REDOX ACTIVE TYROSINES IN PHOTOSYSTEM II: ROLE IN
PROTON COUPLED ELECTRON TRANSFER REACTIONS

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REDOX ACTIVE TYROSINES IN PHOTOSYSTEM II: ROLE IN PROTON COUPLED ELECTRON TRANSFER REACTIONS

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LIST OF SYMBOLS AND ABBREVIATIONS

OEC  Oxygen-evolving complex
PSII  Photosystem II
PSI   Photosystem I
Y_Z  Tyr161-D1
Y_Z^·  Neutral radical of Tyr161-D1
Y_D  Tyr160-D1
Y_D^·  Neutral radical of Tyr160-D1
EPR   Electron paramagnetic resonance
ATP   Adenosine triphosphate
ADP   Adenosine diphosphate
NADPH Nicotinamide adenine dinucleotide phosphate
b_6f  Cytochrome b_6f complex
PQ    Plastoquinone
PQH_2 Plastoquinol
PC    Plastocyanin
P_700 Special pair of photosystem I
P_700^+ Oxidized form of the special pair of photosystem I
Chl a  Chlorophyll a
Chl   Chlorophyll
P_680 Oxidant to tyrosine Z
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$P_{680}^+$</td>
<td>Oxidized form of the oxidant to tyrosine Z</td>
</tr>
<tr>
<td>PCET</td>
<td>Proton coupled electron transfer</td>
</tr>
<tr>
<td>PTET</td>
<td>Proton transfer followed by electron transfer</td>
</tr>
<tr>
<td>ETPT</td>
<td>Electron transfer followed by proton transfer</td>
</tr>
<tr>
<td>CPET</td>
<td>Concerted proton-electron transfer</td>
</tr>
<tr>
<td>KIE</td>
<td>Kinetic isotope effect</td>
</tr>
<tr>
<td>$\text{D}_2\text{O}$</td>
<td>$\text{H}_2\text{O}$</td>
</tr>
<tr>
<td>$S_n$</td>
<td>Oxidation state of the oxygen-evolving complex</td>
</tr>
<tr>
<td>$S_nY_Z$</td>
<td>Tyrosine Z in the $S_n$ state ($n = 0$-4)</td>
</tr>
<tr>
<td>$S_nY_Z^*$</td>
<td>Neutral radical of tyrosine Z in the $S_n$ state ($n = 0$-4)</td>
</tr>
<tr>
<td>KIE</td>
<td>Kinetic isotope effect (defined as the rate $^2\text{H}_2\text{O}/^3\text{H}_2\text{O}$)</td>
</tr>
<tr>
<td>pL</td>
<td>-log[lyonium Ion] where lyonium is $^1\text{H}$, $^2\text{H}$, or $^3\text{H}$</td>
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SUMMARY

The demand for energy has been a growing concern for modern man. The prophetic paper titled “Photochemistry of the Future” by Giacomo Ciamician written in 1912 clearly states the problems that we still face today; “Modern civilization is the daughter of coal, for this offers to mankind the solar energy in its most concentrated form; that is, in a form in which it has been accumulated in a long series of centuries. Modern man uses it with increasing eagerness and thoughtless prodigality”. We as a people have made much progress since then, but the major problem of finding an energy source that is able to meet our demands remains. The sun is our only viable energy source and plants, algae, and cyanobacteria are the only entities on the planet that know how to harvest solar energy efficiently. Unveiling the secrets of photosynthesis is our best chance at discovering how to exploit the sun for our energy needs. Despite over a century of research there is still much to learn of about photosynthesis. It is agreed that the most important process within photosynthesis is the oxidation of water to molecular oxygen and protons. This process occurs within photosystem II where high potentials are harnessed through proton coupled electron transfer reactions. Proton coupled electron transfer reactions often involve tyrosine residues, because when oxidized, the phenolic side chain deprotonates. Tyrosine Z (YZ) is responsible for extracting electrons in a stepwise fashion from the oxygen evolving-complex in order to build enough potential to oxidize water. This process requires that each step YZ must deprotonate and reprotoenate in order to maintain the high midpoint potential that is necessary to oxidize the oxygen-evolving complex, which makes YZ highly involved in proton coupled electron transfer reactions. In this thesis YZ has been studied within oxygen-evolving photosystem II
utilizing electron paramagnetic resonance spectroscopy to monitor the tyrosyl radical that is formed upon light excitation. Kinetic analysis of $Y_Z$ has shed light on the factors that are important for PSII to carry out water oxidation at the oxygen-evolving complex. Most notably the strong hydrogen-bonding network and the midpoint potential of $Y_Z$ have been shown to be integral aspects of the water splitting reactions of PSII. By studying $Y_Z$ within oxygen-evolving PSII, conclusions are readily applied to the native system.
CHAPTER 1
INTRODUCTION

1.1 Demand for Energy

Currently our demand for energy is only met through technological advances that have enabled us to exploit resources outside our capabilities and utilize energy more efficiently. Without continued innovation in the energy sector, the planet will not be able to survive the growing demand for clean energy. Some of the most plentiful sources of energy on the planet are water and sunlight. For this reason, it is not a new idea to use water and sunlight to fuel the planet. Jules Verne recognized that coal supplies were exhaustible and that hydrogen derived from the electrolysis of water could be used as fuel when he stated that “water will be the coal of the future” in 1874. Therefore many efforts to create new technologies are based on duplicating or mimicking structural and functional aspects of photosynthesis. Photosynthesis is among the most fundamental energy conversion process known. Oxygenic photosynthetic processes convert light and CO₂ into an estimated 4 to 17 x 10¹¹ tons of reduced carbon each year, which is the equivalent to harnessing between 1 x 10¹¹ to 5 X 10¹⁴ kJ of energy. This quantity of energy amounts to about 10% of the energy that is consumed on our planet annually. While this quantity seems low, plants, cyanobacteria and algae are not designed to store energy that benefits humankind. Photovoltaics remain expensive when compared to fossil fuels at 25 times the price on a per kilowatt basis, yet it would only require 0.16% of the global land mass with 10% efficient photovoltaics to
produce 20 TW of energy annually.\(^{(4, 8)}\) As long as fossil fuels remain the cheaper option, motivation will be lacking to pay for a cleaner form of energy. For these reasons, we should strive to understand photosynthetic processes in order to create processes that are cheaper and efficient as those found in nature.

1.2 Photosynthesis

Oxygenic photosynthesis is the process used by plants, algae, and cyanobacteria to convert sunlight and carbon dioxide into chemical energy and molecular oxygen.\(^{(5, 9)}\) As shown in Figure 1.1, this process occurs in the thylakoid membranes of all photosynthetic organisms to produce adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH).\(^{(9)}\) The simplest apparatus is found within prokaryotic cyanobacteria where the plasma membrane forms invaginations that contain the photosynthetic proteins (Figure 1.2B). Plants and algae utilize a more complex setup. The thylakoid membrane is stacked organelles within chloroplasts. The stacked regions of thylakoid membranes are called the grana and the regions that connect the grana are termed the lamellae (Figure 1.2A). Plants accumulate different photosynthetic proteins in each of these areas of the thylakoid membrane, whereas the thylakoid membranes of cyanobacteria are more homogeneous. However different these apparatuses may be the functions remain the same. Within the thylakoid membrane are four membrane-bound enzymes: photosystem II (PSII), cytochrome b\(_{6}\)f complex (b\(_{6}\)f), photosystem I (PSI), and ATP synthase.\(^{(10, 11)}\) PSII sequentially absorbs four quanta of light, which in turn oxidizes two molecules of water to yield four protons and one molecule of dioxygen on the lumenal side of the complex. On the stromal side of the complex, within the membrane, two molecules of plastoquinone (PQ) are reduced to two
Figure 1.1. Cartoon depiction of a thylakoid membrane containing the photosynthetic proteins. From left to right the membrane-bound proteins are PSII (aqua), b₆f (blue), PSI (green), and ATP synthase (orange). (12)

molecules of plastoquinol (PQH₂).(13, 14) These PQH₂ molecules diffuse to the b₆f complex where plastocyanin (PC) is reduced.(14) The b₆f complex translocates protons and electrons across the membrane. The b₆f complex oxidizes PSII, reduces PSI, and creates a proton gradient.(15) PC then diffuses to a photoexcited PSI where it reduces the special pair of chlorophyll molecules P₇₀₀⁺ to P₇₀₀.(13) Upon light excitation, PSI reduces ferredoxin (Fd), which in turn reduces ferredoxin:NADP⁺ reductase (FNR).(13) As a result, two NADPH are produced from two molecules of NADP⁺. NADPH is then utilized as an electron source in the Calvin cycle.(11) The proton gradient established by PSII and b₆f are subsequently used by ATP synthase to produce ATP from ADP and inorganic phosphate.(11, 13)
1.3 Photosystem II

At the core of photosynthetic processes is the extraction of electrons and protons from water to form molecular oxygen, which is carried out by PSII. PSII is comprised of over 20 subunits with pseudo-C$_2$ symmetry. The enzyme has 35 chlorophyll $a$ (chl $a$) molecules, 12 carotenoid molecules, 25 integral lipids, and 2-3 chloride ions per monomer (Figure 1.3). Catalysis begins within PSII when the primary chlorophyll (chl) donor is excited by light energy. Subsequent electron transfer oxidizes the special pair of chl molecules known as P$_{680}$ to P$_{680}^+$. Within nanoseconds, P$_{680}^+$ oxidizes the Tyr161-D1 (Y$_Z$), which then oxidizes the oxygen-evolving center (OEC).
1.4 Oxygen Evolving Complex

Within PSII, the OEC, a Mn$_4$CaO$_5$ cluster (Figure 1.4), stores oxidizing equivalents until two molecules of water are oxidized.(21, 22, 29, 30) Each oxidation step represents the transition of a Mn(III) to Mn(IV) molecule until the final oxidation, wherein a Mn(IV) is oxidized to a very short lived Mn(V) state. At the lowest oxidation state of the cluster it is assumed that the oxidation states are III, III, III, and IV for Mn1, Mn2, Mn3, and Mn4 respectively (Figure 1.4).(31, 32) It can easily be seen that there must be three oxidation steps to transition all Mn(III) ions to Mn(IV) and then one final oxidation to a transient Mn(V) oxidation state followed by catalysis. This four period oscillation is described by the Kok cycle where the OEC cycles through S states entitled $S_n$, where n is the number of oxidizing equivalents stored (Figure 1.5).(33, 34) The original experiment used to
determine the S states is shown in Figure 1.5A, where oxygen evolution was measured as a function of microsecond xenon lamp flashes. It was shown that the first three flashes yield oxygen, but that subsequently it required four flashes to evolve oxygen. \(^{(33, 35)}\)

This result led to the conclusion that the OEC rests in the \(S_1\) state. The S states have interesting temperature dependences. \(^{(36-38)}\) Specifically, the \(S_1\rightarrow S_2\) transition has a significantly lower temperature barrier at 135 K than the other transitions. The \(S_0\rightarrow S_1\), \(S_2\rightarrow S_3\), and the \(S_3\rightarrow S_0\) transitions occur at temperatures > 220-225 K, > 230 K, and > 235 K, respectively. \(^{(36, 38)}\) This temperature dependence is shown in Figure 1.5B where the half inhibition temperatures are shown in red for each transition.

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**Figure 1.4.** Putative structure of the catalytic inorganic \(\text{Mn}_4\text{CaO}_5\) cluster at 1.9 Å. Spheres are colored orange, yellow, red, and purple representing water, calcium, oxygen, and manganese respectively. \(^{(22)}\)
1.5 Tyrosine Z and Tyrosine D

Despite the near C$_2$ symmetry of the protein complex, there is only one OEC per PSII monomer. Complementary to the D1 subunit is the D2 subunit (shown in green and cyan respectively within Figure 1.2A), which contains few redox-active cofactors but shares many structural similarities to the D1 subunit. Similar to Y$_Z$ in the D1 subunit, the D2 subunit contains a redox-active tyrosine residue, Tyr160-D2 (Y$_D$), which is also oxidized by P$_{680}^+$. Y$_D$ is located further from the OEC and has a more stable neutral radical than Y$_Z$. Y$_D^\cdot$ decays on the order of minutes to hours, whereas Y$_Z^\cdot$ decays on the microseconds to milliseconds time scale. Both Y$_Z$ and Y$_D$ are in hydrogen bond distance to histidine residues, but the orientation differs between the two sites. Y$_Z$ has been shown as an integral component in the water oxidation process. Y$_D$ is important for the function of photosystem II because it has been implicated in the photoassembly of the OEC, and in the decay of the S$_2$ and S$_3$ states. Y$_Z$ and Y$_D$ are equidistant from P$_{680}^+$, and Y$_Z^\cdot$/Y$_Z$ ($\approx$ 930 mV) has a higher midpoint potential.
Figure 1.6. A comparison of the local hydrogen-bonding environment for \( Y_Z \) (A) and \( Y_D \) (B). (22) Amino acid residues predicted to hydrogen bond with \( Y_Z \) and \( Y_D \) are shown in green (A) and orange (B), respectively. Assigned, bound water molecules are shown in red (A and B), and the calcium ion is in green (A). An assigned water molecule, which is predicted to have partial occupancy, is shown in gray (B). Predicted hydrogen bonds are depicted with black dashed lines and with distances in Angstroms (The Pymol Molecular Graphics System, Version 1.3, Schrödinger, LLC).

Y\(^{-}\)D\(^{-}\) decay is \( \mathrm{pL} \) dependent and that the kinetic isotope effect (KIE) increases with pH. (47) A proton inventory was previously constructed for \( Y_D^{-} \) recombination showing that there are multiple protons involved in the mechanism. (48) \( Y_Z^{-} \) reduction has been shown to be pH independent in both the \( S_0 \) and \( S_2 \) states with a significant \( \mathrm{pL} \) independent KIE. (49)

1.6 Proton Coupled Electron Transfer Reactions

Both \( Y_Z \) and \( Y_D \) are involved in proton coupled electron transfer (PCET) reactions. PCET reactions are of great importance in chemistry and biology. (50, 51) Understanding the nature of these reactions is the key to efficient energy conversion processes. Here we define PCET reactions as the transfer of an electron and a proton regardless of the mechanism. Figure 1.7 demonstrates the possible paths for \( Y_Z^{-} \) decay. Three unique PCET mechanisms exist. The top of Figure 1.7 illustrates a proton first
Figure 1.7. Reaction diagram illustrating three possible mechanisms for the decay of the tyrosyl radical with two competing proton donors. (Top) Illustrates a PTET mechanism where a proton is transferred to create a tyrosyl radical cation followed by an electron transfer to form neutral tyrosine. (Bottom) Shows an ETPT mechanism where an electron is transferred to create a tyrosinate followed by a proton transfer to yield tyrosine. (Middle) Shows a CPET mechanism where an electron and a proton are transferred concertedly to skip high energy intermediates and form tyrosine.
pathway, where a proton is initially transferred, generating the cation radical $Y_Z\text{H}_1^+$ followed by an electron transfer to form $Y_Z$ (PTET). The bottom pathway illustrates an electron transfer that creates $Y_Z^-$, followed by a proton transfer to form $Y_Z$ (ETPT). The middle pathway illustrates the concerted transfer of a proton and an electron in one kinetic step (CPET).

The magnitude of the KIE can be instrumental in determining the type of PCET taking place within a system. For small (<1.3) KIEs the mechanism is expected to be an ETPT, a larger KIE (1.6-81) is exhibited by a CPET mechanism, and PTET will show only a small equilibrium isotope effect (~0.5 $\Delta pK_a$) in $D_2O$ with a pH dependent rate. (53-68)

1.7 Phenol and Tyrosine PCET

PCET reactions involving tyrosine are of great importance to many biological processes including water oxidation, (27) DNA biosynthesis, (69, 70) and oxygen activation. (71-73) For this reason many researchers have turned to biomimetic phenol and tyrosine model compounds to understand these complex processes. (3, 53, 67, 74-77) Most phenol and tyrosine model compounds exhibit CPET mechanisms. (52, 58, 78-81) Intermolecular and intramolecular hydrogen bonds have been shown to facilitate CPET reactions. (52, 58, 59, 76, 78-81) CPET reactions avoid the formation of high-energy intermediates but occur at the expense of higher reorganization energy. (52, 78-80) Hydrogen-bonding facilitates CPET reactions by decreasing the reorganization energy. (76)


1.8 Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance (EPR) spectroscopy is an invaluable tool for the study of photosynthesis, because the technique allows molecules with unpaired electrons to be studied directly. Every unpaired electron has a magnetic moment that will align itself either parallel or antiparallel to an externally applied magnetic field. The application of an external field causes degenerate electrons to split into distinct energy levels.

Equation 1.1 illustrates the resonance condition for an EPR experiment where $\Delta E$ is the energy difference, $h$ is plank’s constant, $v$ is the frequency, $g$ is the $g$-factor, $\beta_e$ is the Bohr magneton, and $B_0$ is the external magnetic field. The $g$-factor is determined by the type of magnetic center that is being studied and is affected by the spin orbit coupling parameters.

**Equation 1.1**

A free electron has a $g$-value of $g_e = 2.0023$, whereas a typical value for a chlorophyll-cation radical is $g = 2.0025$ and a quinone-anion radical is $g = 2.0046$. The difference is attributed to the quinone having more spin density at the oxygen than the chlorophyll radical. Spin orbit coupling parameters increase with the atomic mass of the nuclei. This effect makes the analysis of a $g$-factor extremely useful for determining the type of environment surrounding the electrons being studied. Figure 1.8 illustrates how degenerate electrons can be split into discrete energy levels and that these
discrete energy levels can further split by the magnetic field of surrounding nuclei to create hyperfine couplings represented by $A_0$.

![Energy diagram illustrating the hyperfine splitting for a degenerate electron within a magnetic field.](image)

**Figure 1.8. Energy diagram illustrating the hyperfine splitting for a degenerate electron within a magnetic field.**

In addition to the information that is obtained from the $g$-value, the lineshape that is formed from the hyperfine couplings of the radical species is also instrumental in determining the environment and the orientation of the radical species.\(^{(85-87)}\) The $g$-values for tyrosine radicals are very similar, but their lineshapes differ greatly.\(^{(85)}\) The orientation of the $\beta$-methylenic protons relative to the ring play a significant role in determining the lineshape.\(^{(85)}\) This orientation becomes unique to most tyrosyl radicals in various protein systems, because the protein constrains its movement, making EPR analysis of tyrosyl radicals an excellent probe of local structure.
Figure 1.9. Field swept EPR spectrum of Y$_Z$ in the S$_2$ state at 190 K and pH 6.5. Red arrow indicates the magnetic field monitored for transient spectra.

Conventional EPR is generally conducted by holding the frequency of the microwave constant while the magnetic field is scanned. This convention is more related to the traditional instrumentation than the theoretical limits of the experiment. When studying the decay kinetics of radicals, the resonant magnetic field can be locked and the radical can be monitored transiently. Figure 1.9 shows (A) field sweep EPR spectrum with a red arrow indicating the magnetic field used for (B) time-resolved EPR transient of S2Y$_Z$ decay. This technique can lead to the determination of the reaction rates for radicals of interest.

Figure 1.10. Diagram depicting the oxidation and reduction reactions of Y$_Z$. A two base model for Y$_Z$ PCET reactions in oxygen-evolving preparations of PSII.
1.9 Scope of Thesis

Within this thesis, we test the hypothesis that the oxidation of the OEC modulates the pKₐ of proton donors to YZ⁻. This hypothesis is tested with EPR spectroscopy in the available S states. We show that we are able to unambiguously monitor tyrosyl radicals within PSII at room temperature and 190 K. In Figure 1.10, we show the oxidation and reduction of YZ with hydrogen bond partners His190-D1 and calcium bound water. This work focuses on the reduction reaction highlighted in blue where an electron is transferred from P₆₈₀ to YZ⁻ while His190-D1 and calcium bound water compete for proton donation. We determine that the mechanism in both the S₀ and S₂ states is a CPET despite differences in the rate of recombination and KIE.

1.10 References


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CHAPTER 2
PROTON COUPLED ELECTRON TRANSFER AND REDOX-ACTIVE TYROSINE Z IN THE PHOTOSYNTHETIC OXYGEN-EVOLVING COMPLEX

by

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Abstract: Proton coupled electron transfer (PCET) reactions play an essential role in many enzymatic processes. In enzymatic PCET, redox-active tyrosines may be involved as intermediates when the oxidized phenolic side chain deprotonates. Photosystem II (PSII) is an excellent framework for studying PCET reactions, because it contains two redox active tyrosines, Y_D and Y_Z, with different roles in catalysis. One of the redox active tyrosines, Y_Z, is essential for oxygen evolution and is rapidly reduced by the manganese-catalytic site. In this report, we investigate the mechanism of Y_Z PCET in oxygen evolving PSII. To isolate Y_Z• reactions, but retain the manganese-calcium cluster, low temperatures were used to block the oxidation of the metal cluster, high microwave powers were used to saturate the Y_D• EPR signal, and Y_Z• decay kinetics were measured with EPR spectroscopy. Analysis of the pH and solvent isotope dependence was performed. The rate of Y_Z• decay exhibits a significant solvent isotope effect, and the rate of recombination and the solvent isotope effect are pH independent from pH 5.0 to 7.5. These results are consistent with a rate limiting, coupled proton electron transfer (CPET) reaction and are contrasted to results obtained for Y_D• decay kinetics. This effect
may be mediated by an extensive hydrogen bond network around Y\textsubscript{Z}. These experiments imply that PCET reactions distinguish the two PSII redox active tyrosines.

2.1 Introduction

In this article, EPR spectroscopy is used to investigate Y\textsubscript{Z} PCET reactions in PSII. PSII catalyzes the light driven oxidation of water at a Mn\textsubscript{4}CaO\textsubscript{5}-containing oxygen-evolving center (OEC). The transmembrane electron transfer pathway involves four chlorophylls (chl), two pheophytin, two plastoquinone, and two redox active tyrosines (Figure 1). Four flashes are required to produce oxygen from water. The OEC cycles among five S\textsubscript{n} states, where n refers to the number of oxidizing equivalents stored.\(^{(1)}\) PSII contains two redox active tyrosine residues, Y\textsubscript{D} and Y\textsubscript{Z} (Figure 1),\(^{(2, 3)}\) which are located with approximate C\textsubscript{2} symmetry in the reaction center.\(^{(4, 5)}\) These tyrosine residues are equidistant from the chlorophyll donor, P\textsubscript{680}, and are active in PCET reactions, but play different roles in catalysis. Y\textsubscript{Z}, Y161 of the D1 polypeptide, is essential for oxygen evolution.\(^{(6, 7)}\) Y\textsubscript{D}, Y160 of the D2 polypeptide, is not essential for catalysis, but may be involved in assembly of the OEC.\(^{(6, 8, 9)}\) There are other kinetic and energetic differences between the two tyrosines. For example, Y\textsubscript{D} forms a more stable radical and is easier to oxidize. As shown in Figure 1, the placement of the OEC and neighboring amino acid side chains distinguishes the two redox active tyrosines. In particular, the OEC calcium ion is located 5 Å from Y\textsubscript{Z}, but over 20 Å from Y\textsubscript{D} (Figure 2.1). An extensive set of hydrogen bonds, which link the Mn\textsubscript{4}-Ca site and Y\textsubscript{Z}, is predicted. Hydrogen-bonding distinguishes Y\textsubscript{Z} and Y\textsubscript{D}. 
Figure 2.1. (A) Crystal structure of the charge transfer cofactors within PSII at 1.9 Å. (B) Hydrogen-bonding environment surrounding YZ. (C) Hydrogen-bonding environment surrounding YD. Cyan dashed lines show the predicted hydrogen bonds calculated using the polar contacts function with an edge distance of 3.2 Å and a center distance of 3.6 Å using The Pymol Molecular Graphics System, Version 1.3, Schrödinger, LLC and the 1.9 Å structure (3arc). (5)

YZ mediates electron transfer between the primary chlorophyll donor, P$_{680}$, and the OEC.(10) Photoexcitation of PSII produces a chlorophyll cation radical, P$_{680}^+$, which oxidizes YZ on the nanosecond time scale.(10) If YD is available to act as an electron donor, i.e. after long dark adaptation, YD is also oxidized on the nanosecond time regime by P$_{680}^+$.(11) YZ$^-$ is reduced by the OEC with a microsecond-millisecond rate, which depends on S state.(12) The S$_1$ to S$_2$ transition has a barrier of 100 K, while S$_0$ to S$_1$, S$_2$ to S$_3$, and the S$_3$ to S$_0$ transitions occur at temperatures greater than 225 K.(13)
Figure 2.2. (A) Representative EPR spectrum of the S₂YZ· state at 190 K and pH 6.5. The spectrum was acquired under red-filtered illumination. The red arrow shows the magnetic field used to monitor kinetics. (B) Representative EPR transients reflecting the decay of YZ· at p¹H 6.5 (blue) and p²H 6.5 (red) at 190K.

Thus, at 190 K, the S₂ to S₃ transition cannot occur. Acceptor side quinone molecules, QA and QB, act as electron acceptors. At 190 K, PSII is limited to one charge separation; QB is not functional. EPR signals from the neutral radicals, YD· and YZ·, can be measured and distinguished by their decay kinetics and by their microwave power dependence in the presence of the OEC.

In many previous studies of YZ PCET, the OEC is removed or absent due to biochemical manipulation of PSII. Removal of the OEC slows YZ· reduction. However, removal of the OEC may influence YZ PCET reactions. By EPR spectroscopy, no change in YZ orientation was inferred from study of g tensor orientation. However, changes in the hydrogen-bonding environment around YZ (Figure 2.1) may occur due to removal of calcium and manganese.
In this work, $YZ^\cdot$ PCET was studied in the presence of the OEC. Oxygen-evolving PSII samples were prepared in the $S_2$ state by illumination at 190 K. A saturating 532 nm flash was then used to generate $S_2YZ^\cdot Q_A^-$. The $S_2$ state cannot act as an electron donor at this temperature, and therefore, $YZ^\cdot$ decays by recombination with $Q_A^-$. The rate of the $P^\cdot 680 Q_A^- Q_A^+$ reaction is on the microsecond time scale in plant PSII and is pH and solvent isotope insensitive (see refs (21, 22)). In the absence of the OEC, EPR and optical spectroscopy have reported $YZ$ and $Q_A^-$ recombination reactions with an overall time constant of 100-500 msec.(17, 23) In the presence of the OEC, EPR spectroscopy has also reported $YZ$ and $Q_A^-$ recombination on the millisecond time scale with rate constants of 9.5 (27%) and 0.8 (73%) s$^{-1}$ (pH 6.5, 190 K).(18)

### 2.2 Materials and Methods

PSII was isolated from market spinach.(24) The average oxygen evolution rate was 600 $\mu$mol O$_2$/mg chl-hr.(25) The samples were solvent exchanged by repeated centrifugation at 100,000 x g in a $^1$H$_2$O or $^2$H$_2$O (99% Cambridge Isotopes, Andover, MA) buffer containing 0.4 M sucrose, 15 mM NaCl, and 50 mM buffer at each of the following pH values: pH 5.0 (succinate), 5.5 (succinate), 6.0 (2-(N-morpholino)ethanesulfonic acid) (MES), 6.5 (MES), 7.0 (4-(2-hydroxyethyl)-1-piperazin-1-ethanesulfonic acid) (HEPES), 7.5 (HEPES). Experiments above pH 7.5 were not possible due to inactivation of the OEC and loss of oxygen evolution at these values. The final concentration was 2 mg/mL, and samples were stored at -70 °C. The pH is reported as the uncorrected meter reading, accounting for the solvent isotope dependence of the glass electrode and the compensating change in the pKa of acids and bases in $^2$H$_2$O.(26)
After a 15 min dark adaptation to trap the $S_1$ state, samples were homogenized with 500 µM potassium ferricyanide and transferred to the EPR cavity and allowed to equilibrate at 190 K. The 100 mM ferricyanide stock solution was made either in $^1$H$_2$O or $^2$H$_2$O on the same day as the experiment. The ferricyanide was present to oxidize the quinone acceptors after each flash. A field swept spectrum was recorded to select a field position. The 190 K samples were flashed with a 532 nm flash from a Continuum (Santa Clara, CA) Surelite III Nd:YAG laser. The laser intensity was 40 mJ/cm$^2$, and the beam was expanded with a cylindrical lens. The laser flash generated the $S_2Y_Z^•Q_A^-$ and the decay of the $Y_Z^•$ EPR signal was monitored. Transient data, associated with $S_2Y_Z^•Q_A^-$ decay, were averaged from 3-8 samples, with 15 transients recorded per sample. An offset, averaged from 10 s of data before the laser flash, was subtracted. Flash 1 to 15 yielded similar kinetics. The data from 15 flashes were fit with three exponentials and that fit was used to derive a $t_{1/2}$ value. This $t_{1/2}$ value was derived from multiple samples to give an average (mean) and a standard deviation. The EPR analysis was conducted on a Bruker (Billerica, MA) EMX spectrometer equipped with a Bruker ER 4102ST cavity and Bruker ER 4131VT temperature controller. EPR parameters were: magnetic field: 3375 G, microwave frequency: 9.46 GHz, power: 101 mW, modulation amplitude: 5 G, conversion: 20 ms, time constant: 164 ms. High microwave powers were used in order to saturate the $Y_D^•$ signal.(18)

Following collection of the transient EPR data, EPR spectra were measured by illuminating the sample within the EPR cavity with red filtered light (600 µmol photons/m$^2$-s, Dolan Jenner Industries, Boxborough, MA) at 190 K. To show that the expected $Y_Z^•$ hyperfine splitting is observed, the microwave power and modulation
amplitude were decreased compared to the conditions used for the EPR transients. Because the microwave power is low, this field swept spectrum also has a contribution from $Y_D^\bullet$. The spectra shown are the average of three samples with three spectra obtained for each sample. EPR parameters were: frequency: 9.46 GHz, power: 0.64 mW, and modulation amplitude: 2 G (Figure 2.2) or 5 G (Figure 2.4), conversion time: 41 ms, time constant: 164 ms, sweep time: 21 s.

**2.3 Results**

A representative field sweep spectrum of $Y_Z^\bullet$ in the $S_2$ state at 190 K is shown in Figure 2.2A. $Q_A^\bullet$ gives rise to a spin coupled signal with an acceptor side Fe$^{+2}$ ion and is not detectable under these conditions. The decay of $Y_Z^\bullet$ can be monitored by changes in EPR intensity at fixed magnetic field (Figure 2.2A, arrow). The use of high microwave power saturates the $Y_D^\bullet$ signal. As expected, the g value of the $Y_Z^\bullet$ radical is 2.004. The $Y_Z^\bullet$ spectrum and decay kinetics are similar to previous reports. For example, fits to the $p^{1}H$ 6.5 data in Figure 2.2B with a biexponential function gave $t_{1/2}$ values of 12.2 s (40%) and 1.2 s (60%), in agreement with the results of ref (18).

In Figure 2.3B, the $Y_Z^\bullet$ decay kinetics were monitored, the data were fit, and the overall halftime as a function of $p^{1}H$ was plotted (black line). The halftimes for $Y_D^\bullet$ decay were derived from published rate constants for the majority phase, which are plotted in Figure 2.3A. While the rate of $Y_D^\bullet$ decay accelerates at low $p^{1}H$ (Figure 2.3A), $YZ^\bullet$ decay did not show significant pH dependence (Figure 2.3B, black line). Over this pH range, the steady state rate of oxygen evolution was constant, and the content of extrinsic polypeptides, which interact with the OEC, was similar (Figure A.4). Higher pL
values led to activity loss and depletion of the extrinsic polypeptides (Figure A.4 and Figure A.5).

The solvent isotope effect was calculated from the data in Figure 2.3B. Figure 2.3C shows the $Y_Z$ solvent isotope effect as a function of pL. A significant solvent isotope effect was observed from pL 5 to 7.5 with an average value of ~2. The overall magnitude is consistent with a pH independent, kinetic isotope effect (KIE) on $Y_Z$ recombination with $Q_A^-$. The observation of a significant solvent isotope effect at all pL values implies that the $Y_Z$ pocket is exposed and readily accessible to water, consistent with recent X-ray crystal structures.(4, 5) Thus, the lack of pH dependence (Figure 2.3C) cannot be attributed to lack of accessibility. By contrast, the decay of $Y_D^-$ showed a ~10 fold increase in rate at low pH (Figure 2.3A) and a pH dependent isotope effect, with a maximum value of ~2 at high pL.(26) A proton inventory at high pH was consistent with multiple proton transfer pathways to $Y_D^-$, with histidine and water molecules suggested to be the proton donors on two different pathways.(29)

2.4 Discussion

Phenol and tyrosine model compounds have been used to determine the mechanism of PCET reactions. PCET may occur through a concerted pathway, in which the electron and proton are transferred in one kinetic step (CPET). Alternatively, either the electron or proton may be transferred first (ETPT or PTET, respectively). In tyrosine model compounds, a CPET mechanism has been inferred in some studies.(30-33) The magnitude of the KIE and the pH dependence can be used as a first approach to distinguish these mechanisms.
Figure 2.3. (A) pH dependence of $Y_D^\cdot$ decay, as assessed by EPR spectroscopy, attributed to $Y_D^\cdot Q_A^\cdot$ recombination, and derived from ref.(26) The halftime was derived from the rate constant for the majority phase, corresponding to >85% of the amplitude.  (B) pH dependence of $Y_Z^\cdot$ recombination in the $S_2$ state at 190 K. Data were acquired either in $^1H_2O$ (black) or $^2H_2O$ (red) buffers.  (C) pH dependence of the solvent isotope effect for $Y_Z^\cdot$ recombination, derived from the data in (B).

For $Y_D^\cdot$ reduction, the pH dependence and the kinetic isotope effect were used to argue that the mechanism is PTET at low pH and CPET at high pH.(29) Unlike $Y_D^\cdot$, $Y_Z^\cdot$ recombination does not show a stimulated rate at low pH values. The lack of pH dependence and the isotope effect are consistent with a CPET mechanism for $Y_Z^\cdot$
reduction throughout this pH range. Our data suggest that PCET mechanism distinguishes $Y_D$ and $Y_Z$.

The experiments presented here focus on the mechanism of $Y_Z^*$ reduction. Previous work has concerned the mechanism of $Y_Z$ oxidation. For OEC depleted preparations, optical studies of $P_{680}^+$ reduction were used to argue a CPET mechanism at low pH and a pure ET or a PTET mechanism at high pH.\(^{30, 34}\) A pKa of ~7.5 was derived from the optical data and attributed to titration of a base near $Y_Z$.\(^{34}\) Removal of the OEC may influence PCET reactions. For OEC containing preparations, optical studies of $P_{680}^+$ reduction have shown no significant solvent isotope effect on nanosecond components.\(^{35-37}\) A significant equilibrium isotope effect\(^{38}\) may explain a difference in KIE on the two reactions. Low fractionation factors can occur if $Y_Z$ is involved in a strong hydrogen bond, in which case deuterium will prefer the solvent.\(^{39}\)

Model compound studies have shown that intermolecular and intramolecular hydrogen bonds facilitate CPET reactions.\(^{30-33, 40}\) These reactions avoid the formation of high-energy intermediates, but occur at the expense of high reorganization energies.\(^{30-33}\) Hydrogen-bonding facilitates reactions by decreasing the reorganization energy (for example, see \(^{41}\) and references therein). We propose that the extensive hydrogen-bonding network around $Y_Z^*$ and the calcium ion facilitates CPET at low pH. This hydrogen-bonding network is shown in the 1.9 Å structure of PSII.\(^5\) In this structure, His190 is located 2.5 Å from $Y_Z$ and several water molecules, bound to calcium, are modeled with one at 2.6 Å (Figure 2.1). The observation that recombination of $Y_Z$ is not accelerated at low pH is of significance with regard to PSII activity, which must be maintained under the acidic conditions of the thylakoid lumen.
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2.5 References


CHAPTER 3

REDOX CONTROL AND HYDROGEN-BONDING NETWORKS:
PROTON COUPLED ELECTRON TRANSFER REACTIONS AND
TYROSINE Z IN THE PHOTOSYNTHETIC OXYGEN-EVOLVING
COMPLEX

by

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Abstract: In photosynthetic oxygen evolution, redox active tyrosine Z (YZ) plays an essential role in proton coupled electron transfer (PCET) reactions. Four sequential photooxidation reactions are necessary to produce oxygen at a Mn₄CaO₅ cluster. The sequentially oxidized states of this oxygen-evolving cluster (OEC) are called the Sₙ states, where n refers to the number of oxidizing equivalents stored. The neutral radical, YZ•, is generated and then acts as an electron transfer intermediate during each S state transition. In the X-ray structure, YZ, Tyr 161 of the D1 subunit, is involved in an extensive hydrogen-bonding network, which includes calcium-bound water. Oxidation of the Mn₄CaO₅ cluster may activate these calcium-bound water molecules and alter YZ PCET through changes in the hydrogen-bonding network. We compared the S₀ and S₂ states, which differ in Mn oxidation state, and found a significant difference in the YZ• decay rate (t ½ = 3.3 ± 0.3 s in S₀; t ½ = 2.1 ± 0.3 s in S₂) and in the kinetic isotope effect (KIE) on the reaction (1.3 ± 0.3 in S₀; 2.1 ± 0.3 in S₂). Although the YZ site is known to be solvent accessible, the recombination rate and KIE were pH independent in both S states. To define the origin of these effects, we measured the YZ• recombination rate in the presence of ammonia, which inhibits oxygen evolution and disrupts the hydrogen
bond network. We report that ammonia dramatically slows the $Y_Z^*$ recombination rate in the $S_2$ state, but has a smaller effect in the $S_0$ state. In contrast, ammonia had no significant effect on $Y_D^*$, the stable tyrosyl radical in PSII. Therefore, the alterations in $Y_Z^*$ decay, observed with $S$ state advancement, are attributed to alterations in OEC hydrogen-bonding and consequent differences in the $YZ$ midpoint potential/$pK_a$. These changes may be caused by activation of metal bound water molecules, which hydrogen bond to $YZ$. These observations document the importance of redox control in proton coupled electron transfer react

3.2 Introduction

Photosynthetic oxygen evolution occurs at a $\text{Mn}_4\text{CaO}_5$ cluster in the thylakoid membrane associated reaction center, photosystem II.(1) Each reaction center contains the transmembrane subunits D1, D2, CP43, and CP47, which bind the redox-active cofactors and antenna pigments, chlorophyll (chl) and carotenoids (Figure 3.1A). A photoinduced charge separation is generated between the dimeric chl donor, $P_{680}$, and a bound plastoquinone, $Q_A$. $Q_A^-$ reduces a second quinone, $Q_B$, and $P_{680}^+$ oxidizes a tyrosine residue, $Y_Z$, Y161-D1
Figure 3.1. (A) Redox active cofactors involved in the charge recombination of Q\textsubscript{A} with Y\textsubscript{Z}\textsuperscript{•}. The phytanyl chains of the cofactors have been removed for clarity.\(^{(1)}\) (B) Local environment of Y\textsubscript{Z} with predicted hydrogen bonds shown as black dashes and distance shown in Angstroms (The Pymol Molecular Graphics System, Version 1.3, Schrödinger, LLC). (C) S state cycle showing the reported half inhibition temperatures\(^{(2)}\) in red, and the number of laser flashes required to achieve that state in green.

...polypeptide. The radical, Y\textsubscript{Z}\textsuperscript{•}, is a powerful oxidant.\(^{(3, 4)}\) Under physiological conditions, Y\textsubscript{Z}\textsuperscript{•} oxidizes the Mn\textsubscript{4}CaO\textsubscript{5} cluster, where water oxidation occurs (Figure 3.1B).\(^{(1)}\) Four sequential photooxidations lead to the release of molecular oxygen, and the yield of O\textsubscript{2} fluctuates with period four.\(^{(5)}\) The sequentially oxidized states of the Mn\textsubscript{4}CaO\textsubscript{5} cluster are the S\textsubscript{n} states, where \(n\) refers the number of oxidizing equivalents stored (Figure 3.1C). Y\textsubscript{Z} acts as an electron transfer intermediate between P\textsubscript{680}\textsuperscript{+} and the Mn\textsubscript{4}CaO\textsubscript{5} cluster on each S state transition and is required for oxygen evolution.\(^{(6)}\) A single short laser flash advances the OEC from one S state to the next. For example, a short flash given to the so-called S\textsubscript{1} state (generated by dark adaptation), generates the S\textsubscript{2} state. This S\textsubscript{1} to S\textsubscript{2} transition corresponds to the oxidation of a Mn(III) to Mn(IV).\(^{(7)}\)
During the S state cycle, water oxidation occurs on the microsecond to millisecond time scale, oxygen is produced during the S₃ to S₀ transition.\(^{(5, 8)}\)

Tyrosyl radicals play an important role in proton coupled electron transfer (PCET) in many biological systems.\(^{(9)}\) The tyrosine side chain can participate in PCET reactions when the pH is below the pKa of the phenolic oxygen (\(\sim\)10). Oxidation of tyrosine under these conditions leads to production of a neutral tyrosyl radical, due to the dramatic decrease in pKₐ of the phenolic oxygen in the radical state.\(^{(10)}\) Results from PSII, RNR, and model systems indicate that solvent accessibility and hydrogen-bonding can control the lifetime and midpoint potential of tyrosyl radicals.\(^{(11-15)}\) These studies also suggest that conformational changes of the redox active tyrosine are coupled with the oxidation reactions.\(^{(16, 17)}\)

In PSII, YZ plays a critical role in the water splitting reactions. Mutations at this tyrosine, Y161-D1, inactivate water oxidation.\(^{(18, 19)}\) In the absence of Yₗ, QA⁻ decays by recombination with P₆₈₀\(^{+}\) on the microsecond time scale.\(^{(19)}\) Interestingly, PSII contains a second tyrosyl radical, Y\(₉\)⁺ (Y160-D2), which has different redox chemistry, when compared to YZ in the D1 subunit.\(^{(20, 21)}\) YD forms a much more stable radical (minutes-hours)\(^{(22)}\) and has a lower midpoint potential, compared to YZ.\(^{(23)}\) While YD does not play a direct role in oxygen evolution, this redox-active tyrosine may play a role in assembly of the Mn₄CaO₅ cluster.\(^{(24)}\) The 1.9 Å structure suggests that hydrogen-bonding distinguishes the YD and YZ pockets,\(^{(1)}\) and our previous EPR study concluded that PCET distinguishes the two redox active tyrosines.\(^{(14)}\)

In the X-ray structure of PSII, YZ is 5 Å from the Mn₄CaO₅ cluster and is predicted to participate in a hydrogen bond network, which includes two water molecules
and the side chain of His190 in the D1 subunit (Figure 3.1B).(1) This extensive hydrogen bond network may play a role in catalysis.(1, 14, 25) One of the hydrogen bonded water molecules is directly bound to calcium while the other is in the second coordination sphere of calcium (Figure 3.1B).(1) Changes in OEC oxidation state are likely to alter the acidity of these metal bound water molecules. This change in acidity can then modulate the strength of the hydrogen-bonding network to $Y_Z$, providing a control mechanism over the pK$_a$ and midpoint potential of $Y_Z$. This is potentially important question, because $Y_Z$ must deprotonate and reprotonate on each S state transition in order to have the high midpoint potential that is necessary to oxidize water (see discussion in ref (26)).

In this study, we use electron paramagnetic resonance EPR spectroscopy to investigate the effect of OEC oxidation on $Y_Z^\cdot$ PCET reactions. The term, PCET, is used to refer to any reaction that involves transfer of a proton and an electron, regardless of the detailed mechanism.(27) The mechanism of PCET reactions will be sensitive to changes in distance, angle and strength of hydrogen bonds to $Y_Z/ Y_Z^\cdot$. In principle, EPR is a sensitive method with which to measure the kinetics of light induced paramagnetic species, such as $Y_Z^\cdot$. However, in the presence of the OEC, $Y_Z^\cdot$ is reduced rapidly by the Mn$_4$CaO$_5$ cluster; this fast decay rate complicates its facile detection by EPR spectroscopy. Another complication is that $Y_D^\cdot$ and $Y_Z^\cdot$ give rise to overlapping g=2.0 signals with similar hyperfine splittings, with $Y_D$ forming the more stable radical.

To monitor $Y_Z^\cdot$ specifically with EPR spectroscopy, we used the approaches described previously, in which high microwave powers and low temperatures were employed.(14, 26) Normally, $Y_Z^\cdot$ is reduced on the microsecond to millisecond time
scale by the OEC.(8) The use of low temperature blocks this OEC to YZ reaction (Figure 3.1C), leaving YZ as the terminal electron donor, which can be oxidized by single flash and will decay on the seconds time scale by recombination with Q\textsubscript{A}⁻.(14, 26) The use of high microwave powers saturates the Y\textsubscript{D}⁺ EPR signal and allows Y\textsubscript{Z}⁺ kinetics to be monitored independently of Y\textsubscript{D}⁺, which is more distant (20 Å) from the Mn\textsubscript{4}CaO\textsubscript{5} cluster.(1)

To investigate the dependence of YZ PCET on S state, flash-freeze quench techniques were used to prepare the S\textsubscript{0} state, attributed to (Mn(III))\textsubscript{3}(MnIV)\textsubscript{1}, or the S\textsubscript{2} state, attributed to Mn(III)\textsubscript{1}(Mn(IV)\textsubscript{3}.(28-30) At room temperature, saturating flashes are given to PSII samples, dark adapted in the S\textsubscript{1} state, to generate either the S\textsubscript{0}Y\textsubscript{Z}Q\textsubscript{A} (three flashes) or the S\textsubscript{2}Y\textsubscript{Z}Q\textsubscript{A} state (Figure 3.1C). The samples are then flash frozen to 190 K. At this temperature, the S\textsubscript{0} to S\textsubscript{1}, S\textsubscript{2} to S\textsubscript{3}, and Q\textsubscript{A}⁻ to Q\textsubscript{B} transitions are blocked (Figure 3.1C).(2, 14, 26, 31) After a saturating flash to these samples at 190 K, Y\textsubscript{Z}⁺ is generated and then decays on the seconds time scale by recombination with Q\textsubscript{A}⁻, which is a single electron donor and is not protonated (reviewed in ref (32)). Y\textsubscript{Z}⁺ reduction occurs by a PCET reaction, and the mechanism of the reaction can be interrogated by pH and solvent isotope alterations. This approach has the advantage of preserving the structural interactions between the OEC and Y\textsubscript{Z}, while allowing effects on the rate of Y\textsubscript{Z}⁺Q\textsubscript{A}⁻ recombination to be assessed by EPR spectroscopy in a facile manner.

Here, the dependence of the Y\textsubscript{Z}⁺Q\textsubscript{A}⁻ recombination rate on solvent isotope and pL in two different OEC oxidation states, S\textsubscript{0} and S\textsubscript{2}, is reported. These two states were chosen because their half inhibition temperatures are similar, with a high activation barrier. In addition, ammonia, a substrate analog and PSII inhibitor(25, 33-35) was used
as a probe of YZ hydrogen-bonding interactions with the OEC. Through vibrational spectroscopy, it has been shown recently that ammonia alters the OEC hydrogen-bonding network during the S1 to S2 transition.(25) The results presented here are consistent with a change in YZ PCET mechanism when the S0 and the S2 states are compared. Also, we report a dramatic, S state dependent effect of ammonia on YZ•Q/A- recombination, which is consistent with alterations in YZ•/water hydrogen-bonding.

3.3 Materials and Methods

**PSII Samples.** PSII was isolated from market spinach.(36) The average oxygen evolution rate was 800 μmol O2/mg chl-hr.(37) The samples were solvent exchanged by repeated centrifugation at 100,000 x g in a 1H2O or 2H2O (99% Cambridge Isotopes, Andover, MA) buffer containing 0.4 M sucrose, 15 mM NaCl, and 50 mM buffer at each of the following pH values: pH 5.0 (succinate), 5.5 (succinate), 6.0 (2-(N-morpholino)ethanesulfonylic acid, (MES), 6.5 (MES), 7.0 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, (HEPES), 7.5 (HEPES). Experiments above pH 7.5 were not possible due to inactivation of the OEC and loss of oxygen evolution at these values. The final concentrations were 4 and 2 mg/mL, respectively, for the S2YZ and S0YZ samples, and samples were stored at -70 °C. The pH is reported as the uncorrected meter reading, accounting for the solvent isotope dependence of the glass electrode and the compensating change in the pKa of acids and bases in 2H2O.(38)

**YZ• Experiments.** After a 15 min dark adaptation to trap the S1 state, samples were homogenized with 500 μM potassium ferricyanide and flashed either one or three times at 1 Hz with a 532 nm flash from a Continuum (Santa Clara, CA) Surelite III Nd:YAG laser at room temperature. The laser intensity was 40 mJ/cm², and the beam
was expanded with a cylindrical lens. The 100 mM ferricyanide stock solution was made either in $^1$H$_2$O or $^2$H$_2$O on the same day as the experiment. The ferricyanide was present to oxidize the quinone acceptors after each flash. Samples were immediately submersed in liquid N$_2$ and transferred into the EPR cavity at 190 K. The samples generated with one flash correspond to a dark state of S$_2$Y$_Z$Q$_A$. The samples generated with three flashes correspond to a dark state of S$_0$Y$_Z$Q$_A$. In some experiments, the dark state, S$_2$Y$_Z$Q$_A$ was generated by 190 K illumination.(14) A field swept spectrum was recorded to select a field position. The 190 K samples were flashed with a 532 nm pulse, generating either S$_0$Y$_Z$Q$_A^-$ or S$_2$Y$_Z$Q$_A^-$ and the decay of the Y$_Z^-$ EPR signal was monitored. Transient data, associated with S$_2$Y$_Z$Q$_A^-$ decay, were averaged from 3-8 samples, with 15 transients recorded per sample. Transient data, associated with S$_0$Y$_Z$Q$_A^-$ decay, were averaged from 3-10 samples, with 15 transients recorded per sample. An offset, averaged from 10 s of data before the flash, was subtracted. Flash 1 to 15 yielded similar kinetics. The data from 15 flashes were fit with three exponentials and that fit was used to derive a t$_{1/2}$ value. This t$_{1/2}$ value was derived from multiple samples to give an average (mean) and a standard deviation. The EPR analysis was conducted on a Bruker (Billerica, MA) EMX spectrometer equipped with a Bruker ER 4102ST cavity and Bruker ER 4131VT temperature controller. EPR parameters were: magnetic field: 3375 G, microwave frequency: 9.46 GHz, power: 101 mW, modulation amplitude: 5 G, conversion: 20 ms, time constant: 164 ms. High microwave powers were used in order to saturate the Y$_D^-$ signal.(14, 26)

To estimate the yield of the S$_2$ state, two different methods of preparation were compared. In the first approach, one flash was used to generate the S$_2$ state from a dark-
adapted sample (in S1). In the second approach, the dark-adapted sample was continuously illuminated with red light at 190 K. The second procedure is expected to generate a ~100% yield of S2.(31) The kinetic data obtained were similar. Thus, the percentage of centers in the S2 state is assumed to 100% using either illumination protocol. Data from the two methods were therefore averaged. This prediction concerning the flash induced yield of the S2 state agrees with a recent report, which was consistent with 0% misses (100% yield) on the first flash.(39)

Three flashes were used to generate the S0 state from a dark-adapted sample in the S1 state (Figure 3.1C). The percentage of the S0 state can be estimated by assuming 0% misses on the first flash (see above) and 14% misses on the second and third flash.(5) The expected distribution after three flashes is 0% S1, 2% S2, 24% S3, and 74% S0. Thus, the state generated with three flashes is primarily S0, but has a small contribution from S3 and S2. The S0 and S2 states were chosen for comparison because the barrier heights for the S0 to S1 and S2 to S3 transitions were predicted to be similar (Figure 1C).(2)

The S0 and S2 states were chosen for comparison because the barrier heights for the S0 to S1 and S2 to S3 transitions were predicted to be similar (Figure 3.1C). We were not able to measure YZ′QA− recombination rates in the S1 and S3 states at 190 K because the activation barriers were too low (but see ref (2) for a previous report concerning the S3 state).

As an additional control, alkaline Tris treatment was used to remove the OEC. (40) The procedure incubated the sample at 1.5 mg/mL in Tris buffer (0.8 M Tris, 2 mM EDTA, pH 8.0) for 30 minutes under room light in a shaker. After the incubation, the sample is pelleted by centrifugation. The sample is then resuspended in SH buffer (400
mM sucrose, 50 mM HEPES, pH 8.0), homogenized and pelleted by centrifugation. Following three resuspensions in SH buffer, the sample is resuspended in SH-Cl buffer (400 mM sucrose, 15 mM NaCl, 50 mM HEPES, pH 7.5). EPR spectra were acquired under the same conditions as the oxygen evolving samples.

**Y$_D^*$ Experiments.** Samples were dark adapted up to 24 hours in order to trap the S$_1$ state and generate a dark state in which Y$_D^*$ had decayed. After this 24 hour dark adaptation, samples were still active and evolved oxygen at a rate of 700 μmol O$_2$/mg chl-hr. After dark adaptation, samples were homogenized with 500 μM ferricyanide. A control sample was compared to samples in which either 200 mM NaCl or 200 mM NH$_4$Cl was added. In some experiments, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added from a 1 mM stock solution in ethanol. In these cases, the final concentration of ethanol was 1%. Samples were transferred to a Wilmad Lab Glass (Buena, NJ) WG-808-S-Q flat cell. Experiments were performed at 300 ± 1 K following a single laser flash. Experiments at lower temperatures were not possible due to the extremely long relaxation time of Y$_D^*$. The data were fit with a monoexponential function to derive a t$_{1/2}$. The laser intensity was 40 mJ/cm$^2$, and the beam was expanded with a cylindrical lens. EPR parameters: magnetic field: 3496 G, microwave frequency: 9.85 GHz, power: 2 mW, modulation amplitude: 5 G, conversion: 5.24 s, time constant: 2.62 s, sweep time: 21,400 s.

**Field-swept EPR spectra.** Following collection of the transient EPR data, EPR spectra were measured by illuminating the sample within the EPR cavity with red filtered light (600 μmol photons/m$^2$-s, Dolan Jenner Industries, Boxborough, MA) at 190 K. To show that the expected Y$_Z^*$ hyperfine splitting is observed, the microwave power and
modulation amplitude were decreased compared to the conditions used for the EPR transients. Because the microwave power is low, this field swept spectrum also has a contribution from \( Y_D^\cdot \). The spectra shown are the average of three samples with three spectra obtained for each sample. EPR parameters were: frequency: 9.46 GHz, power: 0.64 mW, and modulation amplitude: 2 G (Figure 3.2) or 5 G (Figure 3.4), conversion time: 41 ms, time constant: 164 ms, sweep time: 21 s.

### 3.4 Results

In Figure 3.2, we present a comparison of the light induced X-band EPR spectra, as detected in the \( S_2 \) state (A) and in the \( S_0 \) state (B). The data in Figures 3.2A and B were recorded at 190 K under illumination at low microwave power and reflect overlapping \( Y_Z^\cdot \) and \( Y_D^\cdot \) contributions. These free radical signals, measured in the \( S_0 \) and \( S_2 \) states, have similar partially resolved hyperfine splittings, a \( \sim 20 \) G overall linewidth, and the expected \( g \)-value of 2.004.(41, 42) A narrow 7-10 G \( g=2 \) signal, which overlaps the \( Y_D^\cdot / Y_Z^\cdot \) signals is attributed to low yield chlorophyll and carotenoid radicals. These species are known to be generated by continuous illumination at low temperatures.(31) Under these conditions, \( Q_A^- \) exhibits a spin-coupled signal with the \( Fe^{2+} \) ion and is not detected.(43)

In Figures 3.2A and B, the red arrow indicates the magnetic field used to obtain the kinetic data. This position allows for unambiguous monitoring of time dependent changes in the \( Y_Z^\cdot \) EPR signal without contribution from narrow chlorophyll and carotenoid radicals. The use of high microwave power for transient data saturates the \( Y_D^\cdot \) signal, which decays on a much longer time scale, compared to \( Y_Z^\cdot \).(26) The kinetic transients in Figures 3.2C and D result from \( Y_Z^\cdot \) recombination with \( Q_A^- \) in \(^1\text{H}_2\text{O} \) and
$^{2}\text{H}_2\text{O}$ buffer at pH 6.5 (black and red respectively). These transients were normalized to their initial amplitude for presentation purposes. As observed, the rate of $Y_Z^\cdot Q_A^-$ recombination slows in $^2\text{H}_2\text{O}$ both in the $S_2$ (Figure 3.2C, red) and the $S_0$ (Figure 3.2D, red) states. Because $Q_A^-$ is not protonated, a significant solvent isotope effect is consistent with a PCET mechanism for $Y_Z^\cdot$ decay in both $S$ states.

![Graphs showing EPR spectra and decay transients for $S_2Y_Z^\cdot$ and $S_0Y_Z^\cdot$.](image)

**Figure 3.2.** Comparison of representative EPR spectra (A and B) and averaged decay transients (C and D) derived for $S_2Y_Z^\cdot$ (A and C) and $S_0Y_Z^\cdot$ (B and D). Spectra were recorded under illumination at 190 K, pH 6.5, and yield a $g$-value of 2.004. The red arrows in A and B indicate the magnetic field position, which were used to monitor the kinetics in C and D. The transients in C and D were acquired either in $^1\text{H}_2\text{O}$ (black) or $^2\text{H}_2\text{O}$ (red), respectively. The transients were normalized for presentation purposes. Triexponential fits to the data and residuals are shown with in red and black.

The data were fit with three exponentials, and the fits were used to derive a $t_{1/2}$ value as a function of pH in $S_2$ and $S_0$ (Figure 3.3A and B, $^1\text{H}_2\text{O}$-black and $^2\text{H}_2\text{O}$-red).
Averaged over pL (where L refers to the lyonium ion), the rates of $Y_Z^+Q^-\cdot$ recombination were slightly different in the $S_0$ and the $S_2$ states, with $t_{1/2}$ values of $3.3 \pm 0.7$ s and $2.1 \pm 0.3$ s in $^1$H$_2$O buffer. The average $t_{1/2}$ value measured in the $S_2$ state is in agreement with a previous report.(14) As shown in Figure 3.3A and B, there was no significant pH dependence in either S state.

The $t_{1/2}$ values in $^1$H$_2$O and $^2$H$_2$O were divided to give a solvent or kinetic isotope effect (KIE). In Figures 3.3C and D, the KIE was plotted as a function of pL in the $S_2$ and $S_0$ states, respectively. The average KIE was higher in the $S_2$ state, when compared to the $S_0$ state ($S_0$, $1.3 \pm 0.3$ and $S_2$, $2.1 \pm 0.3$). The KIE was pL independent (from pL 5.0 to

![Figure 3.3](image-url)

Figure 3.3. pL and solvent isotope dependence of $Y_Z^+$ decay at 190 K, as assessed by the half-time for the overall reaction. (A) pL dependence in the $S_2$ state. The black line shows data acquired in $^1$H$_2$O and the red line shows data acquired in $^2$H$_2$O. (B) pL dependence in the $S_0$ state. The black line shows data acquired in $^1$H$_2$O, and the red line shows data acquired in $^2$H$_2$O. (C) pL dependence of the kinetic isotope effect (KIE) in the $S_2$ state. (D) pL dependence of the kinetic isotope effect (KIE) in
the $S_0$ state. The KIE values were derived by dividing the $^2\text{H}_2\text{O} \ t_{1/2}$ values by the $^1\text{H}_2\text{O} \ t_{1/2}$ values.

7.5) in both S states. The lack of pH dependence for the KIE was reported previously for the $S_2$ state, generated by continuous illumination.(14) In the PSII X-ray structure, the $Y_Z$ pocket is exposed and readily accessible to water.(1) Thus, the lack of pL dependence cannot be attributed to lack of solvent accessibility.

In these studies, we report the effect of pL and deuterium isotope on the overall halftime of the $Y_Z'Q_A^-$ reaction, as a measure of $k_{\text{obs}}$. The individual rate constants and amplitudes derived from the three exponential fits are summarized in the Appendix B. The use of the overall $t_{1/2}$ as a measure of the reaction rate has the advantage that it is independent of assumed mechanism and that this value can be derived from the triexponential fits to the data with high precision. As an alternative, the pL and isotope dependence of the individual rate constants and amplitudes were also evaluated (Appendix B, Figures. B.1-4). Given the standard deviations, no significant pL dependence was observed either in the derived rate constants or amplitudes using this approach (Appendix B, Tables and Figures), consistent with our analysis of the $t_{1/2}$ values. The effect of $^2\text{H}_2\text{O}$ was difficult to evaluate because of the decrease in precision using this alternative approach.

To test the effect of changes in OEC hydrogen-bonding, PSII was treated with either 200 mM NH$_4$Cl or 200 mM NaCl (as a control for the ionic strength and chloride concentration change) at pH 7.5. Ammonia is known to bind to the OEC in the $S_2$ state.(25, 35) In Figure 3.4A, representative EPR spectra, recorded under illumination, are shown for samples prepared in the $S_2$ or $S_0$ state. These spectra yield the expected signal with a g-value of 2.004 and an overall linewidth of 20 G. Small changes in
lineshape are attributed to changes in the yield of narrow signals from chlorophyll or carotenoid radicals, generated under continuous illumination.

Figure 3.4. EPR spectra and transients, showing the effect of ammonia on $Y_Z^\cdot$ decay at 190 K and pH 7.5. (A) Representative EPR spectra for $S_0Y_Z^\cdot$ (black), $S_2Y_Z^\cdot$ (red), $S_0Y_Z^\cdot + 200$ mM NaCl (blue), $S_2Y_Z^\cdot + 200$ mM NaCl (green), $S_0Y_Z^\cdot + 200$ mM NH$_4$Cl (orange), and $S_2Y_Z^\cdot + 200$ mM NH$_4$Cl (purple). Spectra in (A) were recorded under illumination and yield a $g$-value of 2.004. The red arrow in (A) indicates the magnetic field position, used to monitor the kinetics in (B). The transients in (B)
reflect $S_0Y_Z^\cdot$ (black), $S_2YZ^\cdot$ (red), $S_0Y_Z^\cdot + 200$ mM NaCl (blue), $S_2Y_Z^\cdot + 200$ mM NaCl (green), $S_0Y_Z^\cdot + 200$ mM NH$_4$Cl (orange), and $S_2Y_Z^\cdot + 200$ mM NH$_4$Cl (purple). The transients were baseline corrected and normalized for presentation purposes.

In Figure 3.4B, the decay of the $Y_Z^\cdot Q_A^-$ state was monitored at constant field, as described above, either in 200 mM NaCl (green and blue) or in 200 mM NH$_4$Cl (orange and purple). In both the $S_2$ (purple) and $S_0$ (orange) states, the addition of ammonia resulted in a significant decrease in the rate of decay. There was no detectable change induced by the addition of 200 mM NaCl (compare black and red with blue and green, Figure 3.4). The $t_{1/2}$ values were derived from the transient data (Figure 3.4B). The values in the $S_2$ state were $1.4 \pm 0.8$ s in the control 200 mM NaCl sample and $15.3 \pm 4.4$ s in the 200 mM NH$_4$Cl sample. The values in the $S_0$ state were $1.9 \pm 1.4$ s in the control 200 mM NaCl sample and $8.2 \pm 1.9$ s in the 200 mM NH$_4$Cl sample. As controls for irreversible inactivation of the OEC by ammonia, we examined the rate of radical decay in Tris-washed PSII(40), in which the Mn$_4$CaO$_5$ cluster is purposely removed. In this preparation, radical decay was much faster ($t_{1/2} = 0.7$ s) and was on the time scale consistent with oxygen evolving samples (Figure B.6). We conclude that the ammonia-induced slowing of $Y_Z^\cdot$ decay is not due to irreversible removal of the Mn$_4$CaO$_5$ cluster.

As shown in Figure 4, ammonia decreased the rate of decay of the $S_2Y_Z^\cdot Q_A^-$ state by a factor of $\sim 11$, and caused a $\sim 4$ fold decrease in the rate of $S_0Y_Z^\cdot Q_A^-$ decay. This value for the $S_0Y_Z^\cdot Q_A^-$ is an upper limit, because this sample is expected to have a small contribution from the $S_2$ and $S_3$ states (see Materials and Methods).
Figure 3.5. EPR transients, showing the effect of 200 mM NaCl or NH₄Cl on Y₄⁻ decay at 300 K and pH 7.5. The transients were recorded from oxygen evolving PSII and correspond to Y₄⁻ (black), Y₄⁻ + 200 mM NH₄Cl (red), Y₄⁻ + 200 mM NaCl (blue), and Y₄ + 10 µM DCMU (green). The transients were baseline corrected and normalized for presentation purposes.

As an additional control, we investigated the effect of ammonia on Y₄⁻Q₄⁻ recombination. In Figure 3.5, the decay of Y₄⁻ at 300 K is shown in a control (black), 200 mM NaCl (blue), 200 mM NH₄Cl (red), and 10 µM DCMU (green) at pH 7.5. The t½ values were 5300 ± 2000 s, 6200 ± 3000 s, 4400 ± 700 s, 4500 ± 1600 s respectively. As expected, the rate of recombination was insensitive to the addition of DCMU, an inhibitor that binds at the Q₄ site. This result verifies that Y₄⁻Q₄⁻ recombination is monitored in Figure 5. Given the standard deviation, there was no significant effect of ammonia on Y₄⁻Q₄⁻ recombination.

Previous EPR measurements have investigated the pH dependence of Y₄⁻Q₄⁻ decay. In Figure 3.6, we plot the t½ value, derived from those Y₄⁻ data, and compare to the pH dependence of Sₐₙₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐportion
recombination at high pH values. However, the \( \text{Y}_D^\cdot \) rates exhibit more than a 1000 fold stimulation as the pH decreases, while the \( \text{Y}_Z^\cdot \) data do not show a significant pH effect in either S state.

Figure 3.6. Comparison of the pL dependence for \( \text{Y}_D^\cdot \), \( \text{S}_2\text{Y}_Z^\cdot \), and \( \text{S}_0\text{Y}_Z^\cdot \) decay. Both \( \text{Y}_Z^\cdot \) and \( \text{Y}_D^\cdot \) decay by recombination with \( \text{Q}_\lambda^\cdot \). These \( \text{Y}_D^\cdot \) data were acquired in OEC-depleted PSII (14, 38).

Figure 3.7 provides a comparison of the hydrogen-bonding environment of \( \text{Y}_D \) and \( \text{Y}_Z \). (1) As shown, \( \text{Y}_Z \) participates in an extensive hydrogen-bonding network, but \( \text{Y}_D \) does not. In our experiments, addition of ammonia, which decreases hydrogen-bonding in the \( \text{S}_2 \) state, (25) increased the \( \text{Y}_Z^\cdot \) lifetime by a factor of eleven at pL 7.5. Thus, when ammonia displaces bound water, the kinetic behavior of the \( \text{Y}_Z^\cdot \) radical is observed to be more similar to that of the long-lived \( \text{Y}_D^\cdot \) radical.
Figure 3.7. A comparison of the local hydrogen-bonding environment for \( Y_Z \) (A) and \( Y_D \) (B). (1) Amino acid residues predicted to hydrogen bond with \( Y_Z \) and \( Y_D \) are shown in green (A) and orange (B), respectively. Assigned, bound water molecules are shown in red (A and B), and the calcium ion is in green (A). An assigned water molecule, which is predicted to have partial occupancy, is shown in gray (B). Predicted hydrogen bonds are depicted with black dashed lines and with distances in Angstroms (The Pymol Molecular Graphics System, Version 1.3, Schrödinger LLC).

3.5 Discussion

Summary of results. In this paper, we report that \( \text{Mn}_4\text{CaO}_5 \) redox reactions and the addition of ammonia alter the rate of \( Y_Z \) reduction and the average solvent isotope effect on the reduction reaction. We compared two different OEC oxidation states, the \( S_0 \) (three flashes) and the \( S_2 \) state (1 flash or continuous illumination at 190 K). These states are known to differ in Mn oxidation state, with \( S_0 \) assigned to a \( \text{Mn(III)}_3\text{Mn(IV)}_1 \) state and \( S_2 \) assigned to a \( \text{Mn(III)}_1\text{Mn(IV)}_3 \) oxidation state.\( (28, 30, 46) \) Mn oxidation reactions are likely to change hydrogen-bonding interactions among the bound water molecules in the OEC.\( (46, 47) \) We found that the rate of \( Y_Z^{-}/Q_{A}^{-} \) recombination and the average KIE were altered, when the \( S_0 \) and \( S_2 \) states were compared. However, the halftimes and KIE values were pH independent in both the \( S_0 \) and \( S_2 \) states, given the standard deviations of the measurements. This is in contrast to the pronounced pH dependence, previously
reported for \( Y_D^\cdot Q_A^- \) recombination. The addition of ammonia dramatically slowed the rate of \( Y_Z^\cdot \) decay, but had no significant effect on \( Y_D^\cdot \). We attribute this effect to substitution of ammonia into the OEC hydrogen-bonding network, surrounding \( Y_Z \), and the subsequent, expected decrease in hydrogen bond strength.\(^{(25)}\) Taken together, these data suggest that hydrogen-bonding changes are linked with the S state cycle. Such hydrogen-bonding changes would alter the midpoint potential and/or pK\(_a\), and, thereby, could change the rate of \( Y_Z^\cdot \) recombination.

*Overview of PCET reactions and \( Y_Z \).* Figure 8 shows a general scheme for PCET reactions involving \( Y_Z \) and \( Y_Z^\cdot \). In this paper, we focus on the recombination reactions, shown in the blue brackets, in which an electron and proton are transferred orthogonally to the tyrosyl radical.

Tyrosine-based PCET reactions may occur by three different mechanisms.\(^{(12, 48-57)}\) When the proton and electron are transferred together through a single transition state, the reaction is CPET (Figure 3.9). Many tyrosine-based model compounds exhibit a CPET mechanism.\(^{(12, 48, 49, 53, 56, 57)}\) Experimental and theoretical approaches have concluded that intermolecular and intramolecular hydrogen bonds facilitate CPET reactions.\(^{(12, 48, 49, 53, 54, 56-58)}\) For tyrosine redox reactions, CPET reactions avoid the formation of high-energy intermediates, such as a tyrosine cation radical, but occur at the expense of a high reorganization energy.\(^{(12, 48, 49, 53)}\) Alternatively, the electron can be transferred before the proton (ETPT) or the proton can be transferred before the
Figure 3.8. Oxidation (top) and reduction (bottom) reactions for Y\textsubscript{Z}, which is depicted as hydrogen bonded to a calcium-bound water and to the side chain of His190-D1. In the top panel, P\textsubscript{680} oxidizes Y\textsubscript{Z} generating a neutral radical. In the bottom panel, the reduction of Y\textsubscript{Z} and calcium bound water and His190-D1 compete to donate a proton. In this paper, we are monitoring the reduction (bottom) reaction.
Figure 3.9. Reaction diagram illustrating the stepwise and concerted pathways for PCET reactions of tyrosine with histidine acting as a proton donor. The top pathway begins with a proton transfer to create the tyrosyl cation radical followed by an electron transfer to form tyrosine. The bottom pathway starts with and electron transfer that creates tyrosinate followed by a proton transfer to form tyrosine. The middle pathway concertedly transfers an electron and a proton in a single step to transition from a neutral tyrosyl radical to tyrosine.

electron (PTET). In PTET, the intermediate in the two-step reaction is a tyrosine cation radical. In ETPT, the intermediate in the two-step reaction is the tyrosinate singlet state.
**Summary of Previous Results:** Oxidation of YZ. Previous studies have focused on PCET during the oxidation reaction (Figure 3.8, top) in which YZ is both an electron and a proton donor. Electron transfer from YZ to P_{680}^{+} follows the rapid, picosecond time scale, light-induced production of the P_{680}^{+}Q_{A}^{-} state and, then occurs predominantly on the nanosecond time regime.(59-61) This process is complete before the beginning of data acquisition under our conditions. In oxygen-evolving centers, the rate of YZ to P_{680}^{+} electron transfer exhibited no significant isotope effect in most studies ((59-62) and references therein).

**Electron transfer to YZ^{•}**. In this paper, we are studying the recombination reaction in which YZ^{•} accepts an electron and a proton (Figure 3.8, bottom). The detailed mechanism of electron transfer during recombination is not known, but can be imagined to involve a direct one-step recombination or a multistep mechanism.(63, 64) Dutton's ruler, an empirical relationship that estimates the rate of electron transfer (65), can be used to estimate the direct transfer mechanism. In the equation below, the exponential decay of tunneling rate is $k_{et}$ (in s^{-1}), with edge-to-edge distance R (in Å) and a parabolic dependence of log rate on $\Delta G$ and $\lambda$ (in eV).(65)

\[
\text{rate} = k_{et} \exp(-\frac{\Delta G}{k_{B}T}) \frac{1}{R^2}
\]

The YZ midpoint potential has been reported as 1 V (19), the P_{680} potential as 1.26 V (63), and the Q_{A} potential as ~0 V (66). A reorganization energy of 0.7 V is often assumed (65). For the 34.1 Å distance between Q_{A} to YZ, predicted by the X-ray structure (1), a rate of $10^{-6}$ s^{-1} is predicted using these measurements and assumptions. This rate is many orders of magnitude slower than our observed recombination times (~1 s^{-1}). Thus, a direct one step mechanism is not favored.
A multi-step recombination mechanism may occur via the P$_{680}^+$Q$_{A}^-$ couple. A simple mechanism of electron transfer in the back reaction can be written with two elementary steps:

Step 1:

Step 2:

where $k_{in}$ is the intrinsic rate of charge recombination through P$_{680}^+$Q$_{A}^-$ (which occurs on the microsecond time scale). (67-69)

For this pre-equilibrium mechanism, the velocity of the reaction can be written,

which can be rearranged to give:

As shown in the derivation above, for this two-step, pre-equilibrium recombination mechanism, the measured rate constant, $k_{obs}$, is the product of the P$_{680}^+$Q$_{A}^-$ recombination rate constant, $k_{in}$, and the equilibrium constant governing redox reactions between Y$_Z$ and P$_{680}^+$. The $k_{in}$ recombination rate has been reported as $\sim 7 \times 10^2$ s$^{-1}$ (67) or $5 \times 10^3$ s$^{-1}$ (69) and is pH and solvent isotope independent (67, 69). The value of $k_1$ has been reported as $\sim 10^7$ s$^{-1}$ (59). Using our experimentally measured value of $k_{obs}$ ($\sim 0.2-0.3$ s$^{-1}$), the pre-equilibrium two-step mechanism predicts the value of $k_2$ to be $\sim 10^4$ s$^{-1}$. Estimating the distance between Y$_Z$ and P$_{680}$ in the crystal structure (1) as 13.5 Å and using the potentials and reorganization energies given above, Dutton's ruler predicts that
an electron transfer (ET only) rate constant over this distance will be $\sim 10^3 \text{ s}^{-1}$. An adjustment of the reorganization energy to 0.6 V gives a predicted rate of $10^4 \text{ s}^{-1}$. This analysis suggests that the two-step mechanism is a reasonable kinetic scheme for the $YZ^\cdot$ recombination reaction. However, more complex mechanism, involving thermal relaxation events among $YZ^\cdot$ and $P_{680}^+$ substates cannot be excluded. The use of the overall $t_{1/2}$ in our analyses has the advantage that it is independent of assumed mechanism.

*The PSII X-ray structure predicts multiple proton donors to $YZ^\cdot$.* The recent PSII X-ray structure(1) suggests that activated, calcium bound water and protonated histidine side chain compete for proton donation to $YZ$ (Figures 3.1B and 3.7A). It should be noted that it is not known which S state is represented in the X-ray structure.(1) As depicted in Figures 3.1B and 3.7A, $YZ$ is involved a network of strong hydrogen bonds and is 2.5 Å from His190-D1, 2.6 Å from a bound water, and 2.8 Å from a calcium bound water molecule. Calcium acts as a Lewis acid and will decrease the pKa of the water and increase its acidity by $\sim 1000$ fold.(70) As the oxidation of the Mn$_4$CaO$_5$ cluster increases, calcium will become a stronger Lewis acid. Chemical complementation suggested a role for His190-D1 in proton transfer to $YZ$;(71) evidence for another proton donor was obtained from EPR studies of a His190-D1 mutant.(72) Water, especially activated water, and protonated His 190-D1 side chain are both expected to be facile proton donors.(70, 73-75)

*pH independence of the $YZ^\cdot QA^-\cdot$ recombination rate.* The pH independence of the $YZ^\cdot QA^-\cdot$ recombination rate, reported here, is an interesting observation. pH independence for recombination in the $S_0$ state was reported previously, by monitoring the production
of a coupled $Y_Z$-metal signal at liquid helium temperature. (76) In that work, lack of pH dependence was attributed to a lack of solvent exchange and a hydrophobic $Y_Z$ environment. However, the more recent PSII X-ray structure shows that the $Y_Z$ environment is hydrophilic. Also, we observe a significant KIE in both $S_0$ and $S_2$ states, demonstrating that solvent isotope exchange does occur. To explain the lack of pH dependence on recombination in the $S_2$ state, we recently proposed that the network of strong hydrogen bonds, predicted in the X-ray structure, facilitates a coupled proton transfer reaction (CPET). CPET reactions can proceed by a pH independent mechanism. (12, 51, 76) Alternatively, if the two-step preequilibrium model is the correct mechanism, the lack of pH independence could be attributed to compensating pH effects on the forward, $k_1$, and back reaction, $k_2$. However, compensation may not explain the solvent isotope dependence, because $k_1$ and $k_m$ have both been reported to be independent of solvent isotope effects, as described above. (59-61, 67, 69)

$Y_Z$ and $Y_D$ have different PCET mechanisms. Comparison of $Y_Z$ to $Y_D$ aids in the interpretation of these results (Figure 3.7). Previous work used EPR spectroscopy to investigate the mechanism of $Y_D^+$ PCET. (38, 45) The reaction followed was recombination of $Y_D^+$ with $Q_A^-$. Through analysis of the pH and solvent isotope dependence, the $Y_D^+$ reaction was deduced to be PTET at low pL and to be CPET at high pL values, for example pL 8. (38) The PTET reaction showed a dramatic increase in rate as the pH decreased, consistent with titration of the $Y_D^+$ radical and its proton donor. The PTET reaction was not associated with a significant KIE. However, a significant KIE (2.4) was observed at pL 8.0. A proton inventory on the CPET reaction at pL 8.0 was consistent with multiple proton transfer reactions through two different pathways. One
pathway was suggested to involve water and the other pathway was suggested to involve His189-D2 (Figure 3.7B).(45) The 1.9 Å X-ray crystal structure supports this interpretation.(1)

Unlike $Y_D^\cdot$ PCET, $Y_Z^\cdot$ PCET does not show pH dependence. When comparing the environment surrounding $Y_Z$ and $Y_D$ (Figure 3.7), the hydrogen-bonding environment is dissimilar. One difference between the environments of $Y_Z$ and $Y_D$ is the presence of Arg294 in the D2 polypeptide, which is 2.8 Å from His189-D2. $Y_D$ has potential pi-cation interactions with this arginine (at 6.4 Å from YD) and with Arg-272-D2 in the CP47 subunit (at 7.8 Å). The corresponding arginines are not found in the $Y_Z$ environment. Studies of biomimetic PSII-inspired peptides, which conduct a tyrosine based PCET reaction involving a pi-pi stacked histidine, concluded that pi-cation interactions with arginine lower the potential of redox active.(13, 77) This effect may account for the lower reported midpoint potential of $Y_D$ (23); when compared to $Y_Z$. (19) An additional impact of the Arg294/His189 interaction will be to decrease the pK$_a$ of His189-D2, making it a better proton donor, compared to His190-D1.

A second difference between $Y_D$ and $Y_Z$ is the distance between the potential proton donating histidine and the redox active tyrosine. His190-D1 is 2.5 Å from $Y_Z$; the analogous His189-D2 is 2.7 Å from $Y_D$. However, the potential impact of this small change in hydrogen bond length to histidine is not clear. A recent study on a series of ruthenium bound tyrosine complexes with varying hydrogen bond distances from 2.54-2.75 Å was conducted.(57) This study concluded that all of these compounds proceed through a CPET mechanism with a varying KIE. Interestingly, the compound with a hydrogen bond donor-acceptor distance of 2.54 Å exhibited more rapid PCET and was 2-
3 orders of magnitude faster when compared to a compound with a donor-acceptor distance of 2.75 Å. However, the 2.54 and 2.75 Å compounds were found to have KIE values of 1.4 and 4.0 respectively, while Y_Z and Y_D have similar KIE’s on the CPET reaction (at high pL for Y_D). Also, it should be noted that a kinetic study of phenol-amines did not report this exquisite distance dependence.

Another difference between the environments of Y_Z and Y_D is that Y_Z is involved in a network of strong hydrogen bonds involving multiple bound water molecules (Figure 3.7A). Y_D does not participate in this network and is hydrogen bonded just to His 189-D2 and a single bound water molecule with partial occupancy. Y_Z is 2.6 Å from the closest bound water molecule, whereas Y_D is 2.7 Å from the water molecule with partial occupancy. The effects of ammonia on Y_Z• kinetics, but not on Y_D• kinetics, suggest that this hydrogen-bonding network plays a role in control of Y_Z• kinetics (Fig 10).

Y_Z• PCET and the S state transitions. In this paper, we report that the KIE and halftime for Y_Z recombination are distinct in two different S states, corresponding to two different oxidation states of the Mn cluster. We attribute this to oxidation-induced changes in hydrogen-bonding network of the OEC. For example, oxidation of the Mn may decrease the pK_a of metal bound water, making calcium bound water a more effective proton donor in the higher S state. Theoretical studies have suggested that OEC oxidation may control pathways for water transfer by changes in electrostatic interactions.

As discussed above, three different pathways (Figure 3.9) can be imagined for non-synchronous transfer of a proton and electron to Y_Z•. This reaction corresponds to \( k_1 \) and \( k_2 \) in the two-step, pre-equilibrium mechanism (see step 1). This square scheme
(Figure 3.9) is introduced here to account for the kinetic isotope dependence observed in our experiments.

In PTET (Figure 3.9, top), the $k_2$ reaction is written:

\[ \text{The overall rate in PTET is the product of the preequilibrium constant for formation of the tyrosyl cation radical and deprotonation of a histidine side chain (estimated as } 10^{-8}) \text{ and the intrinsic } k_{et}. \] 

Using the estimated value of $10^4$ s for $k_2$, as estimated above, the PTET scheme predicts that the intrinsic ET rate, $k_{et}$, would have to be very rapid, on the order of $10^{12}$ s$^{-1}$. Lower values of the pK$_a$, consistent with activated water serving as the proton donor (estimated as pK$_a = 2(75)$) still give an estimated $k_{et}$ of $10^8$ s$^{-1}$. These ET rate constants are greater than expected for an uphill electron transfer reaction over 13 Å. Moreover, in PTET, pH dependence is expected due to titration of the proton acceptor, $Y_Z^\cdot$, and the proton donor(s) in a solvent accessible environment. Such a pronounced pH dependence was observed for the Y$_D$ PCET reaction at low pH values for example (38, 45), which also involves water and an imidazole side chain (His189-D2) as proton donors. Therefore, a PTET mechanism for $k_2$ is not favored.

In ETPT, the $k_2$ reaction, estimated as $10^4$ s$^{-1}$, also consists of two steps and can be written:

\[ \text{The ETPT reaction will be pH independent and exhibit only a small isotope effect if electron transfer is the rate limiting step.} \]

(12, 49, 50) Rate-limiting ET is
expected in this scenario, because proton transfer from a protonated histidine to tyrosinate will be significantly downhill in free energy. For example, using $pK_a$ values of 6 and 10, respectively, for His190-D1 (or another proton donor) and tyrosine, the intrinsic $k_{et}$ rate would indeed be predicted to be slow ($1 \text{ s}^{-1}$). In fact, this value is much slower than the estimate for pure ET from Dutton's ruler for this reaction (see above). However, the KIE values measured for the $S_2$ (2.1) and $S_0$ (1.3) states are significant and may not be consistent with ETPT. Therefore, an ETPT mechanism is not favored, especially for the $S_2$ state reaction.

In CPET, a significant KIE (>1.6) is expected, and the reaction may be pH independent.(12, 49, 51) Therefore, a CPET mechanism can account for the pH independence and solvent isotope effects on $Y_Z^*$ decay kinetics in both S states. Adiabatic Marcus theory can be used to explain CPET reactions and that these reactions have smaller rate constants than a pure ET reaction, due to a larger activation barrier for these reactions.(50) If the $k_2$ reaction operates by a CPET mechanism, however, a question remains concerning the forward reaction, governed by $k_1$ (see our previous discussion in ref (14)). This nanosecond time-scale reaction was reported to have no solvent isotope effect in some studies, although there are some discrepancies in the literature.(59-62, 67, 69) One possible answer may be that complex protein dynamics accompanies this reaction. In that case, the mechanism for $Y_Z^*$ formation and decay would be more complex than the two-step mechanism, and may involve, for example, additional elementary relaxation events in the $P_{680}^+$ and the $Y_Z^*$ states. Resolving this question will require further experimentation.
Ammonia alters $Y_z$ decay kinetics by replacing water in the OEC hydrogen-bonding network. To explain the S state dependence of $Y_z$ PCET, we propose that the hydrogen-bonding environment of $Y_z$ is altered by changes in Mn oxidation state and activation of calcium bound water. Supporting the interpretation that hydrogen-bonding is important, we found that the effects of ammonia distinguished the $S_0$ and $S_2$ states. Ammonia is an inhibitor of photosynthetic oxygen evolution and has been proposed to act as a substrate-based inhibitor. Magnetic resonance studies have concluded that ammonia binds directly to Mn ions in the Mn₄CaO₅ cluster. FT-IR studies have shown that ammonia displaces bound water molecules in the OEC and disrupts a hydrogen-bonding network. Such a change in hydrogen bond strength is expected. Ammonia binding to the OEC has been monitored previously by Mn EPR spectroscopy, in which a modified $g=2$ multiline signal from the $S_2$ state was detected. These studies concluded that ammonia binds to the OEC in the $S_2$ and $S_3$ states, but not in the $S_0$ and $S_1$ states. Recent FT-IR studies of the $S_1$ to $S_2$ transition have shown that ammonia induces a change in OEC peptide carbonyl frequencies, consistent with a change in hydrogen-bonding in the $S_2$ state. Through comparison with the amtB ammonia transporter, it was proposed that ammonia displaces water molecules in an extended hydrogen-bonding network, which involves peptide carbonyl groups.
Figure 3.10. Proton donation to $Y_Z$ (A) and $Y_D$ (B) in ammonia treated PSII. A two pathway model for proton donation to $Y_D$ was suggested by a proton inventory study. (45)

In these EPR studies, we used ammonia as a probe of $Y_Z$ hydrogen-bonding interactions. Ammonia had differential effects on the $S_2$ and $S_0$ states, and the observed effect of ammonia on the recombination reaction was dramatic. Ammonia is expected to form weaker hydrogen bonds. (80, 81) Therefore, the observed slowing of the reaction rate is attributed to a decrease in $Y_Z/Y_Z^*$ hydrogen-bonding, when water is displaced by ammonia in the OEC hydrogen-bonding network (Figure 10). We observed no significant ammonia effect on the rate of $Y_D^*Q_A^-$ recombination. $Y_D^*$ and $Q_A^-$ are not predicted to interact with water molecules in the hydrogen-bonding network (Figure 10).

The addition of ammonia increased the lifetime of $Y_Z^*$, making its kinetic behavior more similar to the slow kinetic behavior of $Y_D^-$. Ammonia may induce a change in $Y_Z$ midpoint potential, with the direct effect from changes in hydrogen-bonding ($\sim 3-5$ kcal/mole) estimated as $\sim 0.1-0.2$V. Interestingly, the midpoint potential of $Y_D$ has been reported to be 250 mV lower when compared to $Y_Z$ (19, 23) and $Y_D$ is shown to be less extensively hydrogen bonded in the X-ray structure. (1) The slowing of the $Y_Z$ recombination rate in the presence of ammonia is reminiscent of the effects reported
when the OEC is removed by treatment with alkaline Tris. Under those conditions, the midpoint potential $Y_Z^*$ decreased by $\sim$100 mV, and its lifetime increased. These previous reports support the interpretation that a decrease in midpoint potential can decrease the rate of PSII tyrosyl radical decay. It should be noted that ammonia addition is not leading to loss of Mn, because, in a control experiment in which we purposely removed the OEC, the rate of $Y_Z^*$ decay dramatically increased. By contrast, our data show that ammonia slows the rate of $Y_Z^*$ decay in both S states and has a reversible effect on oxygen evolution.

**Implications.** Using $Y_Z^*$ kinetics as a probe, our experiments provide evidence that the hydrogen-bonding environment of $Y_Z$ is altered when the $S_0$ and $S_2$ states are compared. This hydrogen-bonding change is attributed to the accumulation of positive charge, which is expected to activate calcium-bound water molecules and alter the strength of the hydrogen-bonding network with His190-D1. Such hydrogen-bonding changes could potentially play a crucial role in controlling the acid/base chemistry during proton transfer reactions. For example, charge accumulation on the Mn$_4$CaO$_5$ has been predicted to control the movement of water to the active site. Comparing the $S_0$ and $S_2$ states, our kinetic results are consistent with a change in $Y_Z$ midpoint potential or $pK_a$, which is mediated by the Mn oxidation reaction. This dynamic interplay between water, $Y_Z$, and the Mn$_4$CaO$_5$ may regulate the unique and complex chemistry of photosynthetic water oxidation.
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3.6 References


CHAPTER 4
GLOBAL INCORPORATION OF THE NON-NATURAL AMINO ACID 3-FLUORO-L-TYROSINE INTO CYANOBACTERIAL PHOTOSYSTEM II

by

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4.1 Introduction

Photosynthetic oxygen evolution occurs within the protein complex photosystem II (PSII). Proton coupled electron transfer (PCET) reactions act to store energy in an oxygen-evolving complex (OEC) composed by a Mn$_4$CaO$_5$ cluster. A key intermediary in this PCET process is tyrosine Z (Y$_Z$), Tyr161 of the D1 polypeptide. Y$_Z$ is oxidized by a dimer of chlorophyll molecules known as P$_{680}$, which produces a neutral tyrosyl radical (Y$_Z^\bullet$). The photooxidation of tyrosine causes deprotonation because the pKa of the radical is -2.\(^{(1)}\)

Previously we have shown that PCET reactions of Y$_Z$ in the S$_0$ and S$_2$ states are pH independent with significant kinetic isotope effects (KIE). These results were unexpected due to the nature of PCET reactions. The Nernst equation predicts that if there is a change in the protonation state of a molecule during PCET then the rate will show pH dependence. This pH dependence has been shown previously for the decay of Y$_D^\bullet$Q$_A^-$.\(^{(2)}\)

The pKa of tyrosine is ~10 and that of histidine is ~6.\(^{(1, 3)}\) The pH range (5.0-7.5) previously studied is expected to titrate the histidine within this PCET pair, yet no pH dependence has been seen. The pKa of Y$_Z$ is too high to titrate with pH adjustments, because the alkaline conditions would remove the Mn$_4$CaO$_5$ cluster. Removing the OEC drastically affects the kinetics of the reaction and is not comparable to \textit{in vivo} applications. In order to assess the nature of these PCET reactions involving Y$_Z$, we propose lowering the pKa and increasing the midpoint potential by substituting non-natural amino acid derivatives of fluoro-L-tyrosine (FY). FY variants can alter the midpoint potential by up to 300 mV and have pKa varying from 8.4 down to 6.3.\(^{(4)}\)
The global incorporation of fluorinated tyrosines into PSII presents an intriguing and challenging possibility. The allure of fluorinated tyrosine is created by the control that can be exhibited on the midpoint potential and the pKa of the tyrosine by changing the extent and position of the fluorination. Fluorination can alter the midpoint potential by 300 mV and the pKa by up to 5 units. By lowering the pKa of YZ, titration should occur within a pH range that will leave the enzyme in an oxygen evolving state. The challenge presented is that fluorinated tyrosine is toxic to most organisms. Some proteins are more easily manipulated to allow site specific incorporation of fluorinated amino acid than others. Mutation of aminoacyl-tRNA synthetase and intein chemistry has allowed for the incorporation of fluorotyrosines into ribonucleotide reductase. Unfortunately, these techniques do not work for all proteins.

Previously, researchers incorporated 3-fluoro-L-tyrosine (3FY) into photosystem II (PSII). They incorporated 3FY into *Synechocystis* by growing the cyanobacteria in 10 L minimal media until they reached a cellular optical density of 730 nm of ~0.5 abs.
They then added 1 L of 0.5 mM 3FY, 0.5 mM phenylalanine, and 0.25 mM tryptophan bringing the final concentration to 45 μM amino acids. Addition of amino acids to the media induced apoptosis, so the cells were harvested and the proteins were purified. They did not report oxygen evolution for the cells or for the purified PSII. A procedure to remove the Mn₄CaO₅ cluster was performed using hydroxylamine. The PSII was characterized by optical studies and EPR. Undoubtedly, they were able to incorporate 3FY into PSII utilizing this technique. However, the extent of incorporation is questionable. Instead of using mass spectrometry to quantify the percent incorporation, they opted to use EPR simulations. From simulations they estimated the extent of incorporation to be 75%. From this 75% incorporation, they received similar kinetic results to previously published Mn-depleted studies. The applicability of these results to the native system is questionable due to the removal of the Mn cluster. The more interesting experiments would be to study 3FY within oxygen-evolving PSII.

In this study a strain of Synechocystis sp. PCC 6803 with a hexa-histidine tag located on the 3’ terminus of the CP47 subunit is utilized as a host for the incorporation of 3FY into PSII. Synechocystis sp. PCC 6803 is a very sensitive organism. Slight alterations in the media can cause extreme reactions. This sensitivity makes the incorporation of amino acids very difficult. In order to incorporate amino acids into Synechocystis, we start by growing them on a very low concentration (10 μM) to build a tolerance and slowly increase until the desired concentration (250 μM) is reached. Synechocystis is highly adaptable, so adjusting the concentration slowly allows time for the cells to evolve.
4.2 Materials and Methods

*Synechocystis* sp. PCC 6803 cultures were maintained on solid media containing BG-11,\(^{(7)}\) 250 µM 3FY (Synquest Laboratories), phenylalanine, and tryptophan buffered with 5 mM TES-NaOH, pH 8.0, and 6 mM Na\(_2\)S\(_2\)O\(_3\).\(^{(8)}\) Liquid cultures were grown in BG-11, 5 mM TES-NaOH, pH 8.0, and 10 µg/mL kanamycin, and were bubbled with sterile air. Liquid cultures began growing in 50 mL of media and are grown for 7-10 days. These cells were then transferred to 15 L of sterile BG-11 media and allowed to grow for one month. To prepare 15 L of BG-11 media, it is autoclaved in a concentrated form in order to accommodate the sterile addition of amino acids. Due to the low solubility of aromatic amino acids, stock solutions are prepared at 1 mM concentration. To make 15 L of media, 3.75 L of amino acids must be added to 11.25 L of BG-11 that contains enough nutrients for 15 L.

After being grown for a month, the cells were harvested with an Amicon DC-10 hollow fiber concentrator to concentrate the cells to ~ 1 L. The use of the concentrator is necessary with this cell line for multiple reasons. Firstly, the yield is low, so large volumes are employed by growing six 15 L (90 L) carboys. This volume would take days to centrifuge concentrate. Secondly, when these cells are stressed they tend to float more. Stress can cause cyanobacteria to float by either upregulating their gas vacuoles or storing more lipids, either scenario makes centrifugation difficult. Once the cells were concentrated down to ~1 L, they were centrifuged at 30,000 x g for 10 minutes. Once pelleted, the cells were resuspended in freeze buffer (25% glycerol, 20 mM CaCl\(_2\), 20 mM MgCl\(_2\), 20 mM MES, pH 6.0), pelleted again, and frozen at -70 °C.
To purify thylakoid membranes from cells, the pellets were resuspended in 1 L of break buffer (0.8 M sucrose, 50 mM MES, pH 6.0) and pelleted by centrifugation for 10 min at 30,000 x g. The cells were resuspended in approximately 300 mL of break buffer and incubated for 60 min on ice in the dark. They were then pelleted by centrifugation for 10 min at 30,000 x g. After incubating, the cells were resuspended in ~50 mL of break buffer to which was added 0.5 mg of DNase I, 1.5 mg of N-tosyl-L-phenylalanine chloromethyl ketone, 5 mg of phenylmethanesulfonylfluoride, 0.25 mg of pepstatin A, and 1.25 g of bovine serum albumin. The cells were added to a chilled bead beater that was two-thirds full with 0.1 mm diameter glass beads (Bio-spec Products). The chamber was then filled with break buffer, and the outside jacket was filled with an ice/water slurry. The cells were broken in the dark for 7 cycles of 30-s blending and 15-min rest. The lysate was separated from the beads by decanting the fluid off the top. The beads were washed four times with 200 mL of break buffer in each wash. This solution was then centrifuged for 30 min at 7,500 x g. CaCl₂ was added to the supernatant to give a final concentration of 40 mM. Thylakoid membranes were then pelleted by a at 24,000 x g for 30 min. The thylakoid membrane pellet was resuspended in freeze buffer (25% glycerol, 20 mM MES pH 6.0, 20 mM CaCl₂, 20 mM MgCl₂), an aliquot was taken for oxygen assays, and the membranes were pelleted again by centrifugation at 44,000 x g for 30 min. These pellets were stored at -70 °C.

The pellets from the thylakoid membrane preparation contain all of the photosynthetic proteins. In order to separate PSII, thylakoid membrane pellets were thawed and resuspended in minimal his-tag buffer (25% glycerol, 5 mM CaCl₂, 10 mM MgCl₂, 50 mM MES-NaOH, pH 6.0) to 0.6 mg/mL chl, 1% lauryl maltoside, and 0.01
mg/ml phenylmethanesulfonylfluoride while shaking on ice. The sample was shaken for 10 min and then centrifuged at 100,000 x g for 60 min. After centrifuging, the supernatant was gently loaded on to a His-Pur cobalt resin (Fisher Scientific). The sample was allowed to flow through the column until there was just enough liquid to keep the top of the column hydrated. His-tag wash buffer (25% glycerol, 0.04% lauryl maltoside, 5 mM CaCl₂, 10 mM MgCl₂, 50 mM MES-NaOH, pH 6.0) was then run slowly through the column until baseline absorbance at 280 nm was achieved. Once the absorbance was zero, his-tag elution buffer (25% glycerol, 100 mM histidine, 0.04% lauryl maltoside, 5 mM CaCl₂, 10 mM MgCl₂, 50 mM MES-NaOH, pH 6.0) was run through the column. The fractions containing PSII were collected and concentrated with an Amicon 100MWCO concentrator. The sample were washed three times with equal volumes of his-tag resuspension buffer (25% glycerol, 20 mM CaCl₂, 50 mM MES-NaOH, pH 6.0) and an aliquot was taken for oxygen assays.

EPR analysis was conducted on a Bruker (Billerica, MA) EMX spectrometer equipped with a Bruker ER 4102ST cavity and Bruker ER 4131VT temperature controller. EPR spectra were measured in the dark at 190 K. Microwave power and modulation amplitude were set low, to show that the expected Y₂⁻ hyperfine splitting. Because the microwave power is low, this field swept spectrum also has a contribution from Y₃⁻. EPR parameters were: frequency: 9.39 GHz, power: 0.64 mW, and modulation amplitude: 2 G (Figure 4.3), conversion time: 41 ms, time constant: 164 ms, sweep time: 21 s.
4.3 Results

To begin incorporating 3FY, we developed a cell line that was tolerant to tyrosine in the media. We then used this strain to begin incorporating 3FY through various methods. Each attempt we made with this cell line was unsuccessful.

It was concluded that tyrosine tolerant cells were not helpful when trying to incorporate 3FY. This information led to the conclusion that to incorporate 3FY, the cells need to be tolerant to 3FY. We began directing the evolution of cells by adding 10 μM 3FY, phenylalanine, and tryptophan to the solid media of a hexa-histidine tagged strain of *Synechocystis sp.* PCC 6803. At 10 μM amino acids there was no change in the way that the cells grew. We increased the concentration every couple weeks until we reached 50 μM amino acids. Once the concentration reached 50 μM amino acids, the majority of the cells died with only a few colonies surviving. We continued to select the colonies that were thriving on the 50 μM 3FY media and started growing these cells in liquid cultures that contained 250 μM 3FY media. We continued to increase the concentration of amino acids in the solid media to equimolar to the liquid cultures. We transferred the 3FY tolerant cells to solid media containing 250 μM 3FY, 250 μM phenylalanine, and 250 μM tryptophan, and the cells grew well. In Figure 4.1, we show pictures of 3FY tolerant cells growing on solid (A) and in 15 L liquid media (B) with 250 μM 3FY media.
Figure 4.2  A) 3FY tolerant strain of *Synechocystis sp.* PCC 6803 cells growing on a 1.5% agar plate containing BG-11 media with sodium thiosulfate and 250 µM 3FY, tryptophan, and phenylalanine. B) Three 15 L carboys growing 3FY tolerant cells in BG-11 media with 250 µM 3FY, tryptophan, and phenylalanine.

Once the cells have grown for ~1 month in 15 L of media, they were harvested. Figure 4.2 shows the supernatant taken off the cells grown in BG-11 media (A) and (B) BG-11 with 250 µM 3FY media during concentration. It is easy to see the difference in the cyanobacteria’s response to stress. In both the lighter (A) and the darker (B) carboy the unknown pigment exhibits the same absorption spectrum (Figure 4.2C). In Figure 4.2C, an absorption spectrum is shown from 200 to 750 nm of the compound that is excreted during growth. The concentration of this orange compound is extremely high in the cells grown on 3FY media. Each step of the purification process releases more of this compound. Modifying the purification procedure to accommodate this compound is necessary, because the compound binds to the Co-NTA column. To effectively run a his-tag purification on the 3FY labeled PSII the orange pigment should be removed from solution. Running an anion exchange column to separate the photosynthetic proteins from the orange chromophore prior to running a his-tag column should increase the efficacy of the his-tag column.
Cells that have been harvested to date show very similar oxygen evolution rates within the error of the experiments. Control cells were grown in either BG-11 or BG-11 with 250 μM tyrosine, phenylalanine, and tryptophan and 3FY cells grown in BG-11 with 250 μM 3FY, phenylalanine, and tryptophan evolved oxygen at a rate of 480 μmol O₂/hr•mg chl and 450 μmol O₂/hr•mg chl respectively. Oxygen evolution rates after the thylakoid membranes were purified have been within error of the rates in the cells with rates of 490 μmol O₂/hr•mg chl and 450 μmol O₂/hr•mg chl respectively for control and 3FY which is expected.

The yield from these cells and thylakoid membrane preparations has been extremely low. We have run one his-tag purification for the control and 3FY cells. The control cells were purified with low oxygen evolution activity. For purified cyanobacterial PSII, rates should be above 2000 μmol O₂/hr•mg chl, but instead rates were 570 μmol O₂/hr•mg chl. The 3FY his-tag purification led to oxygen evolution rates of 900 μmol O₂/hr•mg chl, but the yield was extremely low (200 μg chl). These rates might be adequate for 3FY-incorporated PSII. Performing this preparation multiple times
will allow us to know what to expect since no one has purified oxygen-evolving 3FY-PSII previously. The low yield was caused by the chromophore produced by the cells (Figure 4.2B). This compound binds to the his-tag column, so it competes for space on the column and possibly has a higher affinity for the cobalt than the his-tag PSII.

Figure 4.4. Dark field sweep EPR spectrum of PSII from *Synechocystis* sp. PCC 6803 HT3A cell line at pH 6.0 and 190 K.

In Figure 4.3, an EPR field sweep spectrum of cyanobacterial PSII is shown. This is a characteristic dark field sweep signal from cyanobacterial PSII with the expected $g$-value of 2.004. This field sweep data is derived from samples with low oxygen evolution rates that dropped after being frozen to 370 $\mu$mol O$_2$/hr•mg chl. This made the analysis of transient kinetics difficult due to inactivation of the Mn$_4$CaO$_5$ cluster.

4.4 Discussion
The successful construction of a 3FY-tolerant strain of *Synechocystis* sp. PCC 6803 is a large step forward. The creation of this strain has led to many other challenges, such as the low yield of PSII and the large quantity of chromophore that is produced under stress by the cyanobacteria. These hurdles will easily be overcome with time and sound experimental practices. We will continue to increase the yield from these cells, by changing growth conditions and optimizing the purification while trying to reduce the cyanobacteria’s response to stress.

Currently, different growth protocols are being attempted to minimize the stress cells undergo during incorporation of 3FY. When previous researchers incorporated 3FY into PSII, they used a much lower concentration (45 μM) than the growth protocol that was employed here (250 μM). Previous studies from our lab have shown that incorporation above 95% is seen with 250 μM concentrations of the desired amino acid. Reducing the concentration of 3FY could lead to higher yields of PSII and reduce production of the orange chromophore.

Future experiments include EPR at both cryogenic and room temperature. In these two different conditions we will perform similar experiments that we have employed for spinach thylakoid membranes. The global incorporation of 3FY will create samples that will be useful for analyzing both Y\textsubscript{Z} and Y\textsubscript{D}. In addition to EPR, fourier transform infrared (FTIR) spectroscopy offers a unique insight into proteins and is capable of probing the electrostatic environment in PSII.(9) FTIR spectroscopy can be performed at room temperature and cryogenic temperature, which will make for a facile comparison between EPR and FTIR results. Characterizing Y\textsubscript{Z}\textsuperscript{•} and Y\textsubscript{D}\textsuperscript{•} in each of the
available S states with 3FY incorporated PSII will continue to shed light on the critical
PCET processes that catalyze water oxidation.

4.5 References

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Formation to the PsAB Chlorophyll of P700 in Photosystem I Mutants of

CHAPTER 5

5.1 Summary

Innovation begins by understanding fundamental processes. Photosynthesis is the most fundamental energy conversion process known. Photosynthesis is driven by the conversion of light energy into chemical energy. This conversion is facilitated by proton coupled electron transfer (PCET) reactions that limit the amount of high energy intermediates accumulated within the protein matrix. An \textit{ab initio} understanding of PCET reactions within Photosystem II (PSII) might enable innovation that will unlock the greatest sources of energy this planet knows.

Within this thesis, we provide useful techniques that are employed to study the nature of PCET reactions involving redox active tyrosines within PSII. First, we expanded a recently developed electron paramagnetic resonance (EPR) spectroscopy technique that exploits the power saturation difference between tyrosine Z (Y\textsubscript{Z}) and tyrosine D (Y\textsubscript{D}) to study the pH dependence and the kinetic isotope effects.\(^{(1)}\) We then utilized cryogenic temperatures to lock the oxygen evolving complex (OEC) in specific oxidation states.\(^{(2)}\) Once the oxidation state of the OEC is locked we utilize time-resolved EPR to monitor kinetics of the recombination of Q\textsubscript{A}\textsuperscript{-} with Y\textsubscript{Z}.\(^{(3)}\)

We began our focus on the reduction of Y\textsubscript{Z}\textsuperscript{•} set in the S\textsubscript{2} state, throughout the pL range of 5.0-7.5 (where oxygen is evolved) in both \textsuperscript{1}\textsubscript{H}\textsubscript{2}O and \textsuperscript{2}\textsubscript{H}\textsubscript{2}O. These experiments showed that the rate of recombination for S\textsubscript{2}Y\textsubscript{Z}\textsuperscript{•}Q\textsubscript{A}\textsuperscript{-} is pL independent and that Y\textsubscript{Z} is solvent exposed due to a significant KIE (~2). These results agreed well with some previously published results.\(^{(1, 4)}\) However, the pL independence was unexpected when
compared to experiments with Y₆.(5, 6) PCET reactions that transfer a proton are expected to yield a rate that is pH dependent.(7) However, hydrogen-bonding facilitates pH independent reactions by decreasing the reorganization energy.(8) We proposed that the hydrogen-bonding network around Y₇ and the calcium ion facilitate a pH independent CPET reaction.(3)

In order to compare Y₇ PCET in different S states we examined the next S state that had similar temperature transition properties, which is the S₀ state. We compared two unique OEC oxidation states, the S₀ and the S₂. These states are known to differ in oxidation state, with S₀ state assigned to a Mn(III)₃Mn(IV)₁ state, while the S₂ state is assigned a Mn(III)₁Mn(IV)₃ oxidation state.(9-11) Oxidation state changes likely manipulate the hydrogen-bonding interactions among water molecules around the OEC.(9, 12) We found that the rate of Y₇Q₇⁻ recombination and the average KIE were altered, when the S₀ and S₂ states are compared. However, the rates and KIE values are pH independent in both the S₀ and S₂ states. This pH independence is dissimilar to the previously reported Y₆Q₆⁻ recombination rates.(5, 6) We showed that the addition of ammonia is a useful probe for the hydrogen-bonding network and that ammonia dramatically slowed the rate of Y₇⁻ decay, but had no significant effect on Y₆⁻. We attribute this to substitution of ammonia into the OEC hydrogen-bonding network, surrounding Y₇, and the subsequently expected decrease in hydrogen bond strength.(13)

To continue studying PCET reactions within oxygen evolving it is necessary to manipulate the system without losing functionality. The incorporation of non-natural amino acids provides the type of alterations that can lead to new understandings of biological and chemical processes. We have successfully evolved a 3-fluoro-L-tyrosine
(3FY)-tolerant strain of cyanobacteria. The incorporation of 3FY into oxygen evolving
PSII should prove an excellent probe for PCET reactions due to calculated the changes in
chemistry.(14)

This work lays a foundation for many experiments to follow. EPR analysis of the
$S_1$ and $S_3$ transitions should be explored at lower temperatures (77-100 K). While
transitioning to lower temperatures Arrhenius plots of the $S_0$ and $S_2$ states could be
constructed. Arrhenius plots would allow for activation and reorganization energies to be
calculated. The $S_3$ state is possibly the most interesting of these S states, because it is the
gateway to water oxidation (see Appendix C for preliminary data on $S_3Y_Z$). Another
intriguing aspect of the $S_3$ state is that ammonia binding has been shown to be dependent
on the timing of flash protocol that is employed.(15) If 2 flashes are administered
quickly (1 Hz) then only one molecule of ammonia binds to the OEC, whereas if two
flashes are given slowly (0.03 Hz) then two molecules will bind to the OEC.(15) (16, 17)
This differential binding of ammonia in the $S_3$ state could prove useful in examining the
hydrogen bond network that facilitates water oxidation.

An excellent complimentary technique to these EPR studies would be fourier
transform infrared (FTIR) spectroscopy, because EPR focuses on electrons while FTIR is
able to detect molecular vibrations that can indicate changes in conformation, protonation
state, and solvent polarization.(18-23) The information from FTIR studies can provide a
more complete understanding of these PCET reactions. 3FY should prove to be an
excellent probe for FTIR experiments due to expected shifts in the vibrational modes.(24, 25)
Utilizing 3FY as a marker, we can construct double difference spectra that highlight
only the features contributed from 3FY in light induced PCET reactions.(13, 26, 27)
Water oxidation is driven by complex PCET reactions that have been evolved over billions of years. Here, we shed light on PCET reaction of redox-active tyrosines within PSII set in the $S_0$ and $S_2$ states. $Y_Z^*$ decay is very sensitive to the oxidation state of the OEC and hydrogen bond disruptors like ammonia while remaining insensitive to pH. PSII is a complex enzyme that hosts two very unique tyrosine residues. We have shown that both $Y_Z$ and $Y_D$ have unique kinetic properties. Ammonia was shown to be a very specific probe of $Y_Z$ while having no effect on $Y_D$ kinetics. This information will help build a fundamental understanding of the processes that are necessary to harness light energy.

5.2 References


Field Sweep Spectra

Figure A.1. Field sweep spectra of $Y_Z^+$ under illumination at 190 K throughout the pH range 5.0-7.5 in $^1\text{H}_2\text{O}$ (blue) and $^2\text{H}_2\text{O}$ (red).
Transient EPR spectra

Figure A. 2. EPR transient spectra for the recombination of $Y_{Z}^\cdot$ at 190 K throughout the pL range 0f 5.0-7.5. The pL is denoted on the left side within each spectra the transient for $^1\text{H}_2\text{O}$ is blue and $^2\text{H}_2\text{O}$ is red.
Figure A.3. SDS-urea denaturing polyacrylamide gel electrophoresis of PSII enriched thylakoid membranes. Thylakoids were exchanged into solvents from pH 5.0 – 9.0 as shown in the gel. Each lane was loaded with 6 µg chlorophyll.
Figure A.4. Rate of oxygen evolution as a function of pH.
APPENDIX B

CHAPTER 3 SUPPORTING INFORMATION

Table B.1. $S_0 Y_2$: Triexponential fits to transient data throughout the pH range in either $^1$H$_2$O or $^2$H$_2$O buffers using the equation $f(t) = A_1e^{(-K_1t)} + A_2e^{(-K_2t)} + A_3e^{(-K_3t)} + C$. Percentage contribution of each phase is shown in parenthesis next to the value for the amplitude. Units of rate constants, s$^{-1}$.

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Table B.2. S₂YZ: Triexponential fits of transient data throughout the pH range in either $^1$H₂O or $^2$H₂O buffers using the equation $f(t) = A_1e^{(-K_1t)} + A_2e^{(-K_2t)} + A_3e^{(-K_3t)} + C$. Percentage contribution of each phase is shown in parenthesis next to the value for the amplitude. Units of rate constants, s⁻¹.

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<td>A3 (phase)</td>
<td>K3</td>
<td>C (phase)</td>
</tr>
<tr>
<td>5</td>
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<td>2.51</td>
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<td>113 (38)</td>
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<td>-6 (2)</td>
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<td>5.5</td>
<td>136 (24)</td>
<td>1.19</td>
<td>211 (37)</td>
<td>0.186</td>
<td>210 (37)</td>
<td>0.027</td>
<td>-13 (2)</td>
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<tr>
<td>6</td>
<td>216 (28)</td>
<td>0.81</td>
<td>252 (32)</td>
<td>0.158</td>
<td>290 (37)</td>
<td>0.029</td>
<td>-17 (3)</td>
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<tr>
<td>6.5</td>
<td>164 (23)</td>
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<td>0.169</td>
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<td>159 (30)</td>
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<td>173 (32)</td>
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<td>-27 (5)</td>
</tr>
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<td>0.221</td>
<td>213 (51)</td>
<td>0.032</td>
<td>-1 (0)</td>
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Figure B.1. $S_{0YZ}$: Plots of rate constants versus pH in $^1\text{H}_2\text{O}$ buffers (A) $K_1$, (B) $K_2$, (C) $K_3$ and $^2\text{H}_2\text{O}$ buffers (D) $K_1$, (E) $K_2$, (F) $K_3$. See Table B.1.
Figure B.2. $S_0YZ$: Plots of the amplitudes of kinetic phases versus pH in (A) $^1$H$_2$O and (B) $^2$H$_2$O buffers. See Table B.1.
Figure B.3. $S_2YZ$: Plots of rate constants versus pL for triexponential fits in $^1H_2O$ buffers (A) $K_1$, (B) $K_2$, (C) $K_3$ and $^2H_2O$ buffers (D) $K_1$, (E) $K_2$, (F) $K_3$. See Table B.2.
Figure B.4. $S_2YZ$: Plots of the amplitudes of kinetic phases versus pH in (A) $^1H_2O$ and (B) $^2H_2O$ buffers. See Table B.2.
Figure B.5. Plots of the total $Y_Z$ spectral amplitude, normalized for total chlorophyll concentration, in the $S_0$ and $S_2$ states as a function of pL and solvent isotope exchange.
Figure B.6. (A) Representative EPR spectrum of Mn-depleted Y_{D•} at 190 K and pH 7.0. The spectrum was acquired in the dark. (B) Representative EPR transient reflecting the decay of Y_{Z•} at pH 7.0 (black) with the fit overlaid (red) and the residual across the bottom (black) at 190K.
APPENDIX C

PROTON COUPLED ELECTRON TRANSFER REACTIONS OF

TYROSINE Z SET IN THE S₃ STATE

by

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Materials and Methods

Sample Preparation. PSII was isolated from market spinach.(1) The average oxygen evolution rate was 800 \( \mu \text{mol O}_2/\text{mg chl-hr} \).(2) The samples were solvent exchanged by repeated centrifugation at 100,000 x g in a \(^1\text{H}_2\text{O}\) or \(^2\text{H}_2\text{O}\) (99% Cambridge Isotopes, Andover, MA) buffer containing 0.4 M sucrose, 15 mM NaCl, and 50 mM buffer at each of the following pL values: pL 5.0 (succinate), 6.0 (2-(N-morpholino)ethanesulfonic acid, (MES), 6.5 (MES), and 7.0 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, (HEPES). The final concentration was 2 mg/mL, and samples were stored at -70 °C. The pL is reported as the uncorrected meter reading, accounting for the solvent isotope dependence of the glass electrode and the compensating change in the pKa of acids and bases in \(^2\text{H}_2\text{O}\).(3)

\( S_3YZ \cdot \text{Experiments} \). After a 15 min dark adaptation to trap the \( S_1 \) state, samples were homogenized with 500 \( \mu \)M potassium ferricyanide and flashed twice at 1 Hz with a 532 nm flash from a Continuum (Santa Clara, CA) Surelite III Nd:YAG laser at room temperature. The laser intensity was 40 mJ/cm\(^2\), and the beam was expanded with a cylindrical lens. The 100 mM ferricyanide stock solution was made either in \(^1\text{H}_2\text{O}\) or \(^2\text{H}_2\text{O}\) on the same day as the experiment. The ferricyanide was present to oxidize the quinone acceptors after each flash. Samples were immediately submerged in liquid N\(_2\) and transferred into the EPR cavity at either 160 or 190 K. A field swept spectrum was recorded to select a field position. Transient data, associated with \( S_3YZ \cdot Q_A^- \) decay at 190 K, were averaged from 2-5 samples, with 15 transients recorded per sample. Transient data, associated with \( S_3YZ \cdot Q_A^- \) decay at 160 K, was averaged from 4 samples, with 15 transients recorded per sample. An offset, averaged from 10 s of data before the flash,
was subtracted. Flash 1 to 15 yielded similar kinetics. The data from 15 flashes were fit with three exponentials and that fit was used to derive a $t_{1/2}$ value. This $t_{1/2}$ value was derived from multiple samples to give an average (mean) and a standard deviation. The EPR analysis was conducted on a Bruker (Billerica, MA) EMX spectrometer equipped with a Bruker ER 4102ST cavity and Bruker ER 4131VT temperature controller. EPR parameters were: magnetic field: 3360 G, microwave frequency: 9.40 GHz, power: 101 mW, modulation amplitude: 5 G, conversion: 20 ms, time constant: 164 ms. High microwave powers were used in order to saturate the $Y_D^*$ signal.\(^{(4, 5)}\)

Two flashes were used to generate the $S_3$ state from a dark-adapted sample in the $S_1$ state. The percentage of the $S_3$ state can be estimated by assuming 0% misses on the first flash (see above) and 14% misses on the second flash.\(^{(6)}\) The expected distribution after two flashes is 0% $S_1$, 14% $S_2$, 86% $S_3$, and 0% $S_0$. Thus, the state generated with two flashes is primarily $S_3$, but has a small contribution from $S_2$.

*Field-swept EPR spectra.* Following collection of the transient EPR data, EPR spectra were measured by illuminating the sample within the EPR cavity with red filtered light (600 µmol photons/m$^2$-s, Dolan Jenner Industries, Boxborough, MA) at 190 K. To show that the expected $Y_Z^*$ hyperfine splitting is observed, the microwave power and modulation amplitude were decreased compared to the conditions used for the EPR transients. Because the microwave power is low, this field swept spectrum also has a contribution from $Y_D^*$. The spectra shown are the average of three samples with three spectra obtained for each sample. EPR parameters were: frequency: 9.46 GHz, power: 0.64 mW, and modulation amplitude: 2 G (Figure 3.2) or 5 G (Figure 3.4), conversion time: 41 ms, time constant: 164 ms, sweep time: 21 s.
Results

In Figure C.1A, we show a representative field sweep spectrum of S₃Y⁺ at pH 6.0 and 190 K. The red arrow in Figure C.1A indicates the magnetic field used to monitor the transient decay depicted in Figure C.1B. Figure C.1B shows kinetic transient data for p¹H 6.0 (black) and p²H 6.0 (red) at 190 K. The data was fit with three exponentials and the fits were overlaid in red and black for the ¹H₂O and ²H₂O data respectively.

Figure C.2A plots the t½ for the decay of S₃Y⁺ at 190 K as a function of pL. Data acquired in ¹H₂O and ²H₂O are shown in black and red respectively. t½ values were calculated from the fitting parameters to yield values of 1.6, 1.3, and 1.2 s for pH 5.0, 6.0, and 7.0 respectively. Data derived from samples in ²H₂O exhibited t½ values of 1.9, 2.5, 2.4 s for pD 5.0, 6.0, and 7.0 respectively. These rates are on a similar timescale to rates reported for the S₀ and S₂ states.(4) Figure C.2B plots the KIE as a function of pL for the decay of S₃Y⁺ at 190 K. The KIEs were determined by dividing the t½ in ²H₂O by the t½ in ¹H₂O illustrated in Figure C.2A. KIEs were calculated to be 1.2, 2.0, and 2.0 for pL 5.0, 6.0, and 7.0 respectively. The KIEs reported here are on similar magnitudes to KIEs previously reported for the S₀ and S₂ states.

In Figure C.3, we plot of the initial intensity of S₃Y⁺ transients as a function of the number of laser flashes at 190 and 160 K in ¹H₂O and ²H₂O. Intensities are shown for 14 laser flashes. p¹H data are shown for 5.0 (black), 6.0 (red), and 7.0 (blue) at 190 K. p²H data are shown for 5.0 (green), 6.0 (orange), and 7.0 (grey) at 190 K. p¹H data is shown for 6.5 (purple at 160 K.)
Figure C.1 (A) Representative EPR spectrum of the $S_z \cdot Y_Z$ state at 190 K and pH 6.0. The spectrum was acquired under red-filtered illumination. The red arrow shows the magnetic field used to monitor kinetics. (B) Representative EPR transients reflecting the decay of $Y_Z \cdot$ at pH 6.0 (black) and pH 6.0 (red) at 190 K. Transient are overlaid with fits to the data in red ($^1\text{H}_2\text{O}$) and black ($^2\text{H}_2\text{O}$). Residual for the fits are shown below the data in the same color as the transient data.
Figure C.2. (A) pL dependence of $Y_Z^*$ recombination in the $S_3$ state at 190 K. Data were acquired either in $^1\text{H}_2\text{O}$ (black) or $^2\text{H}_2\text{O}$ (red) buffers. (B) pL dependence of the kinetic isotope effect for $S_3Y_Z^*$ recombination, derived from the data in (A).
Figure C.3. Plot of the initial intensity of $S_3Y_Z^\cdot$ transient as a function of the number of laser flashes at 190 and 160 K. Intensities are shown for 14 laser flashes with standard deviation shown as plus or minus one standard deviation. $p^{1}H$ data are shown for 5.0 (black), 6.0 (red), and 7.0 (blue) at 190 K. $p^{2}H$ data are shown for 5.0 (green), 6.0 (orange), and 7.0 (grey) at 190 K. $p^{1}H$ data is shown for 6.5 (purple at 160 K).

Figure C.4A shows the field sweep spectrum of $S_3Y_Z^\cdot$ at pH 6.5 and 160 K. The red arrow in Figure C.4A indicates the magnetic field used to monitor the transient decay depicted in Figure C.4B. Figure C.4B shows kinetic transient data for $p^{1}H$ 6.4 (black) at 160 K. The data was fit with three exponentials and the fit was overlaid in red. The $t_{1/2}$ value was derived from the fitting equation for the overall half time for the decay of $S_3Y_Z^\cdot$ at 160 K.
Figure C.4. (A) Representative EPR spectrum of the $S_3 Y_Z \cdot$ state at 160 K and pH 6.5. The spectrum was acquired under red-filtered illumination. The red arrow shows the magnetic field used to monitor kinetics. (B) Representative EPR transients reflecting the decay of $Y_Z \cdot$ at pH 6.5 (black) at 160 K. Transient data is overlaid with the fit to the data in red. Residual for the fit is shown below the data in black.

**Discussion**

Preliminary data presented for the decay of $S_3 Y_Z$ at 190 K potentially has an interesting pH dependence that accelerates the rate at low pH (5.0). This type of pH dependence is similar to the data previously acquired for $Y_D \cdot$.(3) However, given the
error at pL 5.0 it is not currently possible to assert this conclusion. Increasing the data set for S₃ state at pL 5.0 should make analysis facile. At pL 6.0 and 7.0 significant KIEs were recorded with an average value of 1.98. The magnitude of this KIE is indicative of a CPET mechanism.

During data acquisition in the S₃ state at 190 K, a trend in the amplitude of the initial intensity of transients was noticed. The amplitude data is shown in Figure C.3. Most notably the trend in amplitude of the data at 190 K S₃ YZ pD 7.0 (grey data in Figure C.3) caught our attention. The intensity decreased with every laser flash. We were able to reset each of these samples by incubating them in the dark on ice for 30 minutes, which sets the OEC back in the S₁ state. Following this incubation, we administered two laser flashes at 1 Hz and quickly frozen the sample in liquid nitrogen resetting the S₃ state. The samples that were reset into the S₃ state regained 100% of their initial intensity and decreased in amplitude at a similar rate to the previous data acquisition. This caused concern that at 190 K the S₃ to S₀ transition was not fully inhibited.

In order to determine if the S₃ to S₀ transition was inhibited, S₃ YZ samples were run at 160 K. Figure C.3 shows the amplitude for pH 6.5 samples set in the S₃ state as a function of the number of laser flashes administered. The trend at 160 K is similar to the trend at 190 K. The decrease in amplitude is therefore not attributed to the S₃ to S₀ state transition. This data lends confidence to the data at 190 K and begins the exploration of the S states at lower temperatures.

S₃ YZ data was analyzed at 160 K to yield a t ½ value of 9.52 ± 4.75 s. The error in this experiment is higher than desired. Data for 160 K was analyzed for five samples, which is on the same order as samples analyzed in the S₀ and S₂ states at 190 K. It could
be that as the temperature is decreased and the length of the transient decay increases that
the error will increase.

References


VITA
James M. Keough

James M. Keough was born in Stone Mountain, Georgia. He attended public schools in West Lafayette, Indiana and Peachtree City, Georgia. He received a B.S. in Chemistry with a concentration on Biochemistry from the Georgia Institute of Technology in 2006. In 2007, he began his doctoral study at the Georgia Institute of Technology by continuing his undergraduate work in Professor Bridgette Barry’s laboratory. When he is not working, he enjoys travelling and fishing with his wife and friends.