Energetics of the S_2 State Spin Isomers of the Oxygen-Evolving Complex of Photosystem II

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Supporting Information

ABSTRACT: The S_2 redox intermediate of the oxygen-evolving complex (OEC) of photosystem II (PSII), redox intermediates of the Mn_4CaO_5 complex are known as S_i states (i = 0–4). In each turn of the S-state cycle, four oxidizing equivalents generated by photosynthetic charge separation events are used to oxidize two water molecules, forming one molecule of O_2 and reducing two molecules of plastoquinone to plastoquinol. Protons taken from the stroma and released into the lumen contribute to the transmembrane proton-motive force.

The structure of the OEC in the dark-stable S_1 state has been determined using a combination of X-ray crystallography and extended X-ray absorption fine structure (EXAFS) spectroscopy, and quantum mechanical (QM) calculations. Multiple lines of evidence (reviewed in ref 11) suggest that the S_1 state contains two Mn^{3+} and two Mn^{4+}. However, an alternative hypothesis asserts that the S_1 state contains either (Mn^{3+})_4 or (Mn^{2+})(Mn^{3+})_2(Mn^{4+}).

The S_2 state is produced from the S_1 resting state by either continuous illumination at 130–220 K or by a single-turnover flash at ambient temperature. The S_2 state is paramagnetic and has been extensively studied using electron paramagnetic resonance (EPR) and EXAFS spectroscopy techniques. No proton is released during the S_1 to S_2 transition, and one Mn ion is oxidized from Mn^{3+} to Mn^{4+}. The remaining Mn^{3+} center in the OEC in the S_2 state can be present at the Mn1 or Mn4 position (see Figure 1 for numbering). When Mn4 is Mn^{3+}, the OEC has a “closed cubane” motif and the ground spin state (S) is S/2 (Figure 1A). When Mn1 is Mn^{3+}, the OEC is in an “open cubane” form and the ground spin state is 1/2 (Figure 1B). The S = 5/2 spin isomer produces a nearly isotropic EPR signal at approximately g = 4.1, whereas the S = 1/2 spin isomer produces a “multiline” EPR spectrum centered at g = 2.0 (Figure 1C).

Both spin isomers are present in PSII membranes isolated from higher plants. However, the relative intensities of the two EPR signals (g = 4.1 and 2.0) are sensitive to experimental conditions, such as the illumination temperature and choice of cryoprotectant. In native cyanobacterial PSII core complexes, only the S = 1/2 spin isomer is observed.

Whereas the electronic structures of the two S_i spin isomers have been established by both experiment and theory, their relative energetics have not been established. Herein, we experimentally determine that the S_i = 1/2 isomer is approximately 0.7 kcal mol^{-1} more stable than the S_i = 5/2 isomer, consistent with QM/MM calculations, and discuss the chemical mechanism of photosynthetic water oxidation in light of this finding.

METHODS

Spinach PSII membranes were prepared as previously described and suspended to final chlorophyll concentrations of 5–8 mg mL^{-1} in 50 mM MES, 20 mM Ca(OH)_2, 10 mM NaCl, 0.01% Triton X-100, and 400 mM sucrose. The pH was adjusted to 6.0 with NaOH.

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

Received: January 4, 2017
Published: January 12, 2017
For S\textsubscript{2} state conversion experiments, 0.5 mM phenyl-3,80 80benzoquinone (PPBQ) was added to each EPR sample from a 8150 mM stock solution in dimethyl sulfoxide (DMSO). The 82EPR samples were illuminated in a quartz nitrogen flow cell at 83135 K using a white xenon lamp supplemented with a near-IR 84light-emitting diode (LED; \( \lambda_{\text{max}} = 850 \text{ nm} \)). After 4 min, the 85sample was quickly removed to liquid nitrogen (77 K) in 86darkness and transferred to the EPR cryostat. For incubations at 87<190 K, the sample was returned to the nitrogen flow cell. 88For incubations at \( \geq 195 \text{ K} \), the sample was transferred to an 89equilibrated bath with varying ratios of ethanol/ethylene glycol 90in dry ice.

For S\textsubscript{2}QA\textsuperscript{−} decay experiments, 0.5 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to each EPR sample 93from a 50 mM stock solution in DMSO. The EPR samples 94were illuminated in an ethanol/dry ice bath (200 K) using a 95white xenon lamp supplemented with a near-IR LED (\( \lambda_{\text{max}} = 96850 \text{ nm} \)). After 4 min, the sample was quickly removed to liquid 97nitrogen (77 K) in darkness and transferred to the EPR cryostat. For all incubations, the sample was transferred to an 98equilibrated bath of varying ratios of ethanol/ethylene glycol in 99dry ice.

For both experiments, incubations were performed in 100complete darkness and the temperature was continuously monitored using an external thermocouple.

EPR spectra subtractions and curve fittings were obtained using OriginPro 9.1. Temperature fluctuations during EPR measurements were \( \pm 0.1 \text{ K} \), leading to subtraction errors specifically in the \( g \approx 4 \) region due to rhombic iron contamination. These errors may affect EPR signal intensity measurements and are the predominant source of uncertainty in the values that we report. Nonlinear and linear curve fittings were performed in OriginPro using the Levenberg–Marquardt algorithm. Energy-minimized parameter values and associated standard errors are reported. For logarithmic plots, error bars span the natural logarithm of the mean \( \pm \) standard error.

QM/MM calculations were performed as previously described,\textsuperscript{8,19,20,24} using the B3LYP functional \textsuperscript{25,26} with the LANL2DZ pseudopotential\textsuperscript{27,28} for Ca and Mn and the 6-31G* basis set\textsuperscript{29} for all other atoms for geometry optimizations and 6-311+G** for energy evaluations. The high layer and MM layer 120

![Figure 1](image_url)  
**Figure 1.** QM/molecular mechanical (MM) optimized structures of the S\textsubscript{2} state spin isomers with ground states of \( S = 5/2 \) (A)\textsuperscript{19} and \( S = 1/2 \) (B)\textsuperscript{20} (see Pantazis et al.\textsuperscript{9}). Mn\textsuperscript{3+} ions are shown in purple, Mn\textsuperscript{4+} in lavender, Ca\textsuperscript{2+} in orange, and O\textsuperscript{2−} in red. (C) The \( S = S/2 \) isomer is characterized by a broad EPR signal at \( g = 4.1 \), and the \( S = 1/2 \) isomer gives rise to a multiline EPR signal centered at \( g = 2.0 \). The intensity of the \( g = 4.1 \) signal is measured as the peak-to-peak height, indicated by the red arrow. The intensity of the \( g = 2.0 \) signal is determined by summing the peak-to-peak heights of the four features indicated by blue arrows.

![Figure 2](image_url)  
**Figure 2.** Kinetics of S\textsubscript{2} state conversion. (A) Only the \( g = 4.1 \) EPR signal is formed upon illumination at 135 K, as shown in the light – dark spectrum (top trace). During incubation in darkness at the representative temperature of 170 K in the presence of PPBQ, the \( g = 4.1 \) EPR signal decreases in intensity and the \( g = 2.0 \) EPR signal increases in intensity. Unsubtracted spectra are shown in Figure S1 of the Supporting Information. (B) Normalized peak-to-peak height of the \( g = 4.1 \) EPR signal vs. incubation time. Data were fit to a single exponential decay function (\( y = y_0 + A \exp(-x/t) \)) (dotted lines). (C) Arrhenius treatment of the data in (B). Error bars represent standard error of the fit. \( E_a = 6.7 \pm 0.5 \text{ kcal mol}^{-1}, A = 1.6 \pm 0.2 \times 10^6 \text{ s}^{-1}, R^2 = 0.9867. \)
were chosen according to the scheme described in our previous model. The AMBER force field was used for all MM layer atoms.

**RESULTS AND DISCUSSION**

To study the kinetics of conversion of the \( S = S/2 \) isomer to the \( S = 1/2 \) isomer, we illuminated dark-adapted PSII membranes isolated from spinach, containing the exogenous electron acceptor PPBQ and succrose as a cryoprotectant, at 135 K. Under these conditions, only the \( g = 4.1 \) EPR signal is observed (Figure 2A) because of the presence of near-IR light during illumination (see Methods). After an initial EPR spectrum was obtained, the same sample was incubated in total darkness at 150–242 K, which led to conversion of the \( g = 4.1 \) to the \( g = 2.0 \) signal. For temperatures \( \leq 170 \) K, the initial rate of conversion could be determined using this method (Figure 2B).

Arrhenius analysis of the rate constants predicts an activation barrier of \( 6.7 \pm 0.5 \) kcal mol\(^{-1} \) (Figure 2C). This barrier is in close agreement with that in a previous study by de Paula et al., who reported an activation barrier of \( 7.9 \pm 1.4 \) kcal mol\(^{-1} \) for this conversion under similar conditions. For all tested temperatures, the \( g = 4.1 \) EPR signal decreased and the \( g = 2.0 \) EPR signal increased during dark incubation. This behavior has been previously reported by multiple groups. The \( g = 4.1 \) signal intensity did not approach zero for extended incubation times (Figure 2A,B). Instead, the data suggest that a temperature-dependent equilibrium is established (see refs 31 and 34). For temperatures \( > 170 \) K, the \( g = 4.1 \) signal decayed to a steady-state level in less than \( 300 \) s (first time point in Figure 2B). Although we could not determine the rates of \( S_1 \) state conversion in this temperature range due to fast conversion, equilibrium constants (defined here as \( K_{eq} = [g = 2.0]/[g = 4.1] \)) could be measured. As shown in Figure 3, \( K_{eq} \) is biphasic with respect to temperature. At temperatures \( \leq 170 \) K, the van’t Hoff treatment of the data provides a \( \Delta H^\circ \) of \( +31 \) kJ mol\(^{-1} \) and a \( \Delta S^\circ \) of \( +190 \) J mol\(^{-1} \) K\(^{-1} \). At temperatures \( \geq 195 \) K, the conversion of the \( S = 5/2 \) isomer to the \( S = 1/2 \) isomer is thermodynamically less demanding (\( \Delta H^\circ = +0.40 \) kJ mol\(^{-1} \) and \( \Delta S^\circ = +11 \) J mol\(^{-1} \) K\(^{-1} \)) and becomes exergonic for temperatures \( > 160 \) K (Figure S2). When the data are extrapolated to 298 K, the equilibrium constant is estimated to be \( 3.2 \pm 0.4 \), corresponding to \( \Delta G^\circ = -0.69 \pm 0.14 \) kcal mol\(^{-1} \).

The biphasic temperature dependence of the \( S_2 \) state spin isomer conversion may be caused by the surrounding protein environment. Extensive hydrogen-bonding networks involving water molecules, chloride, amino acid side chains, and the protein backbone amides surround the OEC and influence its properties. For example, a second-shell water ligand, labeled Wx, is proposed to play a key role in water delivery to the OEC upon formation of the \( S_1 \) state. As shown in Figure 4, Wx is a hydrogen-bond donor to the OEC in QM/MM structures of the \( S_1 \) state and the \( S_2 \) state \( S = 5/2 \) isomer. However, at elevated temperatures above the glass-transition temperature, the hydrogen-bonding network can rearrange to accommodate the thermodynamically preferred \( S = 1/2 \) isomer. An H/D kinetic isotope effect of 2.5 associated with the conversion of the \( S_2 \) states at 160 K (Figure S3) further supports this hypothesis. Previous studies by Bousquet and co-workers have shown that the \( g = 4.1 \) EPR signal formed by near-IR illumination at 77–160 K is different from the \( g = 4.1 \) EPR signal formed by 200 K illumination in terms of its temperature stability. We propose that the changes in hydrogen-bonding networks above the observed glass transition may be responsible for this behavior.

To study the kinetics of decay of the \( S_2 \) state to the \( S_0 \) state by charge recombination, dark-adapted PSII samples were illuminated at 200 K. Under these conditions, both the \( g = 4.1 \) and 2.0 EPR signals were observed (Figure S4). After an initial EPR spectrum was obtained, the same sample was incubated in total darkness at 218–258 K and the rates at which the \( g = 4.1 \) and 2.0 EPR signals decay were determined (Figure S4 in the Supporting Information). The \( g = 4.1 \) EPR signal was found to decay faster than the \( g = 2.0 \) signal. Arrhenius analysis of the rate constants provides an activation barrier for charge recombination of \( 6.3 \pm 0.3 \) kcal mol\(^{-1} \) for the \( S_2 \) state \( g = 4.1 \) spin isomer and \( 10.5 \pm 0.9 \) kcal mol\(^{-1} \) for the \( S_2 \) state \( g = 2.0 \) spin isomer (Figure S5).

These experiments revealed two key energetic features of the \( S_2 \) state spin isomers: (1) conversion of the \( S = 5/2 \) isomer to the \( S = 1/2 \) isomer is exergonic at temperatures \( > 160 \) K and (2) the \( S = 1/2 \) isomer has an activation barrier approximately 67% higher for charge recombination from \( S_2 \) to \( S_0 \) than that of the \( S = 5/2 \) isomer.

Several computational chemistry groups have estimated the relative energetics of the \( S_2 \) state spin isomers. Pantazis, Neese, and co-workers first used QM calculations to determine that the \( S = 1/2 \) isomer was more stable than the \( S = 5/2 \) isomer by 0.42–1.64 kcal mol\(^{-1} \). Similar results have been shown by Yamaguchi and co-workers (1.3 kcal mol\(^{-1} \)) and Kaila and co-workers (1.1 kcal mol\(^{-1} \)). Guidoni et al. have used QM/MM–molecular dynamics methods to determine that the \( \Delta G \) at 298 K between the \( S_2 \) state isomers is 1.1 kcal mol\(^{-1} \), with 220
Using QM/MM methods and the models described in the Supporting Information, we find that the energy of the $S = 1/2$ isomer is 0.84 kcal mol$^{-1}$ lower than that of the $S = 5/2$ isomer. All of these theoretical findings are in excellent agreement with the experimental results in this work ($\Delta G = -0.69 \pm 0.14$ kcal mol$^{-1}$ at 298 K).

The kinetics of formation of the $S_2$ state and its conversion from the $S = 1/2$ spin isomer to the $S = 5/2$ spin isomer and vice versa have been harder to determine computationally, as the transition involves a change in both the spin state and geometry. The reaction barriers that we measure provide this vital information needed to delineate the PSII water-oxidation reaction coordinate.

These data suggest that in higher plant PSII at ambient temperature (298 K) approximately 75% of the $S_2$ state population is in the $S = 1/2$ spin isomer form, whereas approximately 25% is in the $S = 5/2$ spin isomer form. The excess $S_2$ state population in the $S = 1/2$ isomer form decays more slowly to $S_1$ via charge recombination with $Q_A^-$. Previously, we and others have suggested that the $S_3$ state is formed via the $S_2$ state $S = 5/2$ isomer. This seems to be in contradiction to the fact that the equilibrium between the $S_2$ spin isomers clearly favors the $S = 1/2$ isomer at 298 K. However, depending on environmental light conditions, cyanobacteria, algae, and plants must carefully balance the thermodynamics of OEC advancement and charge recombination. The distribution of $S_2$ state spin isomers may thus play a role in regulating photosynthetic efficiency under light-limited conditions. The majority of the $S_2$ state population remains in the stabilized low-spin isomer form that has a slower $S_2QA^-\text{charge recombination}$, whereas a minority of the $S_2$ state population is in the reactive high-spin isomer form that has a faster $S_2QA^-\text{charge recombination}$ but can advance to the $S_3$ state. Balancing these two populations can thus tune the overall efficiency of PSII.

Figure 4. QM/MM optimized structures of the $S_1$ state and the two spin isomers of the $S_2$ state. Only the $g = 4.1$ EPR signal corresponding to the $S = 5/2$ spin isomer is formed at 135 K, but it spontaneously converts to the $S = 1/2$ spin isomer at temperatures $>160$ K. The second-shell water molecule, $W_x$, is circled in each structure. $W_x$ is a hydrogen-bond donor to the OEC in the $S_1$ state and the $S = 5/2$ spin isomer of the $S_2$ state but not to the $S = 1/2$ spin isomer of the $S_2$ state. Mn$^{3+}$ ions are shown in purple; Mn$^{4+}$, in lavender; Ca$^{2+}$, in orange; and O$_2^-$, in red.

Figure 5. Kinetics of decay of $S_2Q_A^-$ to $S_1Q_A^-$. (A) Both the $g = 4.1$ and 2.0 EPR signals are formed upon illumination at 200 K, as shown in the light−dark spectrum (top trace). During incubation in darkness at the representative temperature of 218 K in the presence of DCMU, the $g = 4.1$ EPR signal decreases faster than the $g = 2.0$ EPR signal. Unsubtracted spectra are shown in Figure S5 of the Supporting Information. (B) Arrhenius analysis of the decay kinetics of $S_2Q_A^-$ to $S_1Q_A^-$. For the $g = 4.1$ EPR signal (circles), $E_a = 6.3 \pm 0.3$ kcal mol$^{-1}$, $A = 3.6 \pm 0.3 \times 10^3$ s$^{-1}$, and $R^2 = 0.9924$. For the $g = 2.0$ EPR signal (squares), $E_a = 10.5 \pm 0.9$ kcal mol$^{-1}$, $A = 8.8 \pm 1.1 \times 10^6$ s$^{-1}$, $R^2 = 0.9699$. The Journal of Physical Chemistry B
We have determined the energetics of the $S_2$ state spin isomers (summarized in Figure 6). The $S_2$ state spin isomer conversion

![Figure 6. Compiled energetics schemes for the (A) conversion of the $S_2$ state $S = 5/2$ isomer to the $S_2$ state $S = 1/2$ spin isomer and (B) decay of the $S_2$ state $S = 5/2$ isomer and temperature dependence of the free-energy di

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

The authors acknowledge the 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. The experimental work was funded by Grant DE-FG02-05ER15646 (G.W.B.), and the 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 for generous computer time allocations. We thank Prof. Marilyn Gunner for helpful discussions.

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