figure 3, spectrum C). When the sample is annealed at 273 K, an altered $S_2$ state $g = 2$ multiline spectrum is observed that closely matches that of the WT (Figure 3, spectrum D). The absence of a clear $S_2$ state $g = 4.1$ EPR spectrum in K317A PSII suggests that ammonia does not bind to the secondary site, while ammonia is still capable of binding to the primary site.

The observation of a significant decrease in the magnitude of the $S_2$ state $g = 4.1$ EPR signal in K317A compared to that in WT PSII in the presence of 100 mM NH$_4$Cl led to the ammonia-binding model shown in Figure 4. While ammonia binding in the second coordination sphere of the OEC is competitive with that of chloride, we hypothesized that it does not bind at the same site. Instead, the occupancy of the high-affinity chloride site indirectly affects ammonia binding. As shown in Figure 4B, when chloride is depleted, D61 moves away from W1 to form an ion pair with K317.11 Ammonia can then bind as a hydrogen bond acceptor to W1. In K317A PSII (Figure 4C), chloride does not bind and D61 is unable to form a salt bridge with K317. Therefore, D61 remains a strong hydrogen bond acceptor to W1 and blocks ammonia from binding.

To test this hypothesis, we used QM/MM calculations to determine an energy-minimized structure of ammonia bound in the proposed site (Figure 5A, a PDB file is available in the Supporting Information). This structure represents the $S_2$ state $S = 5/2$ spin isomer (oxidation states IV, IV, IV, and III) and was prepared by removing chloride and adding ammonia (as neutral NH$_3$) near W1. Intriguingly, calculations show that the terminal aqua ligand W1 is deprotonated by ammonia to form a terminal hydroxo ligand and ammonium. This finding is consistent with both biochemical studies that suggest the secondary site binding is proportional to the concentration of ammonia and FTIR studies that identified ammonium interacting with one or more carboxylate groups near the OEC.38

The secondary ammonia-binding site reported here is adjacent to our previously reported primary ammonia-binding site (Figure 5B). As discussed above, dark-adapted WT PSII samples illuminated at 200 K in the presence of NH$_4$Cl exhibit $g = 4.1$ ($S = 5/2$ $S_2$ state spin isomer, ammonia bound in the secondary site) and $g = 2$ ($S = 1/2, S_2$ state spin isomer, ammonia not bound in the secondary site) EPR signals. When the sample is annealed in darkness at >250 K, the $S_2$ state $g = 4.1$ EPR signal is lost and the $S_2$ state $g = 2$ signal is altered, indicating that ammonia has bound directly to the OEC. In our models, ammonia binds to the secondary site outside the OEC in some fraction of centers in the $S_1$ intermediate and is protonated to form ammonium. Those sites that have ammonia/ammonium bound advance to the $S_2$ state upon illumination at 200 K. When the temperature is increased to >250 K, ammonium migrates to form a direct ligand to the dangle Mn$_4$ (Figure 5), leading to additional 8.7 kcal/mol stabilization (see Figure S5). This change involves the transfer of a proton from ammonium to W1 and the transfer of a proton from W2 to O$_5$. The Mn oxidation states of the OEC change from IV, IV, IV, and III ($S = 5/2$) to III, IV, IV, and IV ($S = 1/2$).

The binding of ammonia in the secondary site is not a requirement of ammonia binding in the primary site, because the latter is not chloride-dependent. Instead, we propose that the reaction shown in Figure 5 is the most efficient (lowest-barrier) route for ammonia to bind to the primary site. Because the annealing step involves an elevated temperature and is relatively slow (>10 s), ammonia is able to move through the extensive hydrogen bonded networks connecting the OEC to the lumen and bind to the OEC. For the case in which ammonia is bound in the secondary site or the case in which it must enter via a channel,
its binding directly to the OEC is thermodynamically driven by the >120 mV (>2.7 kcal mol⁻¹) stabilization of S₂ when ammonia is bound.14

In conclusion, we have used mutagenesis, EPR spectroscopy, and QM/MM calculations to provide the first structural model of the secondary ammonia-binding site in the outer coordination sphere of the OEC. Ammonia competes with D61 as a hydrogen bond acceptor to W1. Upon chloride depletion, D61 moves away from W1 due to formation of a salt bridge with K317.20 (C) In K317A PSII, chloride does not bind. D61 remains a strong hydrogen bond acceptor to W1, and ammonia is unable to bind.

![Figure 4](image1.png)

**Figure 4.** Proposed model of chloride-competitive ammonia binding outside the OEC. (A) Native structure of the S₂ state S = 5/2 spin isomer.10 (B) Ammonia competes with D61 as a hydrogen bond acceptor to W1. Upon chloride depletion, D61 moves away from W1 due to formation of a salt bridge with K317.20 (C) In K317A PSII, chloride does not bind. D61 remains a strong hydrogen bond acceptor to W1, and ammonia is unable to bind.

![Figure 5](image2.png)

**Figure 5.** (A) QM/MM-optimized structure of ammonia bound in the secondary site in the S₄ state intermediate of the OEC (S = 5/2 with Mn oxidation states IV, IV, IV, and III). Above approximately 250 K, ammonia migrates to the primary binding site (B) (S = 1/2 with Mn oxidation states III, IV, IV, and IV).10 See Figure S5 for additional information about primary/secondary site energetics. Selected distances are shown in angstroms.

Three figures showing EPR spectra, one figure showing the dependence of ammonia binding on the choice of cryoprotectant, one figure showing primary and secondary site energetics, and a PDB file with the QM/MM coordinates of the secondary ammonia-binding model.

---

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00543.

Three figures showing EPR spectra, one figure showing the dependence of ammonia binding on the choice of cryoprotectant, one figure showing primary and secondary site energetics, and a PDB file with the QM/MM coordinates of the secondary ammonia-binding model.

---

**AUTHOR INFORMATION**

**Corresponding Author**
E-mail: gary.brudvig@yale.edu. Phone: (203) 432-5202. Fax: (203) 432-6144.

**Funding**
The authors acknowledge support by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, Division of Chemical Sciences, Geosciences, and Biosciences, Photosynthetic Systems. Experimental work was funded by Grant DE-FG02-05ER15646 (G.W.B.), mutant construction was funded by Grant DE-FG02-10ER16191 (R.J.D.), and computational work was funded by Grant DESC0001423 (V.S.B.). We thank the National Energy Research Scientific Computing Center (NERSC) and Shanghai Jiao Tong University II High Performance Computation Center for generous computer time allocations.

**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**
We thank Prof. Marilyn Gunner and Dr. Sahr Khan for helpful discussions.

**ABBREVIATIONS**
DM, n-dodecyl β-D-maltoside; EDTA, (ethylenedinitrilo)-tetraacetic acid; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kDa, kilodalton; MES, 2-(N-morpholino)ethanesulfonic acid; NTA, nitrilotriacetic acid; OEC, oxygen-evolving complex; PSII, Photosystem II; QM/MM, quantum mechanics/molecular mechanics; Sₙ, S state intermediate (n = 0–4); WT, wild type.

**REFERENCES**
Although the specific content of the image is not provided, the text appears to be a scientific document related to biochemistry, possibly discussing topics such as oxygen evolution, ammonium binding, and related mechanisms. The text includes references to various authors and publications, indicating a detailed and scholarly discussion of the subject matter.