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Article

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

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ABSTRACT

The S_2 redox intermediate of the oxygen-evolving complex in Photosystem II is present as two spin isomers. The S = 1/2 isomer gives rise to a multiline EPR signal at g = 2, while the S = 5/2 isomer exhibits a broad EPR signal at g = 4.1. The electronic structures of these isomers are known, but their role in the catalytic cycle of water oxidation remains unclear. We show that formation of the S = 1/2 state from the S = 5/2 state is exergonic at temperatures above 160 K. However, the S = 1/2 isomer decays to S_1 more slowly than the S = 5/2 isomer. These differences support the hypotheses that the S_3 state is formed via the S_2 state S = 5/2 isomer and that the stabilized S_2 state S = 1/2 isomer plays a role in minimizing $S_2Q_A^-$ decay in light-limiting conditions.

INTRODUCTION

In the catalytic cycle 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).[1] In each turn of the S-state cycle, four oxidizing equivalents generated by photochemical 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 to the lumen contribute to the trans-membrane proton-motive force.[2]

The structure of the OEC in the dark-stable S_1 state has been determined using a combination of X-ray crystallography,[3, 4] extended X-ray absorption fine structure (EXAFS) spectroscopy,[5, 6] and quantum mechanical (QM) calculations.[7-10] Multiple lines of evidence (reviewed in reference [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 $(Mn^{2+})(Mn^{3+})_2(Mn^{4+})$.[12, 13]

The S_2 state is produced from the S_1 resting state by either continuous illumination at 130-220 K[14, 15] or by a single-turnover flash at ambient temperature.[1, 16] The S_2 state is paramagnetic and has been extensively studied by using electron paramagnetic resonance (EPR) and EXAFS spectroscopies.[17] 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 positions (see Figure 1 for numbering). When Mn4 is Mn^{3+}, the OEC has a "closed cubane" motif and the ground spin state (S) is 5/2 (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).[9] The S = 5/2 spin isomer produces a nearly isotropic EPR signal at approximately g = 4.1,[14, 18] while the S = 1/2 spin isomer produces a "multiline" EPR spectrum centered at g = 2.0 (Figure 1C).[16]

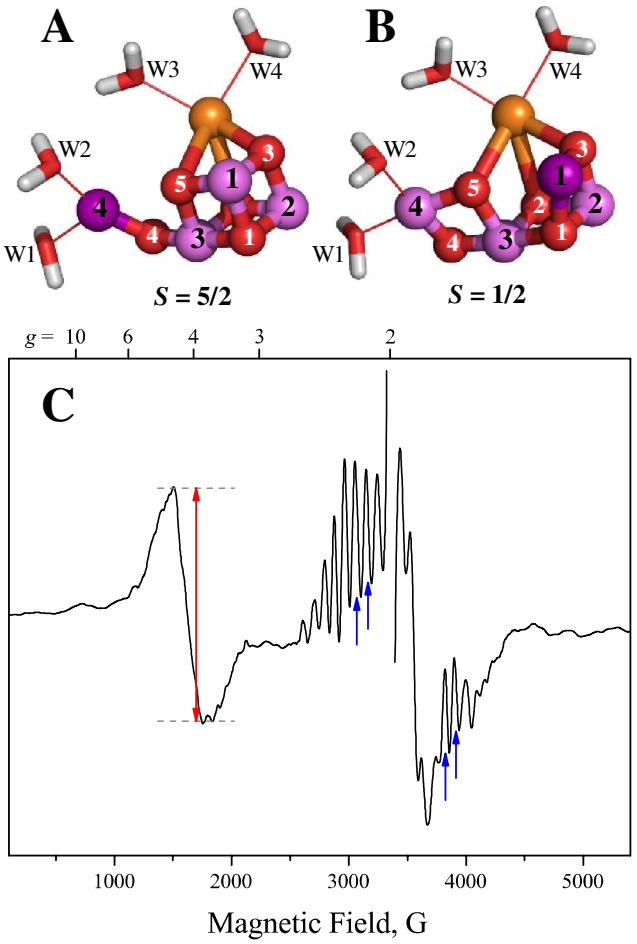

Figure 1. QM/MM optimized structures of the S_2 state spin isomers with ground states of $S = 5/2$ (A)[19] and $S = 1/2$ (B)[20] (see Pantazis *et al.*[9]) Mn^{3+} ions are shown in purple, Mn^{4+} in lavender, Ca^{2+} in orange, and O^{2-} in red. (C) The $S = 5/2$ isomer is characterized by a broad EPR signal at $g = 4.1$ and the $S = 1/2$ isomer gives rise to a multiline EPR signal centered at $g = 2$. 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 $g = 2$ signal is determined by summing the peak-to-peak heights of the four features indicated by blue arrows.

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 $g = 2.0$) are sensitive to experimental

conditions such as the illumination temperature and choice of cryoprotectant.[17] In native cyanobacterial PSII core complexes, only the $S = 1/2$ spin isomer is observed.

While the electronic structures of the two S_2 spin isomers have been established by both experiment[21] and theory,[9] the relative energetics have not been established. Herein, we experimentally determine that the $S = 1/2$ isomer is approximately 0.7 kcal mol^{-1} more stable than the $S = 5/2$ 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[22, 23] 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 a SHQ cavity and an Oxford ESR-900 helium flow cryostat at 6-7 K. Instrument parameters were 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.

For S_2 state conversion experiments, 0.5 mM phenyl-*p*-benzoquinone (PPBQ) was added to each EPR sample from a 50 mM stock solution in dimethyl sulfoxide (DMSO). EPR samples were illuminated in a quartz nitrogen flow cell at 135 K using a white xenon lamp supplemented with a near-IR LED (λ_{max} = 850 nm). After four minutes, the sample was quickly removed to liquid nitrogen (77 K) in darkness and transferred to the EPR cryostat. For incubations < 190 K, the

sample was returned to the nitrogen flow cell. For incubations ≥195 K, the sample was transferred to an equilibrated bath of varying ratios of ethanol/ethylene glycol in dry ice.

For $S_2Q_A^-$ decay experiments, 0.5 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to each EPR sample from a 50 mM stock solution in DMSO. EPR samples were illuminated in an ethanol/dry ice bath (200 K) using a white xenon lamp supplemented with a near-IR LED (λ_{max} = 850 nm). After four minutes, the sample was quickly removed to liquid nitrogen (77 K) in darkness and transferred to the EPR cryostat. For all incubations, the sample was transferred to an equilibrated bath of varying ratios of ethanol/ethylene glycol in dry ice.

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

EPR spectra subtractions and curve fittings were performed in OriginPro 9.1. Temperature fluctuations during EPR measurements were ± 0.1 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 (Figure 1C) and are the predominant source of uncertainty in the values we report. Non-linear 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 ± standard error.

QM/MM calculations were performed as previously described[8, 19, 20, 24] using the B3LYP functional[25, 26] with the LANL2DZ pseudo-potential[27, 28] for Ca and Mn and the 6-31G* basis set[29] for all other atoms for geometry optimizations and 6-311+G** for energy evaluations. The choice of the high layer and the MM layer was done according to the scheme described in our previous models.[20] The AMBER force field was used for all MM layer atoms.[30]

RESULTS AND DISCUSSION

To study the kinetics of conversion of the $S = 5/2$ isomer to the $S = 1/2$ isomer, we illuminated dark-adapted PSII membranes isolated from spinach containing the exogenous electron acceptor PPBQ and sucrose as a cryoprotectant at 135 K. Under those conditions, only the $g = 4.1$ EPR signal is observed (Figure 2A)[14] because of the presence of near IR light during illumination[31] (see Methods). After an initial EPR spectrum was obtained, the same sample was incubated in total darkness at 150-242 K, which led to the conversion of the $g = 4.1$ to the $g = 2$ signal. For temperatures ≤ 170 K, the initial rate of conversion could be determined using this method (Figure 2B). The Arrhenius analysis of the rate constants predicts an activation barrier of 6.7 ± 0.5 kcal mol^{-1} (Figure 2C). This barrier is in close agreement with a previous study by de Paula *et al.* that reported an activation barrier of 7.9 ± 1.4 kcal mol^{-1} for this conversion under similar conditions.[32]

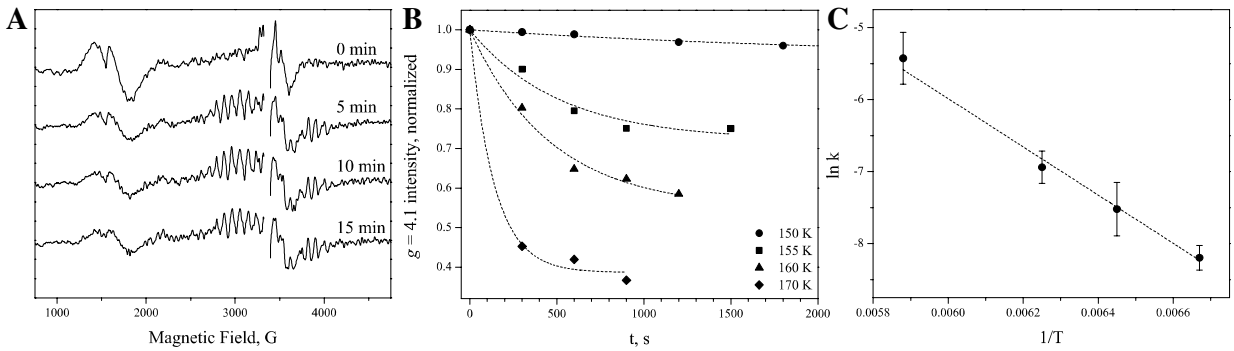

Figure 2. Kinetics of S_2 state conversion. (A) Only the $g = 4.1$ EPR signal is formed upon illumination at 135 K as shown in the light-*minus*-dark spectrum (top trace). During incubation in darkness at the representative temperature of 170 K in the presence of PPBQ, the $g = 4.1$ EPR signal decreases in intensity and the $g = 2$ 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 panel B. Error bars represent standard error of the fit. $E_a = 6.7 \pm 0.5$ kcal mol^{-1}, $A = 1.6 \pm 0.2 \times 10^6$ s^{-1}, $R^2 = 0.9867$.

For all tested temperatures, the $g = 4.1$ EPR signal decreased and the $g = 2$ EPR signal increased during dark incubation. This behavior has been previously reported by multiple groups.[14, 31-33] The $g = 4.1$ signal intensity did not approach zero for extended incubation times (Figures 2A and 2B). Instead, the data suggest that a temperature-dependent equilibrium is established (see [31, 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). While we could not determine the rates of S_2 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 < 170 K, the van't Hoff treatment of the data provides a $\Delta H°$ of +31 kJ mol^{-1} and a $\Delta S°$ of +190 J mol^{-1} K^{-1}. At temperatures ≥ 195 K, the conversion of the $S = 5/2$ isomer to the $S = 1/2$ isomer is thermodynamically less demanding ($\Delta H° = +0.40$ kJ mol^{-1} and $\Delta S° = +11$ 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 ± 0.4, corresponding to $\Delta G° = -0.69 \pm 0.14$ kcal mol^{-1}.

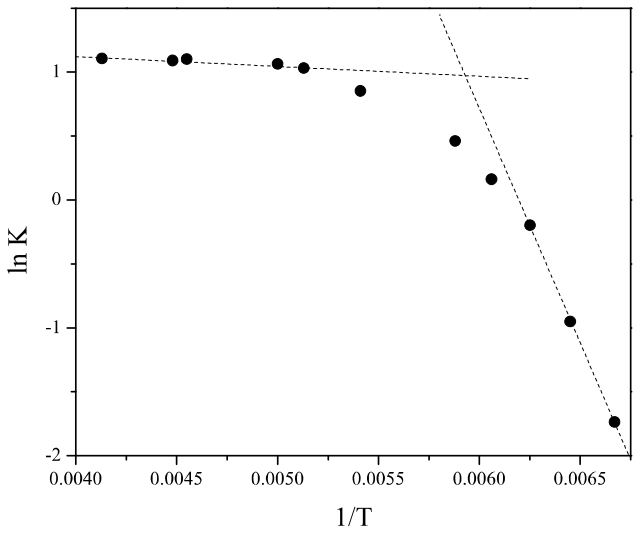

Figure 3. Temperature dependence of the equilibrium between S_2 states. van't Hoff treatment of K_{eq} (defined as [g = 2.0]/[g = 4.1]) shows two distinct linear regions. Fitted parameters are described in the text.

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.[35] 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_3 state.[19, 36] 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, this hydrogen bond is broken upon formation of the S_2 state S = 1/2 isomer due to a dramatic decrease of the μ-oxo (O4) pK_a upon oxidation of Mn4 from Mn^{3+} to Mn^{4+}. We propose that the curvature in the data in Figure 3 reveals a 'glass transition' of the local environment around the OEC. At low temperatures, hydrogen-bonding interactions are frozen in place and only the S = 5/2 isomer can be formed from the S_1 state. However, at elevated temperatures above the glass transition, 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 spin states at 160 K (Figure S3) further supports this hypothesis. Previous studies by Boussac and coworkers 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.[31, 33, 34, 37] We propose that the changes in hydrogen-bonding networks above the observed glass transition may be responsible for this behavior.

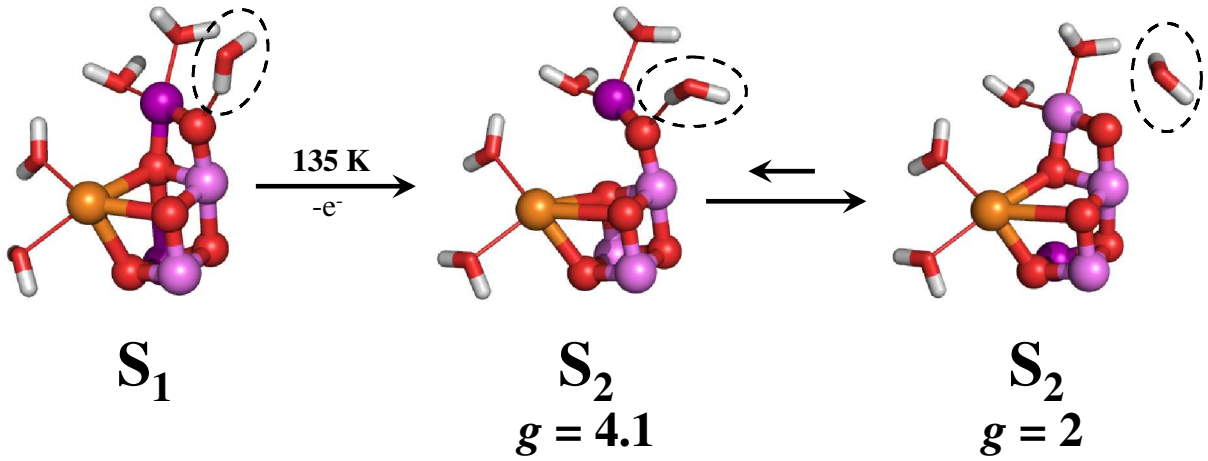

Figure 4. QM/MM optimized structures of the S_1 state and the two spin isomers of the S_2 state. Only the $g = 4.1$ EPR signal corresponding to the $S = 5/2$ spin isomer is formed at 135 K, but it spontaneously converts to the $S = 1/2$ spin isomer at temperatures > 160 K. The second shell water molecule, Wx, is circled in each structure. Wx 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 in 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.

To study the kinetics of the $S_2Q_A^-$ state decaying to S_1Q_A by charge recombination, dark-adapted PSII samples containing the secondary acceptor, Q_B site inhibitor DCMU were illuminated at

200 K. Under these conditions, both the $g = 4.1$ and $g = 2.0$ EPR signals were observed (Figure 5A).[15] 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 $g = 2.0$ 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 ± 0.3 kcal mol^{-1} for the S_2 state $g = 4.1$ spin isomer and 10.5 ± 0.9 kcal mol^{-1} for the S_2 state $g = 2.0$ spin isomer (Figure 5B).

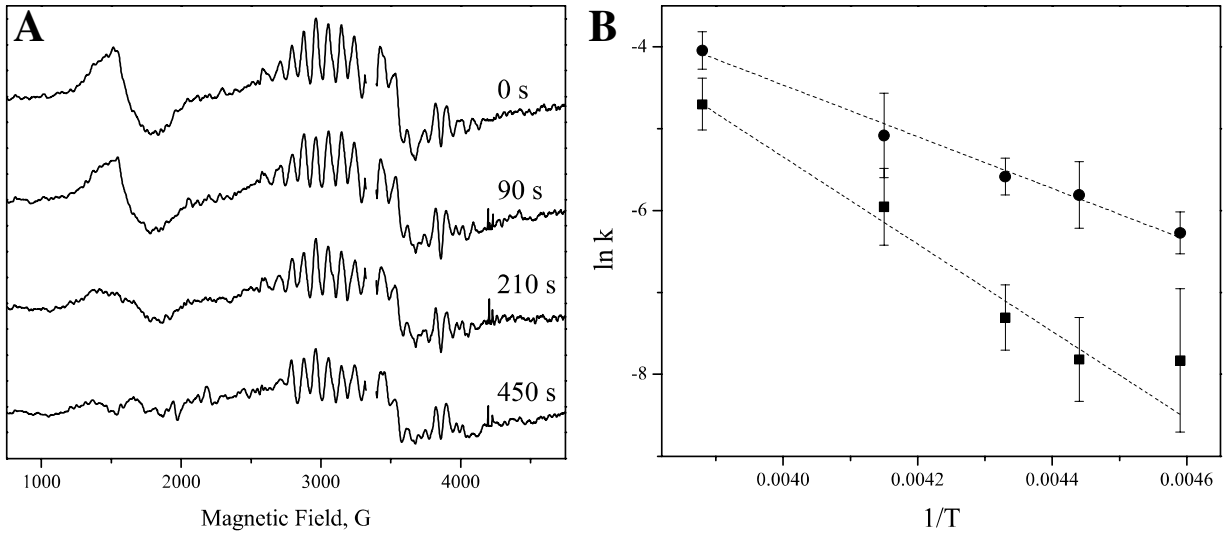

Figure 5. Kinetics of $S_2Q_A^-$ decay to S_1Q_A. (A) Both the $g = 4.1$ and the $g = 2$ EPR signals are formed upon illumination at 200 K as shown in the light-*minus*-dark spectrum (top trace). During incubation in darkness at the representative temperature of 218 K in the presence of DCMU, the $g = 4.1$ EPR signal decreases faster than the $g = 2$ EPR signal. Unsubstracted 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},

$R^2 = 0.9924$. For the $g = 2$ EPR signal (squares), $E_a = 10.5 \pm 0.9$ kcal mol^{-1},

$A = 8.8 \pm 1.1 \times 10^6$ s^{-1}, $R^2 = 0.9699$.

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 $S_2Q_A^-$ to form S_1Q_A than the $S = 5/2$ isomer.

Several computational chemistry groups have estimated the relative energetics of the S_2 state spin isomers. Pantazis, Neese and coworkers 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}.[9] Similar results have been shown by Yamaguchi and coworkers (1.3 kcal mol^{-1})[38] and Kaila and coworkers (1.1 kcal mol^{-1}).[39] Guidoni *et al.* have used QM/MM-MD methods to determine that the ΔG at 298 K between the S_2 state isomers is 1.1 kcal mol^{-1} with the $S = 1/2$ isomer being more stable.[40] Using QM/MM methods and 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 $S = 1/2$ spin isomer to the $S = 5/2$ spin isomer and vice versa have been harder to determine computationally as the transition involves both a change in the spin state and geometry. The reaction barriers we measure provide this vital information needed to delineate the PSII water-oxidation reaction coordinate.

These data suggest that at ambient temperature (298 K) in higher plant PSII, approximately 75% of the S_2 state population is in the S = 1/2 spin isomer, while approximately 25% is in the S = 5/2 spin isomer. The excess S_2 state population in the S = 1/2 isomer 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.[19, 41] 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.[42] The distribution of S_2 state spin isomers may thus play a role in regulating photosynthetic efficiency under light-limited conditions.[43] A majority of the S_2 state population remains in the stabilized low-spin isomer that has slower $S_2Q_A^-$ charge recombination. A minority of the S_2 state population is in the reactive high-spin isomer that has faster $S_2Q_A^-$ charge recombination, but can advance to the S_3 state. Balancing these two populations can thus tune the overall efficiency of PSII. Pantazis and coworkers and Guidoni and coworkers have used computational methods to show that the relative S_2 spin isomer populations change when Y_Z is oxidized.[41, 44] While the influence of Y_Z is outside the scope of this study, it is likely an important factor in the S_2 to S_3 transition.

CONCLUSIONS

We have determined the energetics of the S_2 state spin isomers (summarized in Figure 6). The S_2 state spin isomer conversion experiments reveal that the free energy difference between the two ground spin states is 0.69 ± 0.14 kcal mol^{-1} at 298 K with the S = 1/2 spin isomer being more stable. $S_2Q_A^-$ decay experiments reveal that the S = 5/2 spin isomer has a lower activation barrier

for charge recombination to form S_1Q_A than the S = 1/2 spin isomer. These differences support the hypotheses that the S_3 state is formed from the S_2 state S = 5/2 spin isomer and that the stabilized S_2 state S = 1/2 spin isomer plays a role in minimizing $S_2Q_A^-$ decay in light-limiting conditions. These findings provide experimental insights into the mechanism of natural water oxidation.

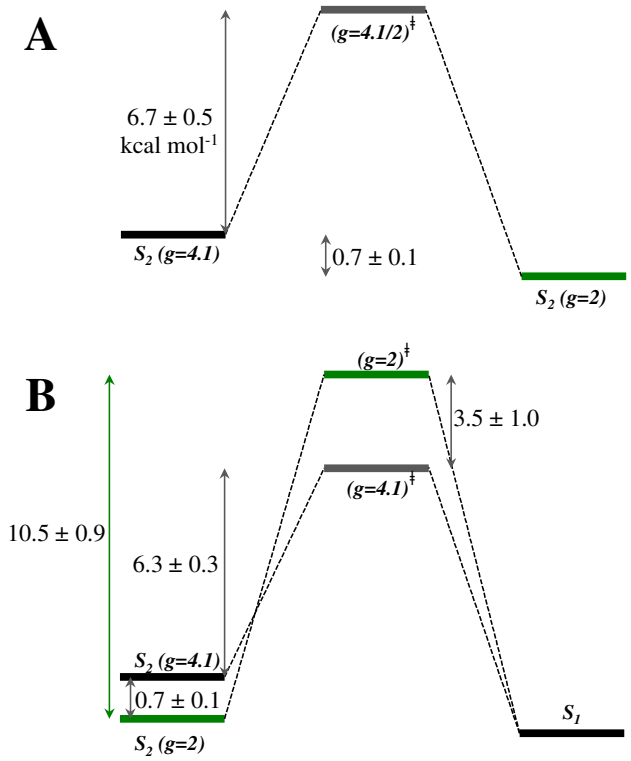

Figure 6. Compiled energetics schemes for (A) the conversion of the S_2 state S = 5/2 isomer to the S_2 state S = 1/2 isomer and (B) decay of the $S_2Q_A^-$ state to S_1Q_A for both S_2 state spin isomers.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website (http://pubs.acs.org). This information includes figures showing

unsubtracted EPR spectra for Figures 2A and 5A, temperature-dependence of the free energy of S_2 state spin isomer conversion, S_2 state spin isomer conversion in D_2O, and $S_2Q_A^-$ decay data at 218 – 258 K.

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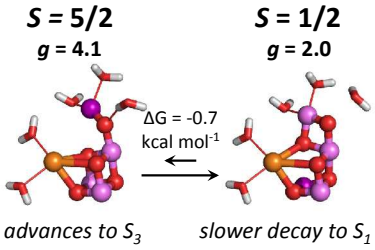
TOC GRAPHIC


S = 5/2
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S = 1/2
g = 2.0
ΔG = -0.7
kcal mol-1
advances to S3
slower decay to S1