Photosynthesis produces dioxygen at photosystem II (PSII), which is located in the thylakoid membrane of plant chloroplasts. Chloride (Cl\textsuperscript{-}) is required for oxygen evolution activity and other anions are known to activate or inhibit O\textsubscript{2} evolution. In this study, the inhibitory effects of fluoride (F\textsuperscript{-}) and nitrite (NO\textsubscript{2}\textsuperscript{-}) were investigated to gain insight into the requirement for Cl\textsuperscript{-}. The way the pH dependence is affected might reveal the type of residue that the Cl\textsuperscript{-} binds to. In the first part of the study, a simplified model followed by a comprehensive kinetic model for the pH dependence of O\textsubscript{2} evolution under F\textsuperscript{-} inhibition in intact PSII was built based on the data of T. Delaney Santoro from the same lab, which included several F\textsuperscript{-} concentrations. The results show that the comprehensive model fit the experiment data quite well. The dissociation constants between enzyme E and its complexes EH\textsuperscript{+} and E(H\textsuperscript{+})\textsubscript{2}, pK\textsubscript{1} and pK\textsubscript{2}, were found to be 5.1 and 7.2. The inhibition constants of F\textsuperscript{-} binding to E, EH\textsuperscript{+} and E(H\textsuperscript{+})\textsubscript{2} were 15.8 mM, 381 mM and 2.0 mM, respectively. In the second part of the study, the NO\textsubscript{2}\textsuperscript{-} dependence of O\textsubscript{2} evolution by PSII was characterized. The investigation showed that NO\textsubscript{2}\textsuperscript{-} activated
O$_2$ evolution at low concentrations and inhibited at the higher concentrations in both NaCl-washed PSII and intact Cl$^-$ depleted PSII. This characteristic of nitrite is similar to that of iodide (I$^-$) and nitrate (NO$_3^-$). The kinetics of NO$_2^-$ action in O$_2$ evolution was modeled as substrate inhibition, in which NO$_2^-$ activates from the Cl$^-$ site and inhibits from a second site. It also suggested that the inhibition due to NO$_2^-$ in PSII lacking extrinsic subunits PsbP and PsbQ was primarily uncompetitive with uncompetitive inhibition constant $K_i$' of 0.60 mM, while the NO$_2^-$ inhibition in intact PSII was probably in an uncompetitive mode with inhibition constant $K_i$' of 14 mM. However, the competitive inhibition constants $K_i$ for both types of PSII were not well determined due to large error in the data. The Michaelis constants $K_m$ for NO$_2^-$, Cl$^-$ and NO$_3^-$ were found to be 0.33 mM, 0.54 mM and 0.16 mM for intact Cl$^-$ depleted PSII. The $K_m$ values for NO$_2^-$, Cl$^-$, and NO$_3^-$ without substrate inhibition were 2.1 mM, 5.0 mM, and 5.5 mM for NaCl-washed PSII, respectively. These values are fairly close and all are within the error range for each type of PSII.
MATHEMATICAL MODELS FOR THE pH DEPENDENCE OF OXYGEN EVOLUTION UNDER FLUORIDE INHIBITION AND EFFECTS OF NITRITE ON OXYGEN EVOLUTION IN PHOTOSYSTEM II

by

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CHAPTER I
INTRODUCTION

It has been well known that photosynthesis produces oxygen. Almost all molecular oxygen or dioxygen we breathe is produced by photosynthesis. As a plentiful energy source, the sun gives off light that is absorbed by plants. Plants transform light energy into chemical energy in the process that converts water into oxygen. This process is a part of the photosynthesis. Chloroplasts are the subcellular organelles where photosynthesis occurs, and many molecular and biochemical details of photosynthesis are known. However, the precise molecular mechanism of oxygen evolution is still unclear.

The oxygen-evolving complex (OEC) in photosystem II (PSII) can extract electrons from water and produce molecular oxygen as a byproduct. Scientists have found that different kinds of anions and molecules have different effects on the function of the OEC: anions such as chloride (Cl⁻) are essential and accelerate the oxygen evolution process;¹ but fluoride (F⁻), amines, and some other anions and molecules inhibit this process.² The topic of Cl⁻ effects on oxygen evolution of PSII is still of interest in the field.³ The effects of other anions on the oxygen evolution process have also been studied by scientists. For
instance, anions like Br\(^{-}\) activate the oxygen evolving process, but a few chemicals and anions such as F\(^{-}\), CH\(_3\)COO\(^{-}\), N\(_3\)\(^{-}\), Tris, NH\(_3\), etc. are inhibitory to the process. Some studies confirmed that I\(^{-}\) and NO\(_3\)\(^{-}\) activated the oxygen evolution process at lower concentrations and acted as an inhibitor at higher concentrations in intact Cl\(^{-}\) depleted PSII and NaCl-washed PSII.\(^{[4,5]}\) Meanwhile, the pH of the medium in PSII also has effects on the oxygen producing process.\(^{[6]}\)

This research focused on two topics. The first topic was the mathematical models for the pH dependence of oxygen evolution rate under fluoride inhibition in PSII. The relationship between anion effects and pH effects is complex. The pH effects are often related to proton transfer and/or H-bond systems at the catalytic site. Anions may promote the correct participation of the proton in catalysis. So far, the mathematical models of anion inhibition with pH dependence have not been well developed. The second topic was the effects of NO\(_2\)\(^{-}\) on the oxygen evolution rate in PSII. Some previous studies suggested that NO\(_2\)\(^{-}\) may have similar effects as I\(^{-}\) on the oxygen evolution rate of PSII with both activation and inhibition characteristics.\(^{[4]}\) The mode of the effect of nitrite (NO\(_2\)\(^{-}\)) on oxygen evolution has not previously been fully studied.
CHAPTER II
BACKGROUND AND LITERATURE REVIEW

Photosynthesis is a process that uses light energy to convert carbon dioxide and water into carbohydrates and molecular oxygen. The overall process can be expressed as

\[6\text{CO}_2 + 6\text{H}_2\text{O} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 \quad \text{Reaction 1}\]

where \(\text{C}_6\text{H}_{12}\text{O}_6\) represents a sugar molecule such as glucose. The reaction requires energy to proceed \((\Delta G^\circ = 2870\ \text{kJ/mol})\). Photosynthesis can be broken down into two sets of reactions: the light reactions and the dark reactions.

**Overall Light Reactions**

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

**Overall Dark Reactions**

\[\text{CO}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow (\text{CH}_2\text{O})_n + \text{H}_2\text{O} \quad \text{Reaction 3}\]

These reactions are carried out in chloroplasts that can be found in mesophyll cells under the surface of plant leaves. Chloroplasts enclose a network of thylakoid membranes, which contain all of the proteins and pigments (chlorophylls and carotenoids) required for photosynthesis. Light-trapping pigments along with associated proteins are
organized into large membrane-embedded complexes called photosystems. Each photosystem is responsible for absorbing a light photon and converting some of its energy into a chemical form.\[9\]

**Photosystems**

Two kinds of photosystems are involved in photosynthesis in plants: photosystem I (PSI) and photosystem II (PSII). PSI has light absorbance up to 700 nm and PSII has light absorbance up to 680 nm. The two systems are linked in a series to carry out the electronic transfer of the light reactions (Figure 1). The overall reactions of the photosystems are

\[
\text{PSII: } 2\text{H}_2\text{O} + 2\text{Q} \xrightarrow{\text{light}} \text{O}_2 + 2\text{QH}_2
\]

**Reaction 4**

\[
\text{PSI: } \text{NADP}^+ + \text{H}^+ + 2\text{PCred} \xrightarrow{\text{light}} \text{NADPH} + 2\text{PCox}
\]

**Reaction 5**

in which PC is plastocyanin.
Electrons transfer from H₂O to QH₂ in PSII while electrons transfer from PC_red to NADPH in PSI. PSII plays the key role in oxygen evolution. The oxygen evolution process depends on the light harvesting complexes, the photochemical reaction center, and the oxygen-evolving complex. In PSII, after light energy is absorbed by light harvesting complexes containing chlorophylls and carotenoids, the excitation energy is transferred to the photochemical reaction center for charge separation. At the same time, oxidizing equivalents in the oxygen-evolving complex are accumulated and four of the equivalents are used to convert two molecules of water to one molecule of oxygen. Several electron transfer components are involved in the process of photochemical reaction in PSII. In the photochemical
reaction, P680 (PSII reaction center chlorophyll) is excited upon light absorption and transfers an electron to pheophytin (Ph, a chlorophyll molecule without magnesium). The electron is then transferred to plastoquinone Q₆. Ultimately, two electrons are sent to a second plastoquinone Q₆, with concurrent binding of two protons. After leaving PSII, the reduced plastoquinone, plastoquinol (QH₂), diffuses through the membrane and interacts with cytochrome b₆f (Cyt b₆f), which is a membrane-bound complex of cytochromes and iron-sulfur proteins. Cyt b₆f catalyzes the transfer of the electrons to plastocyanin (PC) which passes the electrons to the P700 reaction center of PSI.[7, 10]

**Protein Subunits of PSII**

PSII is a multi-protein complex containing intrinsic subunits including D1, D2, CP43, CP47, psbl gene product, cytochrome b₅₅₉, and extrinsic subunits including 33 kDa, 23 (or 24) kDa, 17 (or 18) kDa, 12 kDa, and Cytochrome b₅₅₀, depending on the species (Figure 2). The crystal structure of oxygen-evolving PSII has been studied by Zouni et al. in 2001,[11] by Kamiya et al. in 2003,[12] and by Loll et al. in 2005.[13] The intrinsic subunits are made up mostly of α-helical transmembrane structure. D1 (Psba) and D2 (Psbd) subunits are the core of the PSII reaction center, and bind
a variety of cofactors. Most electron transfer occurs in the D1 and D2 proteins. CP47 (PsbB) and CP43 (PsbC) transfer excitation energy from the light harvesting chlorophylls to the chlorophylls in the PSII reaction center. Cyt b_{559} (PsbE and PsbF) is a redox center.\[^{14}\]

The extrinsic subunits enhance oxygen evolution activity at physiological concentrations of inorganic cofactors, and appear to regulate the access of water and ions at the OEC. Without the 33 kDa (PsbO) subunit, the manganese cluster is unstable under low chloride conditions. Removal of the 23 kDa (PsbP) and 17 kDa (PsbQ) proteins increases the amount of calcium and chloride required for optimal oxygen evolution. Therefore, these two subunits function in the regulation of Cl\(^-\) and Ca\(^{2+}\) in OEC. It is known that all PSII contains the 33 kDa protein. However, the higher plant PSII also contains the 17 kDa and 23 kDa subunits, whereas the cyanobacteria PSII has the Cyt c\(_{550}\) (PsbV) and 12 kDa (PsbU) subunits.\[^{11, 15, 16}\] The presence of the 33 kDa subunit is a necessary condition for the binding of the 17 and 23 kDa subunits on the PSII membrane. They are the three largest extrinsic subunits. Washing with 1.0 M NaCl can remove the 17 and 23 kDa subunits, while washing with 1.0 M CaCl\(_2\) removes all three subunits.\[^{1}\] The overall architecture for OEC is now well known.\[^{17}\] Figure 2 presents a schematic depiction of the subunit arrangement in PSII.
Figure 3 shows a schematic view of the OEC based on the finding of Ferreira et al.\textsuperscript{[17]} In the figure, residues Q165, H190, D170, E333, D61, E65, E189, H337, H332 and D342 as well as TyrZ and C-term are located on the D1 intrinsic subunit; residues E312 and K317 are on the D2 intrinsic subunit; residues R357 and E354 are on the CP43 intrinsic subunit; X\textsubscript{11}, X\textsubscript{21} and X\textsubscript{22} are possible substrate water bound to Mn4 (X\textsubscript{11}) and to Ca\textsuperscript{2+} (X\textsubscript{21} and X\textsubscript{22}). W indicates the possible water molecules that are not visible at the current resolution. The dotted lines are hydrogen bonds.
Franzén and coworkers studied the roles of the extrinsic subunits in PSII using EPR. Their research showed that removal of the 16 and 24 kDa proteins by treating with 1 M NaCl solution inhibited the oxygen evolution activity, but rapid electron transfer to Z+ was still observed. Removal of the 33 kDa subunit with MgCl₂, however, almost completely inhibited the oxygen evolution process, indicating that the 33 kDa subunit was necessary for oxygen evolution. Many other papers also addressed this topic.
Oxygen Evolution Process

After P680 is oxidized to P680⁺, it is electron deficient and regains electrons from water. A protein containing a cluster of four Mn atoms accepts the electrons from water. This Mn cluster cycles through five oxidation states in what is called the Kok cycle or S-state cycle (Figure 4), and passes the electrons to P680⁺ via an intermediate tyrosine radical (Yz·).[7]

Photon absorption and electron transfer from P680 (or charge separation) cause formation of P680⁺ which then oxidizes Yz to Yz·. Then Yz· extracts an electron from the OEC and induces the S₀ → S₁ transition. Subsequent photon absorptions by P680 drive the S₁ → S₂, the S₂ → S₃ and the S₃ → S₄ transitions, where each is one oxidation step. The S₄ state, the highest oxidation state, is unstable and releases dioxygen, resetting the cycle.[18]

There are two charge recombination processes in PSII, one involves the S states of the water-oxidizing complex at the electron donation side and the other is in the plastoquinone molecules (Qₐ and Qₐ) at the electron acceptor side.[19]
So far, two water oxidation mechanisms have been proposed, the metal cluster mechanism and the metallo-radical mechanism.\cite{18, 20} The metal cluster mechanism has been described by Ferreira et al. while the research on metallo-radical mechanism was described by Hoganson and Babcock.\cite{17, 21}

**Effects of Inorganic Cofactors on PSII**

Manganese, calcium and chloride are three primary inorganic cofactors of PSII that are vital to oxygen evolution. Four Mn ions are needed for the conversion of
water into oxygen. These Mn ions comprise the manganese complex or cluster that is the core of the OEC.\cite{17, 21}

Calcium, a cationic activator of \( O_2 \) evolution, is required at the oxidizing side of PSII, and is now required to be a part of the manganese cluster as \( \text{Mn}_4\text{Ca} \).\cite{14, 22, 23} Calcium-depleted PSII loses oxygen evolving ability. Without the 17 and 23 kDa proteins in PSII membrane (NaCl-washed PSII), a higher amount of \( \text{Ca}^{2+} \) is needed for maximal oxygen evolution activity. Calcium is essential for the Kok cycle to proceed beyond the \( S_2 \) state. A wide range of other metal ions has been tested to replace \( \text{Ca}^{2+} \), however, only strontium (\( \text{Sr}^{2+} \)) can promote OEC activity, but with about 50\% as much as that of \( \text{Ca}^{2+} \). It was found that the substitution of lanthanides for \( \text{Ca}^{2+} \) inhibited oxygen evolution activity, which indicates that the binding site of \( \text{Ca}^{2+} \) is the active site of the \( O_2 \) evolving reaction. Analyses and studies strongly support the viewpoint that there is one \( \text{Ca}^{2+} \) ion binding in the OEC and that one or more \( \text{Ca}^{2+} \) ions are bound to other sites of PSII membranes. Recent research has showed that \( \text{Ca}^{2+} \) resides near the Mn cluster at a distance of 3.4-3.5 Å, and is a part of the \( \text{Mn}_4\text{-Ca} \) cluster in the OEC. Most recently, it was confirmed by X-ray diffraction studies that \( \text{Ca}^{2+} \) was ligated at least partly by amino acid side chain residues of the 33 kDa subunits (PsbO).\cite{23, 24} Another binding site for \( \text{Ca}^{2+} \) is in
the antenna protein ensemble, and the other sites are unknown. So far as we know, none of the 17 and 23 kDa extrinsic subunits can retain tightly bound to Ca\(^{2+}\).

As an activator of oxygen evolution, chloride plays an important and specific role in the regulation of redox reaction in PSII.\(^{[14, 25]}\) Cl\(^{-}\) activation has been widely studied and is still a subject of active experimentation. PSII samples without Cl\(^{-}\) show inactivation of the OEC. Chloride facilitates oxidation of the Mn cluster by the Tyr-Z\(^{+}\) radical through its activation effect.\(^{[26]}\) The maximal oxygen evolution activity of PSII can be reversibly decreased by the depletion of Cl\(^{-}\) using a variety of methods such as washing with Cl\(^{-}\) free buffers, washing at alkali pH and dialysis in the presence of other anions. PSII membranes lacking the 17 and 23 kDa proteins can be easily depleted of Cl\(^{-}\). These PSII membrane preparations can still reach S\(_{1}\) and S\(_{2}\) states in the absence of Cl\(^{-}\), indicating that chloride apparently binds at the S\(_{2}\) or earlier S state. The depletion of it inhibits the advancement of the OEC beyond S\(_{2}\), that is, Cl\(^{-}\) is necessary for the S-cycle going beyond S\(_{2}\) state. In fact, it is required for the S\(_{2}\) \(\rightarrow\) S\(_{3}\) transition, and is also required for the transition from S\(_{3}\) to S\(_{0}\). Like calcium, however, the mechanistic details for the way that Cl\(^{-}\) affects the redox reaction in the OEC
remain obscure. Chloride has been proposed to bind to the manganese cluster or a site very close to manganese.

**Effects of Other Anions and Amines on Oxygen Evolution**

Anions and amines can have a variety of effects on the oxygen evolving process ranging from activation to inhibition. Cl\(^-\), Br\(^-\), I\(^-\) and NO\(_3\)\(^-\) activate the oxygen evolving process. Also, Lindberg et al. found that bromide binds in a manner similar to that of Cl\(^-\) in PSII and activates oxygen evolution,\(^{[27]}\) and that removal of the slowly exchanging chloride lowers the stability of PSII, which means that Cl\(^-\) is vital for oxygen evolution activity.\(^{[28]}\) On the other hand, F\(^-\), NH\(_3\), amines, N\(_3\)\(^-\) (azide), Tris etc. inhibit the process. The mechanism for inhibition can be competitive with Cl\(^-\) activation and/or uncompetitive, depending on the ion type. Fluoride inhibits the oxygen evolution process completely by preventing the S\(_2\) → S\(_3\) transition by Cl\(^-\) competitive inhibition. It was found that declining enzymatic activity of the PSII reaction center resulted from increasing the F\(^-\) concentration in an EPR study of the signals from the Mn\(_4\)-Ca cluster.\(^{[29]}\) N\(_3\)\(^-\) inhibits oxygen evolution activity because it is a competitor of Cl\(^-\).\(^{[30, 31]}\) However, some ions such as I\(^-\) can be either activators or inhibitors under certain conditions.\(^{[2]}\) I\(^-\) inhibits oxygen evolution from an uncompetitive site, but
activates the process from the Cl⁻ site. The overall order of oxygen evolution suppression or inhibition ability was reported as NO₃⁻ < I⁻ < F⁻ < CH₃COO⁻.

**Oxygen Evolution Rate in PSII**

Intact PSII preparations contain membrane fragments with membrane-embedded PSII complexes of complete sets of subunits. They can be prepared from spinach thylakoid membranes or other plants. Intact PSII shows high oxygen evolution activity with little or no apparent dependence on Ca²⁺ or Cl⁻. It is difficult to remove Cl⁻ from intact PSII, since the best Cl⁻ depletion methods still leave 30% or more activity. By removing the extrinsic 17 and 23 kDa proteins from the intact PSII, the PSII complexes may lose most of their oxygen evolution activities in the absence of Ca²⁺ or Cl⁻. These preparations are often called “salt-washed PSII” or NaCl-washed PSII, since they are made from the treatment of intact PSII with 1-2 M NaCl solution thereby removing the 17 (or 18) and 23 (or 24) kDa subunits. Calcium and chloride are required for NaCl-washed PSII to maintain oxygen evolution activity because the 17 and 23 kDa subunits probably regulate the local concentrations of Ca²⁺ and Cl⁻. To help understanding the details of anion activation and inhibition, intact and NaCl-washed PSII preparations can be compared. The use of NaCl-washed PSII
facilitates Cl\(^-\) depletion, which will help to reveal the real tendencies and effects of Cl\(^-\) and other anion activators and inhibitors on the oxygen evolution activity.

**pH Dependence of the Oxygen Evolution in PSII**

The pH of the medium has important effects on the oxygen evolution activity of PSII as for other enzymes. The oxygen evolution rate increases with pH until a peak range around pH 6.3-6.5 is reached. At higher pHs, the oxygen evolution rate decreases with the pH value.\(^6\) Most enzymes also show this type of pH dependence with some optimal pH. The pH effect can be expressed in the following reactions.

\[
\begin{align*}
E(H^+)_2 & \overset{K_1}{\underset{K_2}{\rightleftharpoons}} EH^+ + H^+ & \overset{K_2}{\underset{K_1}{\rightleftharpoons}} E + 2H^+
\end{align*}
\]

in which E is the enzyme. Here EH\(^+\) is the active form, but E(H\(^+\))\(_2\) and E are not. The kinetic model can be written as

\[
A = \frac{A_{\text{max}}}{1 + \frac{[H]}{K_1} + \frac{K_2}{K_1}[H]}
\]

Equation 1

where A is the oxygen evolution activity, \(A_{\text{max}}\) is the maximum activity when all enzyme is in the EH\(^+\) complex form, \(K_1\) and \(K_2\) are proton dissociation constants, and [H] stands
for the concentration of $\text{H}^+$. The presence of other ions, however, can affect the apparent $pK_a$'s.

**Kinetics of Oxygen Evolution in PSII**

The Cl$^-$ activation of oxygen evolution of PSII can be modeled using the Michaelis-Menten equation.\textsuperscript{[27]} The Michaelis-Menten equation expresses the kinetics of reactions between enzymes and substrates, and it has the form of

$$ v = \frac{V_{\text{max}}[s]}{K_m + [s]} $$

\textbf{Equation 2}

Here $v$ is the reaction rate, $V_{\text{max}}$ is the maximal reaction rate or the limit of the reaction velocity, $K_m$ is the Michaelis constant or the substrate concentration at which half-maximal rate is achieved, and $[s]$ is the substrate concentration.

Lineweaver and Burk converted this equation to

$$ \frac{1}{v} = \frac{K_m}{V_{\text{max}}[s]} + \frac{1}{V_{\text{max}}} $$

\textbf{Equation 3}

The plot of $1/v$ vs. $1/[s]$ can be used to find $K_m$ and $V_{\text{max}}$ since the line intersects with the $1/[s]$ axis at $-1/K_m$.\textsuperscript{[35]}

In the presence of inhibitors, the interaction process can be represented as
where \( E, I, S, P, ES \) or \( ESI \) expresses enzyme, inhibitor, substrate, product, enzyme-substrate complex, or enzyme-substrate-inhibitor complex, respectively. \( K_i \) is the dissociation constant of the \( EI \) complex (or competitive inhibition constant), and \( K_i' \) is the dissociation constant of the \( ESI \) complex (or uncompetitive inhibition constant).

We have

\[
Ki = \frac{[E][I]}{[EI]}
\]

Equation 4

\[
K_i' = \frac{[ES][I]}{[ESI]}
\]

Equation 5

When a competitive inhibitor is present, the Michaelis-Menten equation becomes

\[
v = \frac{V_{\text{max}}[s]}{K_m \left(1 + \frac{[I]}{K_i} \right) + [s]}
\]

Equation 6

where \([i]\) is the concentration of inhibitor. Equation 6 can be converted to Equation 7.
The plot of $1/v$ vs. $[i]$ is known as the Dixon plot.$^{[35]}$ The intersection point of the straight lines for various $[s]$ values provides a measure of $K_i$. The Dixon model is useful for competitive inhibition, in which only the $EI$ complex forms, but it does not distinguish between competitive and mixed inhibitors. For mixed or uncompetitive inhibitors, it provides no measure of enzyme-inhibitor-substrate (EIS) complex.$^{[36]}$

If mixed, uncompetitive and non-competitive inhibitors are also considered, the inhibition kinetic model becomes

$$v = \frac{V_{\text{max}} [s]}{K_m \left[ 1 + \frac{[i]}{K_i} \right] + [s] \left[ 1 + \frac{[i]}{K_i'} \right]}$$

Equation 8

Cornish-Bowden developed an analysis method based on this equation:

$$\frac{[s]}{v} = \frac{K_m}{V_{\text{max}}} \left( 1 + \frac{[i]}{K_i} \right) + \frac{[s]}{V_{\text{max}}} \left( 1 + \frac{[i]}{K_i'} \right)$$

Equation 9

A plot of $[s]/v$ vs. $[i]$ is a Cornish-Bowden plot.$^{[36]}$ The intersection point of the straight lines for different $[s]$ values provides a measure of $K_i'$. The Dixon plots and the
Cornish-Bowden plots for the various inhibition types or modes are shown in Figure 5.

The mathematical models for the effects of ions on oxygen evolution activity of PSII have an important role in the research of oxygen evolution. They have been widely used in the study of the effects of anion activators and inhibitors. Chloride can be treated as a substrate in these models. In these treatments, Cl$^-$ is actually an activator, not a substrate; but the treatment works anyway.
Figure 5 Dixon plots and Cornish-Bowden plots for different inhibition modes [36]
**Literature Review**

Many studies of the kinetic models of the effects of anions, small molecule effectors and pH in the oxygen evolving process have been done. The studies of the pH dependence of oxygen evolution activity for Cl\(^-\) activation began decades ago. In a review article published in 1985, Christa Critchley summarized the research on the pH dependence of oxygen evolution activity with or without added Cl\(^-\) in intact Cl\(^-\) depleted PSII up until that time.\(^{[15]}\) These studies included those of Gorham et al. in 1952, the Izawa et al. in 1969, the Theg et al. in 1982, and the Critchley in 1983. The studies were carried out using Cl\(^-\) deficient thylakoid membranes, and showed that the optimal pH was 6.0-7.5 without added Cl\(^-\) and the optimal pH was 6.5-8.0 in the presence of added Cl\(^-\).

Early in 1984, Sandusky and Yocum carried out experiments on NH\(_3\) and Tris inhibition of oxygen evolution activity in spinach PSII membranes (they used a PSII preparation.) at various chloride concentrations and presented both Dixon plots and Cornish-Bowden plots of the data.\(^{[37]}\) From the plots, \(K_i\) and \(K'_i\) were found to be 0.22 mM \(\text{NH}_3\) and 0.58 mM \(\text{NH}_3\) respectively. The two values were close but not equal, which indicated that \(\text{NH}_3\) was a mixed competitive inhibitor of Cl\(^-\) activation, with one site probably identified with the Cl\(^-\) binding site. Therefore, the severity of \(\text{NH}_3\)
inhibition of photosynthetic oxygen evolution was attenuated in the presence of Cl⁻. The higher concentrations of Cl⁻ resulted in lower inhibition levels.

Later on, Sandusky and Yocum published their research on F⁻ inhibition of oxygen evolution on PSII in 1986.[38] They studied the effect of F⁻ at various concentrations of Cl⁻ and found that F⁻ was also an inhibitor of oxygen evolution in competition with chloride at pH 7.5. The Dixon and the Cornish-Bowden plots showed that Kᵢ was about 4 mM and Kᵢ’ was greater than 60 mM. The Kᵢ’ value was 10 fold more than the Kᵢ value which indicated that F⁻ was a competitive inhibitor of Cl⁻ in oxygen evolution.

In 1988, Peter Homann published his studies of the pH effects on the Cl⁻ and Ca²⁺ requirements of photosynthetic water oxidation.[6] First, he investigated the pH dependence of the Cl⁻ activation of intact Cl⁻ depleted PSII and 17 and 23 kDa polypeptide-depleted PSII from Phytolacca for the pH range between 4.3 and 7.4. He found that the residual activity was considerably higher at lower pH, and that the concentration of Cl⁻ had little effect on the oxygen evolution activity for both types of PSII when pH < 5. The Lineweaver-Burke plots (1/v versus 1/[Cl⁻]) showed that the oxygen evolution activity was more sensitive to the change of Cl⁻ concentration at higher pH than at lower pH for PSII both with and without the extrinsic 17 and 23 kDa
polypeptides in the pH range of 5.1-7.4. After that, Homann studied the pH dependence of the activation of the 17 and 23 kDa polypeptide-depleted PSII by Ca$^{2+}$ using *P. americana* and *S. oleracea* PSII membranes, in the presence of 35 mM NaCl. It was evident that O$_2$ evolution activity reaches its peak value in the pH range of 6.0-7.0. In the case of the 17 and 23 kDa protein-depleted PSII, the overall activity decreased substantially at all pHs. The O$_2$ evolution activity increased with the addition of CaCl$_2$, but was still below the activity of PSII containing the 17 and 23 kDa subunits. These results indicate that the O$_2$ evolution activity is reduced after removing the subunits, and that CaCl$_2$ stimulates the activity, which is a typical effect observed for NaCl-washed PSII. The studies also showed that there was no difference in the oxygen evolution activity in intact *P. americana* PSII with or without added CaCl$_2$ (2 mM). The pH value for optimal activity was 6.0-6.5 for intact PSII. The peak pH value for NaCl-washed PSII was 6.5-7.0 in the absence of CaCl$_2$, but shifted back to 6.0-6.5 in the presence of CaCl$_2$. The pH dependence plots for both *P. americana* and *S. oleracea* PSII membranes revealed that the plot shapes at higher pH values (the right side of the peaks) did not change and only those at lower pH values (the left side of the peaks) changed after the extrinsic subunits were removed.
On the other hand, studies related to the effects of NO$_2^-$ of oxygen evolution of PSII were conducted in only a few research groups. In a study on the photosynthetic nitrite reduction by dithioerythritol and the effect of nitrite on electron transport,[39] Spiller and Böger found that, in a medium containing ferricyanide, 72% inhibition of oxygen evolution rates was reached by adding 10 mM NO$_2^-$ in the fragmented chloroplasts isolated from the heterocont alga Bumilleriopsis filiformis. They also found that NO$_3^-$ inhibited the oxygen evolution process. The plot of % inhibition vs. log [NO$_2^-$] gave a straight line with a positive slope, which meant the inhibition increased with the concentration of NO$_2^-$; the plot of % inhibition vs. log [NO$_3^-$] gave a concave exponential, which also meant that the inhibition effect enhanced with the concentration of NO$_3^-$.

Moreover, NO$_2^-$ was a much stronger inhibitor, compared to NO$_3^-$.

In 1985, Stemler and Murphy reported that oxygen evolution rates could be inhibited by 60% in maize chloroplasts in the presence of 20 mM NO$_2^-$. [40] This investigation further confirmed that NO$_2^-$ inhibited oxygen evolution activity in PSII.

Recently, Sahay et al. reported their studies on the site of action of nitrite inhibition of oxygen evolution in PSII.[19] Using thermoluminescence in spinach thylakoid
membranes under ion-deficient and ion-sufficient conditions, they found that the site of action of NO$_2^-$ was at the side associated with electron donation in PSII, which was unique to NO$_2^-$ inhibition. Other anions tested in their studies, such as formate, fluoride and nitrate, could not replace NO$_2^-$ at the side associated with electron donation for producing the same inhibitory effect.

The effects of Br$^-$, I$^-$, NO$_3^-$, F$^-$ and CH$_3$COO$^-$ on oxygen evolution activity of Cl$^-$ depleted PSII were studied by Hasegawa and coworkers who used FTIR spectroscopy. Their results revealed that the overall features of spectra from Br$^-$, I$^-$, and NO$_3^-$ substituted PSII were similar to those of Cl$^-$, which confirmed their ability to support oxygen evolution. However, the spectra of F$^-$ and CH$_3$COO$^-$ substituted PSII were rather different, which was related to the suppression of oxygen evolution by these ions. They also investigated the dependence of oxygen evolution rates on the concentrations of all the anions under saturating and limiting light conditions. The dependence plots showed that under either light condition Br$^-$ and NO$_3^-$ had activating effects similar to Cl$^-$, and that their activating ability had the order of Cl$^- >$ Br$^- >$ NO$_3^-$. Meanwhile, I$^-$ behaved like an activator at low concentrations and an inhibitor at higher concentrations; F$^-$ and CH$_3$COO$^-$ were only inhibitory, and their inhibiting effects were very close
with CH$_3$COO$^-$ slightly stronger under the limiting light conditions. In general, the activity in the presence of all related anions under saturating light was much higher than those under limiting light (over 7 fold higher in the case of Cl$^-$).
CHAPTER III
MATERIALS AND METHODS

This thesis focuses mainly on analysis of complex enzyme kinetic data that were collected by previous students in the Haddy laboratory. Therefore, only a few of the experiments presented were performed by the author. T. Delaney Santoro performed the experiments on the pH dependence of oxygen evolution under F\(^-\) inhibition in intact PSII, based on the method of Thomas Kuntzleman. Madhu Kumar collected all of the data from the experiments on NO\(_2^-\) inhibition in the presence of Cl\(^-\). The author completed the experiments on NO\(_2^-\), NO\(_3^-\) and Cl\(^-\) dependence of oxygen evolution in intact Cl\(^-\) depleted PSII and NaCl-washed PSII. The intact Cl\(^-\) depleted PSII membranes were prepared by Xiaoming Li. The NaCl-washed PSII was prepared by Alice Haddy and the author, using intact PSII membranes provided by Alice Haddy.

I. The Methods for Experiments

Preparation of Intact PSII

The original preparation method of intact PSII-enriched thylakoid membranes was described by Berthold and coworkers in 1981.\(^{[33]}\) The preparation used here was modified from the original by others a few years later.\(^{[16, 42]}\) The PSII
was generally prepared by other members of the laboratory and stored as pellets at about 10 milligrams of chlorophyll per mL (mg Chl/mL) in Buffer I (0.4 M sucrose, 20 mM MES and 15 mM NaCl, pH = 6.3, adjusted with NaOH) in liquid nitrogen.

**Preparation of NaCl-washed PSII**

NaCl-washed PSII-enriched membranes were prepared from intact PSII, by a method similar to that reported by Miyao and Murata in 1983.[34] It was slightly changed by the author’s research advisor Alice Haddy, and consists of the following procedure.

About 5-10 mg Chl of intact PSII was suspended into 30 mL of Buffer III (0.4 M sucrose, 20 mM MES, and 1.5 M NaCl, pH = 6.3, adjusted with NaOH) using a small brush. The mixture was incubated on ice for 30-60 minutes, and then centrifuged at 13 K rpm in a Beckman JA 20 rotor for 8 minutes. The pellets were resuspended in 30 mL of Buffer II (0.4 M sucrose and 20 mM MES, pH = 6.3, adjusted by using NaOH) and the mixture centrifuged 8 minutes at 13 K rpm in a Beckman JA 20 rotor. This step was repeated twice. After resuspending the NaCl-washed PSII to about 1.5 mg Chl/mL in Buffer II, it was stored as 1.5 mL aliquots in liquid nitrogen for future use.
Chloride Depletion Method of Intact PSII

Removal of Cl\textsuperscript{-} from intact PSII was carried out by dialysis, which was conducted by Xiaoming Li as described in his thesis\cite{5}. This technology was based on the method presented by Lindberg et al.,\cite{28} and modified by pretreating the intact PSII with Br\textsuperscript{-}.

Determination of Chlorophyll Concentration

Before measuring oxygen evolution rates, the concentration of chlorophyll was determined according to the method of Arnon (1949).\cite{43} A PSII suspension was diluted using a solution of 80% (v/v) acetone and 20% (v/v) demineralized water. The diluted mixture was centrifuged to remove the starch as a pellet. The absorbance was measured at 645 nm and 663 nm using a UV-Vis spectrophotometer (Shimazu UV-1201). Three duplicate measurements were averaged.

The chlorophyll concentration of the diluted mixture was calculated by using the following equations.\cite{43} For chlorophyll a and chlorophyll b in mg/L, we have

\[ C_a = -2.69A_{645} + 12.7A_{663} \] \hspace{1cm} \text{Equation 10}

\[ C_b = 22.9A_{645} - 4.68A_{663} \] \hspace{1cm} \text{Equation 11}

\[ C_{\text{total}} = 20.2A_{645} + 8.0A_{663} \] \hspace{1cm} \text{Equation 12}
where $C_a$, $C_b$ and $C_{total}$ are the concentrations of chlorophyll a, chlorophyll b, and all chlorophylls, and $A_{645}$ and $A_{663}$ are the absorbance values at 645 nm and 663 nm, respectively. The actual concentration of chlorophyll in the PSII suspension was determined by considering the dilution factor from dissolving in the 80% acetone solution.

**Assay of Oxygen Evolution Activity**

Oxygen evolution activity was measured using a Clark-type O$_2$ selective electrode (Yellow Spring Instruments model 5331) equipped with a temperature-controlled water bath, a glass-jacketed reaction cell, a signal processor, saturating light sources, a stirrer, and a computer with data collection software programmed by Sergei Baranov. The temperature of the water bath, the reaction cell, and assay buffers was kept at 25°C during the assays.

The PSII sample to be measured was chilled on ice. The oxygen electrode was calibrated using demineralized water and water saturated with O$_2$ (about 260 µM O$_2$). Before the assay, the buffer was purged with N$_2$ gas to remove O$_2$. The PSII sample was incubated with the buffer in the reaction cell for 2 minutes before the light sources were turned on. As electron acceptor, 1 mM phenyl-p-benzoquenone (PPBQ) was added from a 50 mM stock solution in dimethyl sulfoxide. The maximum slope of voltage due to O$_2$ reduction at the
electrode versus time was taken, and using the relevant input parameters, the oxygen evolution rate was calculated by the program. All of the given rates represent the averages of three or more measurements.

The assay buffers contained the specified concentrations of F⁻, NO₂⁻, NO₃⁻ or Cl⁻. They were prepared by adding NaF, NaNO₂, NaNO₃ or NaCl from stock solutions to Buffer II or Buffer IV (0.4 M sucrose and 20 mM MES, pH = 6.3, adjusted with Ca(OH)₂), depending on the application. The assay buffers for the experiments of NO₂⁻, NO₃⁻ and Cl⁻ dependence of oxygen evolution were prepared by the author, and the rest of the assay buffers were prepared by other students during their own experiments.

**pH Dependence of O₂ Evolution under Fluoride Inhibition in Intact PSII**

The pH dependence data were collected by T. Delaney Santoro according to the experimental method presented by Thomas Kuntzleman. A series of buffers from pH 4 to pH 8 was made using glutaric acid (pH 4.01-4.61), malic acid (pH 4.81-5.41), itaconic acid (pH 5.15-5.75), MES (pH 5.79-6.39), PIPES (pH 6.46-7.06), MOPS (pH 6.90-7.50), and HEPES (pH 7.20-7.80). Each buffer solution contained 20 mM buffer, 1.0 mM NaCl, 0.4 M sucrose, and the specified amount of NaF. A control measurement in 0.4 M sucrose, 20
mM MES, 1.0 mM NaCl, and 30 mM NaF (pH = 6.3) was used to normalize the measurements from the various pHs.

II. Data Analysis Methods

Tools for Mathematical Modeling

Sigma Plot 8.0 was used as a tool for mathematical modeling. This software is a state-of-the-art technical graphing program designed for the Windows platform.[45] After the data for oxygen evolution rates were averaged or converted to the data for Dixon plots and Cornish-Bowden plots, they were input into the worksheets. Then the equations used to fit the data were set up as subroutines based on the related equilibria among enzymes, substrates, inhibitors, and their combinations. The initial parameters were set and the data fitted to the equation using a nonlinear least squares routine, giving optimized values for the dissociation constants. The fitted curves were plotted on the graphs along with the original data.[46]

Mathcad 2000 was used as a tool to assist in the data treatment and analysis, to complement the Sigma Plot calculations.
Calculation of the pH Dependence of Oxygen Evolution Activity under F⁻ Inhibition

For each pH dependence curve, the oxygen evolution activity was initially modeled using Equation 1 to find apparent dissociation constants in the presence of varying amounts of F⁻. This analysis provided a starting point before extending the model to more complex equilibria.

\[
A = \frac{A_{\text{max}}}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}}
\]

where \(K_1\) is the apparent proton dissociation constant for an acidic residue, \(K_2\) is the apparent proton dissociation constant for a basic residue, \(A_{\text{max}}\) is the theoretical maximum activity as the acidic residue is deprotonated and the basic residue is protonated optimally. The pH dependence curve in the absence of F⁻ was taken to represent true values of \(K_1\) and \(K_2\), the protonation and deprotonation constants of the residues that determine the uninhibited pH dependence.

For the pH dependence of oxygen evolution under F⁻ inhibition, two models were used to account for the effect of the inhibitor. The simpler model assumed binding of F⁻ to two protonation states of PSII. This simplified model can be expressed as
and the pH dependence equation from the above scheme is

\[ A = \frac{A_{\text{max}}}{1 + \frac{[H]}{K_1} + \frac{K_2}{[H]} + [F]\left(\frac{1}{K_{i1}} + \frac{[H]}{K_{i1}K_{i2}}\right)} \]

Equation 13

Here \([F]\) is the concentration of \(F^-\). \(K_{i1}\) and \(K_{i2}\) are the dissociation constants of fluoride inhibition for \(EH^+F^-\) and \(E(H^+_2)F^-\) respectively.

The modeling process was carried out in two stages. (1) \(A_{\text{max}}, K_1\) and \(K_2\) were found first, by fitting the data of 0 mM \(F^-\) (the sample without \(F^-\)) using Sigma Plot. Using the values of \(A_{\text{max}}, K_1\) and \(K_2\), the values of \(K_{i1}\) and \(K_{i2}\) were determined by separately fitting the data for 5, 10, 15 and 20 mM of \(F^-\). The values obtained for \(K_{i1}\) or \(K_{i2}\) are expected to be the same for all concentrations of \(F^-\), but actually they were somewhat different for various concentrations of \(F^-\). (2) Using the value obtained for \(A_{\text{max}}\) as a constant, and using the obtained values of \(K_1\) and \(K_2\) and the selected values of \(K_{i1}\) and \(K_{i2}\) from Step (1) as initial guesses for parameters, the curve fitting was carried out for all \(F^-\).
concentrations in one regression. The best values of $K_1$, $K_2$, $K_{i1}$ and $K_{i2}$ for all curves were reached through adjusting the parameters within the Sigma Plot program. Along with $A_{max}$, these best $K$ values were the constants for the simplified model.

For the comprehensive model, $F^-$ was assumed to bind to three protonation states of PSII. In this case the reaction scheme is

\[
\begin{align*}
F^- & \quad F^- \\
E + H^+ & \quad EH^+ + H^+ \\
K_{i0} & \quad K_{i1} \\
EF^- & \quad EH^+ F^- \\
K_{i2} & \quad K_{i2}
\end{align*}
\]

Equation 9

and the pH dependence equation is

\[
A = \frac{A_{max}}{1 + \frac{[H]}{K_1} + \frac{K_2}{[H]} + [F] \left( \frac{1}{K_{i1}} + \frac{[H]}{K_1K_{i2}} + \frac{K_2}{K_{i0}[H]} \right)}
\]

Equation 14

which now includes $K_{i0}$, the dissociation constant for $EF^-$. As in the simplified model, the values for $A_{max}$, $K_1$ and $K_2$ were determined from the 0 mM $F^-$ data, followed by the determination of the dissociation constants for inhibition ($K_{i0}$, $K_{i1}$, and $K_{i2}$) for each concentration of $F^-$. Then, using $A_{max}$ as a constant and the obtained $K_1$, $K_2$, $K_{i0}$, $K_{i1}$ and $K_{i2}$
values as initial guesses for parameters, all Ks were fitted in one regression by adjusting the parameters. Eventually, a single set of constants was found using the same method as described above. The set of constants fits all fluoride concentrations simultaneously.

*Kinetic Models of NO$_2^-$ or NO$_3^-$ Dependence of O$_2$ Evolution in PSII*

After removal of the 17 and 23 kDa subunits, PSII is expected to have no O$_2$ evolving activity in the absence of added activating anions such as Cl$^-$. However, the NaCl-washed PSII may still contain very small amount of residual Cl$^-$ even though it has been washed in Cl$^-$ free buffer. Meanwhile, NO$_2^-$ or NO$_3^-$ has both activating and inhibiting effects, so the expression (Equation 15) for the NO$_2^-$ or NO$_3^-$ dependence of O$_2$ evolution rate in NaCl-washed PSII consists of two parts: the first part is based on a substrate inhibition model including activation as well as inhibition, and the second is a constant, $V_0$, that accounts for the low residual activity.$^{[4]}$ The substrate inhibition model can be expressed by the scheme of Reaction 10, in which E and S are enzyme and substrate respectively.
In Equation 15, $K_m$ is the Michaelis constant for activation, $[s]$ is the concentration of substrate (NO$_2^-$ or NO$_3^-$ here), $K_i$ is the dissociation constant for inhibition, $V_{\text{max}}$ is the maximum reaction velocity, and $V_0$ is the residual activity at 0 mM NO$_2^-$ or NO$_3^-$. For each experiment, the control experiment for activation of oxygen evolution by chloride was performed. Chloride activation of O$_2$ evolution is well known, and follows the Michaelis-Menten equation, Equation 16, including the residual activity that is probably due to bound Cl$^-$.\textsuperscript{[5]}

\[
v = \frac{V_{\text{max}}[s]}{K_m + [s]} + V_0
\]  Equation 16
where \([s]\) is the concentration of substrate, which is chloride here. The values of \(K_m\), \(V_{\text{max}}\), and \(K_i\) in Equations 15 and 16 were found using the Sigma Plot program, by curve fitting of the experimental data points. Equation 16 is Equation 2 with the addition of \(V_0\).

In the case of intact PSII, the model is essentially the same model as that for NaCl-washed PSII except that there is a high amount of residual activity at zero added anion. The high residual activity is because PSII dialyzed against a Cl^- free buffer to remove bound Cl^- loses only about 65% of the control activity.\(^{[27]}\) Moreover, the Cl^- depletion was done by Br^- pretreatment, so residual activity may be due to bound Cl^- or Br^-.

Equation 17 below is for the NO_2^- or NO_3^- dependence of O_2 evolution in intact Cl^- depleted PSII. It is similar to Equation 15, except that the residual activity is also subject to inhibition.\(^{[4]}\)

\[
v = \frac{V_{\text{max}}[s]}{K_m + [s] + \frac{[s]^2}{K_i}} + \frac{V_0}{1 + \frac{[s]}{K_i}}
\]

Equation 17

The control experiments to measure Cl^- activation were also carried out using intact Cl^- depleted PSII. The exact constant values were determined as for NaCl-washed PSII.
CHAPTER IV

RESULTS

I. Mathematical Models for pH Dependence of Oxygen Evolution Rates under F⁻ Inhibition in Intact PSII

pH Dependence under F⁻ Inhibition of Oxygen Evolution in Intact PSII

In previous experiments carried out in the Haddy lab, T. Delaney Santoro investigated the pH dependence of F⁻ inhibition of O₂ evolution in intact PSII in the presence of 1 mM NaCl. The data showed a clear changing pattern of O₂ evolving activity vs. pH under the various concentrations of F⁻ (Figure 6), and they represent a complex equilibrium between PSII, F⁻, and H⁺. The maximum in the O₂ evolution activities shifted to higher pH while the overall activity decreased. The peak values were about 110%, 90%, 80%, 75%, and 65% activity for 0 mM, 5 mM, 10 mM, 15 mM, and 20 mM F⁻ concentrations respectively, corresponding to the optimal pHs of 6.2, 6.3, 6.4, 6.5, and 6.6.

To approach the problem, the data were analyzed using increasing levels of complexity. The equations of the kinetic models for the pH dependence of F⁻ inhibition of O₂ evolution in intact PSII were derived according to the related reaction schemes shown as follows. First, the individual curves were fit to a model assuming two protonation events (Reaction 6), as given in Equation 1.
Figure 6  pH dependence of O₂ evolution rate in intact PSII under F⁻ inhibition. The data were collected by T. Delaney Santoro, and 1 mM Cl⁻ was present in each sample. The curves represent fits assuming only 2 acid dissociation events.
This model assumes that EH\(^+\) is the active species. By analyzing the data using Sigma Plot, the values of \(A_{\text{max}}\), \(K_1\) and \(K_2\) for 0 mM F\(^-\) were found to be 130\%, 7.5\times10^{-6} \text{ M} \) and 6.0\times10^{-8} \text{ M}, where \(A_{\text{max}}\) is the maximum activity or the activity when all E related species are in the form of EH\(^+\). The y-scale for the figure is expressed in percentage, where 100\% was set to an observed rate at pH 6.3 and the value was used as a reference. Using \(A_{\text{max}} = 130\%\) as a constant and fitting to Equation 1, the \(pK_1\) and \(pK_2\) values for 5, 10, 15 and 20 mM fluoride concentrations were obtained using Sigma Plot in separate fits (Table 1). In Table 1, the values of \(pK_1\) and \(pK_2\) are those of the acidic dissociation constant \(pK_a\) and the basic dissociation constant \(pK_b\).

<table>
<thead>
<tr>
<th>Constant</th>
<th>0 mM F(^-)</th>
<th>5 mM F(^-)</th>
<th>10 mM F(^-)</th>
<th>15 mM F(^-)</th>
<th>20 mM F(^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pK_1)</td>
<td>5.12±0.053</td>
<td>5.66±0.047</td>
<td>5.89±0.057</td>
<td>6.06±0.046</td>
<td>6.21±0.046</td>
</tr>
<tr>
<td>(pK_2)</td>
<td>7.23±0.056</td>
<td>7.13±0.051</td>
<td>7.05±0.058</td>
<td>7.00±0.048</td>
<td>6.85±0.047</td>
</tr>
</tbody>
</table>

The acidic \(pK_a\) (or \(pK_1\) increased and the basic \(pK_a\) (or \(pK_2\) decreased with increasing F\(^-\) concentration. Because these \(pK_a\) values are only apparent values, further analysis
was undertaken to determine inherent dissociation constants for F⁻ binding.

**A Simplified Model of the pH Dependence of O₂ Evolution under F⁻ Inhibition**

Based on the above analysis, a kinetic model was built. This model includes equilibria for both protonation and F⁻ binding to two forms of the enzyme, EH⁺ and E(H⁺)₂, as shown in Reaction 8. EH⁺ is still assumed to be the active species, while all other forms of the enzyme are inactive. Although F⁻ is a competitive inhibitor of Cl⁻, the equilibrium with Cl⁻ is not shown and sufficient Cl⁻ is assumed to be present.

In the scheme of Reaction 8, EH⁺ complex was again assumed to be the active center that can give product (O₂). The mathematical expression is given in Equation 13. The model presented by Reaction 8 and Equation 13 is a simplified model since the binding of F⁻ and E is not included. The reason for this was to limit the number of parameters that must be fitted. It can be seen that the greater effect of F⁻ was on the protonated forms, and thus we simplified the modeling process.

During the curve-fitting process using Sigma Plot, three steps were carried out: (1) A_max, K₁ and K₂ were determined with the data for 0 mM F⁻; (2) A_max, K₁ and K₂ were used as constants to find K₁ and K₂ for 5, 10, 15 and 20 mM F⁻ in
separate fits, but these $K_{i1}$ and $K_{i2}$ were somewhat different for the different $F^-$ concentrations; (3) As parameters, $K_1$, $K_2$, $K_{i1}$ and $K_{i2}$ were fitted for all $F^-$ concentrations in one step. During the fitting process, the $A_{max}$ value obtained in Step (1) was used as a constant, the $K_1$ and $K_2$ values obtained in Step (1) were used as initial parameters for $K_1$ and $K_2$, one of the $K_{i1}$ values obtained in Step (2) was selected as the initial parameters for $K_{i1}$, and the same method was used to select the initial parameter for $K_{i2}$. After the initial parameters were input into the Sigma Plot routine, the fitted parameters of $K_1$, $K_2$, $K_{i1}$ and $K_{i2}$ were found by adjusting the initial parameters to make $R^2$ optimal. This resulted in one unique value for each of $K_1$, $K_2$, $K_{i1}$ and $K_{i2}$, which fit all concentrations of $F^-$ and represented the simplified model. Generally, the existence of $F^-$ would not impact the protonation and deprotonation equilibria. Therefore, $A_{max}$, $K_1$ and $K_2$ would be unchanged with the concentration of $F^-$. After fitting the data for 0 mM $F^-$, it was found that $A_{max}$, $K_1$, and $K_2$ were 130%, $7.5 \times 10^{-6}$ M, and $6.0 \times 10^{-8}$ M, and certainly, the values were the same as in the last section. In the next step, $K_{i1}$ and $K_{i2}$ for 5, 10, 15 and 20 mM $F^-$ were determined separately. The values are listed in Table 2, where $K_{i1}$ and $K_{i2}$ are slightly different for the different concentrations of fluoride and range from 33-61 mM for $K_{i1}$.
and 2.3-2.7 mM for $K_{i2}$. It must be mentioned that $K_{i1}$ or $K_{i2}$ should have only one value for each and does not change with fluoride concentration. The individual fits were designed to find a reasonable value range for $K_{i1}$ or $K_{i2}$ respectively. This made the modeling process easier. The plots of activity vs. pH are given in Figure 7 through Figure 11.

Table 2  $K_{i1}$ and $K_{i2}$ of the simplified model for different F$^-$ concentrations by individually curve-fitting

<table>
<thead>
<tr>
<th>Inhibition Constant</th>
<th>5 mM F$^-$</th>
<th>10 mM F$^-$</th>
<th>15 mM F$^-$</th>
<th>20 mM F$^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{i1}$ (mM)</td>
<td>61 ± 45</td>
<td>50 ± 26</td>
<td>60 ± 32</td>
<td>33 ± 11</td>
</tr>
<tr>
<td>$K_{i2}$ (mM)</td>
<td>2.3 ± 0.5</td>
<td>2.5 ± 0.7</td>
<td>2.4 ± 0.7</td>
<td>2.7 ± 1.0</td>
</tr>
</tbody>
</table>
Figure 7  Individual fitted plot of the simplified model for pH dependence of O$_2$ evolution of PSII for 0 mM F$^-$. The data were collected by T. Delaney Santoro, and 1 mM Cl$^-$ was present in each sample. $A_{max}$, $K_1$ and $K_2$ were fitted here. $A_{max} = 130\%$, $K_1 = 7.5 \times 10^{-6}$ M, and $K_2 = 6.0 \times 10^{-8}$ M.
Figure 8 Individual fitted plot of the simplified model for pH dependence of O₂ evolution of PSII for 5 mM F⁻. The data were collected by T. Delaney Santoro, and 1 mM Cl⁻ was present in each sample. Aₘₐₓ, K₁ and K₂ were set, and K₁₁ and K₁₂ were fitted here. Aₘₐₓ = 130%, K₁ = 7.5x10⁻⁶ M, and K₂ = 6.0x10⁻⁸ M. K₁₁ and K₁₂ are shown in Table 2.
Figure 9 Individual fitted plot of the simplified model for pH dependence of O₂ evolution of PSII for 10 mM F⁻. The data were collected by T. Delaney Santoro, and 1 mM Cl⁻ was present in each sample. Aₘₐₓ, K₁ and K₂ were set, and Kᵢ₁ and Kᵢ₂ were fitted here. Aₘₐₓ = 130%, K₁ = 7.5×10⁻⁶ M, and K₂ = 6.0×10⁻⁸ M. Kᵢ₁ and Kᵢ₂ are shown in Table 2.
Figure 10 Individual fitted plot of the simplified model for pH dependence of O₂ evolution of PSII for 15 mM F⁻. The data were collected by T. Delaney Santoro, and 1 mM Cl⁻ was present in each sample. $A_{\text{max}}$, $K_1$ and $K_2$ were set, and $K_{i1}$ and $K_{i2}$ were fitted here. $A_{\text{max}} = 130\%$, $K_1 = 7.5 \times 10^{-6}$ M, and $K_2 = 6.0 \times 10^{-8}$ M. $K_{i1}$ and $K_{i2}$ are shown in Table 2.
Figure 11 Individual fitted plot of the simplified model for pH dependence of O₂ evolution of PSII for 20 mM F⁻. The data were collected by T. Delaney Santoro, and 1 mM Cl⁻ was present in each sample. \( A_{\text{max}} \), \( K_1 \) and \( K_2 \) were set, and \( K_{i1} \) and \( K_{i2} \) were fitted here. \( A_{\text{max}} = 130\% \), \( K_1 = 7.5 \times 10^{-6} \) M, and \( K_2 = 6.0 \times 10^{-8} \) M. \( K_{i1} \) and \( K_{i2} \) are shown in Table 2.
A single set of values for $K_1, K_2, K_{i1}$ and $K_{i2}$ was found by combining the data for all F$^-$ concentrations into one regression analysis using the Sigma Plot program. Again, the value for $A_{\text{max}}$ was set to 130%, but all four equilibria were allowed to vary. This represents the final fit to the simplified model, with equilibria as in Reaction 8 and kinetic equation as in Equation 13. All parameter values and their standard errors are presented in Table 3. $R^2$ for the regression or the curve fitting equals 0.9509, where $R$ is correlation coefficient. The plots of the final simplified model are given in Figure 12.

Table 3  Equilibrium constants and their standard errors of the final simplified model found from a simultaneous fit of all curves

<table>
<thead>
<tr>
<th>Constant</th>
<th>Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pK_1$</td>
<td>5.11</td>
<td>0.053</td>
</tr>
<tr>
<td>$pK_2$</td>
<td>7.12</td>
<td>0.036</td>
</tr>
<tr>
<td>$K_{i1}$ (mM)</td>
<td>78</td>
<td>30</td>
</tr>
<tr>
<td>$K_{i2}$ (mM)</td>
<td>2.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Figure 12 Plots for the simplified model of pH dependence of O₂ evolution rate under F⁻ inhibition in intact PSII. The data were collected by T. Delaney Santoro, and 1 mM Cl⁻ was present in each sample. All curves were fitted simultaneously with A_max set and K₁, K₂, K₁₃, and K₁₂ allowed to vary. Refer to Table 3 for the values found.
Two interrelated observations about this model were revealed. First, the model did not include the equilibrium for the binding of F⁻ and OEC (E). Therefore, the model did not fit the experimental data well at the higher pH. A more complex model, which includes the equilibrium for the binding between F⁻ and E, was then developed.

**A Comprehensive Model of the pH Dependence of O₂ Evolution under F⁻ Inhibition**

The more complex (or the comprehensive) model included three equilibria for F⁻ binding to E, EH⁺ and E(H⁺)₂. Therefore, three parameters (K₁₀, K₁₁ and K₁₂) had to be fit at once, which was more difficult. The key to successfully fitting the data to the comprehensive model mainly depends on the method to find all dissociation constants for all related equilibriums in one regression using Sigma Plot program. Like the simplified model, EH⁺ was treated as the only active center. From the equilibrium scheme of the comprehensive model (see Reaction 9), the kinetic equation was derived as shown in the Appendix A. The equation is similar to that of the simplified model, but with an additional term involving the inhibition dissociation constant for the EF⁻ complex, K₁₀ (Equation 14).

All dissociation constants were fitted in one regression using Equation 14 on the Sigma Plot program. A_{max} was set to
130%, as in the simplified model. Setting \([F] = 0\) mM, \(K_1\) and \(K_2\) were found to be \(7.5 \times 10^{-6}\) M and \(6.0 \times 10^{-8}\) M. These two values were used as initial parameters for \(K_1\) and \(K_2\). \(K_{i1}\) (\(7.8 \times 10^{-2}\) M) and \(K_{i2}\) (\(2.2 \times 10^{-3}\) M) from the combined simplified model were used as the initial parameters for \(K_{i1}\) and \(K_{i2}\). The initial parameter for \(K_{i0}\) was guessed to be \(1.5 \times 10^{-2}\) M. By adjusting the initial parameters for \(K_1\), \(K_2\), \(K_{i0}\), \(K_{i1}\) and \(K_{i2}\) as well as the tolerance and the step size on the Sigma Plot program, the highest possible \(R^2\) (0.9647) for the regression and the lowest possible standard errors for \(K_1\), \(K_2\), \(K_{i0}\), \(K_{i1}\) and \(K_{i2}\) were reached. The results for the fit are shown in Table 4 and Figure 13.

<table>
<thead>
<tr>
<th>Dissociation Constant</th>
<th>Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pK_1)</td>
<td>5.13</td>
<td>0.045</td>
</tr>
<tr>
<td>(pK_2)</td>
<td>7.24</td>
<td>0.043</td>
</tr>
<tr>
<td>(K_{i0}) (mM)</td>
<td>15.8</td>
<td>5.3</td>
</tr>
<tr>
<td>(K_{i1}) (mM)</td>
<td>381</td>
<td>183</td>
</tr>
<tr>
<td>(K_{i2}) (mM)</td>
<td>2.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Figure 13 Plots of the comprehensive model for pH dependence of O₂ evolution in intact PSII under F⁻ inhibition. The data were collected by T. Delaney Santoro, and 1 mM Cl⁻ was present in each sample. All curves were fitted simultaneously with $A_{\text{max}}$ set and $K_1$, $K_2$, $K_{i0}$, $K_{i1}$, and $K_{i2}$ allowed to vary. Refer to Table 4 for the values found.
In summary, the comprehensive model is much better than the simplified model, especially on the higher pH side. This can be seen by comparing Figure 13 with Figure 12, and indicates that the comprehensive model predicts the experimental data quite well.

II. Nitrite Dependence of O₂ Evolution in Intact and NaCl-washed PSII

Nitrite Mode of Inhibition of O₂ Evolution

Some studies on nitrite (NO₂⁻) inhibition of oxygen evolution activity in intact and NaCl-washed PSII were carried out previously in the Haddy lab by M. Kumar in 2001 (unpublished). The author analyzed the data from those experiments and found the competitive inhibition constant, Kᵢ, and the uncompetitive inhibition constant, Kᵢ', for NO₂⁻ for each PSII preparation.

Figure 14 and Figure 15 present the Cornish-Bowden and the Dixon plots of the NO₂⁻ inhibition in intact PSII and corresponding fits. The fitting routine forced the lines to pass through a common point for each case. The intersection points on the Cornish-Bowden plot and the Dixon plot are at (-13.70, 0.000) and (-28.76, -0.001761), respectively. This means that Kᵢ and Kᵢ' for intact PSII were about 29 mM and 14 mM, respectively. In addition, Figures 16 and 17 present the Cornish-Bowden and the Dixon plots of the NO₂⁻ inhibition of oxygen evolution in NaCl-washed PSII.
Intersection points at (-0.5863, 0.008404) and (-11.66, -0.03557) indicate that $K_i$ and $K'_i$ for NaCl-washed PSII were about 12 mM and 0.6 mM, respectively. The $K_i$ and $K'_i$ values including their standard errors are given in Table 5. It can be seen from the table that the standard errors of the competitive constant $K_i$ for both types of PSII are very high. This means that values are essentially undetermined. Examination of the plots reveals that the lines in the Dixon plots for both types of PSII are not well separated and could easily be parallel. Hence the $K_i$s are probably actually very large, so there is very little competitive inhibition.

<table>
<thead>
<tr>
<th></th>
<th>$K_i$ (mM)</th>
<th>$K'_i$ (mM)</th>
<th>Standard error of $K_i$</th>
<th>Standard error of $K'_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intact PSII</strong></td>
<td>29</td>
<td>14</td>
<td>243</td>
<td>12</td>
</tr>
<tr>
<td><strong>NaCl-washed PSII</strong></td>
<td>12</td>
<td>0.60</td>
<td>247</td>
<td>0.72</td>
</tr>
</tbody>
</table>
Figure 14 Cornish-Bowden plot for nitrite inhibition of oxygen evolution in intact PSII. The lines represent the fit to the data. The intersection point is (-13.70, 0.000) and $K_i = 14$ mM. The data were from M. Kumar.
Figure 15 Dixon plot for nitrite inhibition of oxygen evolution in intact PSII. The lines represent the fit to the data. The intersection point is (-28.76, -0.001761) and $K_i = 29$ mM. The data was from M. Kumar.
Figure 16 Cornish-Bowden plot for nitrite inhibition of oxygen evolution in NaCl-washed PSII. The lines represent the fit to the data. The intersection point is (-0.5863, 0.008404) and $K_i = 0.6$ mM. The data were from M. Kumar.
Figure 17 Dixon plot for nitrite inhibition of oxygen evolution in NaCl-washed PSII. The lines represent the fit to the data. The intersection point is (-11.66, -0.03557) and $K_i = 12$ mM. The data were from M. Kumar.
By comparing the results for intact PSII and NaCl-washed PSII, we observe that $K'_i$ decreased after the loss of the subunits, which means that removal of the 17 and 23 kDa subunits affects the constant. This observation suggests that the uncompetitive site in PSII is revealed more by the removal of the subunits and therefore must be located near the oxygen-evolving complex.

The interesting thing revealed in Figures 16 and 17 is the non-linearity of the data for NaCl-washed PSII at higher NO$_2^-$ concentrations. It is noteworthy that, on the plots of the figures, the data points increase and then slip down with increasing concentration of NO$_2^-$, which might indicate that NO$_2^-$ is both an inhibitor and an activator depending on the concentration of NO$_2^-$.

If this is true, the kinetic model for NO$_2^-$ is one for inhibition and activation by the same ion, which is similar to the effect of I$^-$. In fact, a previous study by Wincencjuz et al. suggested that both nitrite and iodide activate oxygen evolution activity.[47] Another research study on I$^-$ effects on O$_2$ evolution was carried out by Bryson et al. and indicated that I$^-$ had both inhibition and activation effects on intact PSII.[4] The Cornish-Bowden plot from that study revealed that the data points for I$^-$ curved upward instead of downward, as for NaCl-washed PSII for NO$_2^-$ shown in Figures 16 and 17. It has been known that I$^-$ is an
activator when its concentration is lower and an inhibitor when its concentration is higher in intact PSII. NO$_2^-$ may be comparable to I$^-$ with NO$_2^-$ acting as an inhibitor at its lower concentration and an activator at its higher concentration in NaCl-washed PSII. We concluded that more studies were needed on the NO$_2^-$ dependence mode of oxygen evolution due to the non-linearity of the inhibition data.

**NO$_2^-$ Dependence of O$_2$ Evolution in PSII**

We carried out an investigation of the NO$_2^-$ activation of oxygen evolution in the absence of added Cl$^-$ for both NaCl-washed PSII and intact Cl$^-$ depleted PSII, from 0 mM through 50 mM nitrite. The results are presented in Figures 18 and 19, which show that NO$_2^-$ has both activation and inhibition effects for each type of PSII. The plots for O$_2$ evolution rate vs. NO$_2^-$ concentration have a similar appearance to that of I$^-$,[4] except with a lower level of activation.

Figure 18 represents NO$_2^-$ dependence of oxygen evolution in NaCl-washed PSII. The oxygen evolution rate starts from 20.5 µmol O$_2$/mg Chl/hr at 0 mM NO$_2^-$ and increases to about 35.7 µmol O$_2$/mg Chl/hr at 2 mM NO$_2^-$, and then it decreases to 23.6 µmol O$_2$/mg Chl/hr at 50 mM NO$_2^-$.

This indicates that NO$_2^-$ activates O$_2$ evolution when its concentration is less than about 2 mM and inhibits the
process when its concentration is greater than about 2 mM. Figure 19 shows NO$_2^-$ dependence of oxygen evolution in intact Cl$^-$ depleted PSII. Because of the high residual Cl$^-$ and/or Br$^-$ that is apparently bound to PSII, the rate begins from about 330 µmol O$_2$/mg Chl/hr at 0 mM NO$_2^-$ and reaches the peak value of 355 µmol O$_2$/mg Chl/hr between 0.4 and 1.0 mM NO$_2^-$ (around 0.7 mM), and thereafter, reduces to 88 µmol O$_2$/mg Chl/hr at 50 mM NO$_2^-$. It has a similar response mode as in the case of NaCl-washed PSII, but the activation reaches the peak at a lower concentration of nitrite than for NaCl-washed PSII.
Figure 18  NO$_2^-$ dependence of O$_2$ evolution rate in NaCl-washed PSII
Figure 19  NO$_2^-$ dependence of O$_2$ evolution rate in intact Cl$^-$ depleted PSII
NO$_2^-$ acts in PSII by substrate inhibition (activation, and then inhibition), which explains both figures. The reaction scheme for substrate inhibition is shown as in Reaction 11. There are two possible activators (Cl$^-$ and NO$_2^-$) and one inhibitor (NO$_2^-$). In the presence of Cl$^-$, activation by NO$_2^-$ is not likely to be observed. For simplicity, the binding of the true substrate, H$_2$O, is not shown in the scheme. In the oxygen evolution process, water is present at extremely high concentrations in the medium of PSII (56 M), so is not
limited in availability; Cl\(^-\) and NO\(_2\)- bind to the oxygen-evolving reaction sites, resulting in increased activities. As activators, these two anions can be treated as substrates, and consequently, the activation and inhibition kinetics can be treated using the substrate activation and inhibition model with the first substrate (Cl\(^-\) or NO\(_2\)-) activating and the second substrate (NO\(_2\)-) inhibiting.\(^{[48, 49]}\) The model explains the activation data from this section without Cl\(^-\) and the inhibition data from the last section. From this point of view and the equilibria in Reaction 11, the kinetic equations were derived (see Appendix B). The equations refer to the bottom half of the reaction scheme in the absence of Cl\(^-\).

The mathematical expression for the kinetic model of O\(_2\) evolution rate with NO\(_2\)- inhibition in NaCl-washed PSII should be composed of two terms, based on Equation 15:

\[
v = \frac{V_{\text{max}}[s]}{K_m + [s] + \frac{[NO_2^-]^2}{K_i}} + V_0
\]

Equation 18

where [NO\(_2\)-] is the concentration of NO\(_2\)-, \(V_{\text{max}}\) is the maximum evolving velocity promoted by NO\(_2\)-, \(K_m\) is the Michaelis constant for activation, and \(K_i\) is the dissociation constant for inhibition, and \(s\) can be either Cl\(^-\) (if it exists) or NO\(_2\)-. The first term represents NO\(_2\)- activation and
inhibition (substrate inhibition). \( V_0 \) represents a constant background rate that probably results from residual bound \( \text{Cl}^- \). For analysis, \( V_0 \) was set to the rate measured at \([\text{NO}_2^-]\) = 0 mM. The values found for \( V_{\text{max}} \), \( K_m \) and \( K_i \) are shown in Table 6. The optimal match was reached at \( R^2 = 0.9204 \) for the curve fitting.

For intact \( \text{Cl}^- \) depleted PSII (pretreated with \( \text{Br}^- \)), because of the high residual \( \text{O}_2 \) evolving activity from bound \( \text{Cl}^- \) and/or \( \text{Br}^- \), the kinetic model must be treated differently. The high \( \text{Cl}^- \) and/or \( \text{Br}^- \) residual activity is inhibited by \( \text{NO}_2^- \) progressively, so an inhibition term needs to be included in the second term to fully consider the fact. This change matches the data points in Figure 19 where the rate at high \([\text{NO}_2^-]\) is much lower than \( V_0 \). The complete model gives the equation

\[
v = \frac{V_{\text{max}}[s]}{K_m + [s] + \frac{[\text{NO}_2^-]^2}{K_i}} + \frac{V_0}{1 + \frac{[\text{NO}_2^-]}{K_i}}
\]

\text{Equation 19}

Again, \( s \) can be \( \text{Cl}^- \) or \( \text{NO}_2^- \) here. The results of the data analysis are also shown in Table 6. The results showed lower standard errors than those obtained from the analysis for \( \text{NaCl} \)-washed PSII. \( R^2 \) for the curve fitting for intact \( \text{Cl}^- \) depleted PSII was 0.9991.
Table 6 Comparison of $V_{\text{max}}$, $K_m$ and $K_i$ values for different PSII preparations under NO$_2^-$ inhibition (refer to Figures 18 and 19)

<table>
<thead>
<tr>
<th>Constant</th>
<th>Intact Cl$^-$/Depleted PSII</th>
<th>NaCl-washed PSII</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ ($\mu$mol O$_2$/mg Chl/hr)</td>
<td>62 ± 6</td>
<td>38 ± 17</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>0.33 ± 0.09</td>
<td>2.1 ± 1.4</td>
</tr>
<tr>
<td>$K_i$ (mM)</td>
<td>14.5 ± 0.6</td>
<td>4.5 ± 3.1</td>
</tr>
</tbody>
</table>

From the comparison of NO$_2^-$ dependence of O$_2$ evolving activity between intact Cl$^-$ depleted and NaCl-washed PSII (Figure 20 and Table 6), we know that the oxygen evolution activity in intact Cl$^-$ depleted PSII is much higher than the activity in NaCl-washed PSII and the inhibition in NaCl-washed PSII is much more evident. The maximum activity for intact Cl$^-$ depleted PSII was about 355 $\mu$mol O$_2$/mg Chl/hr at 0.7 mM NO$_2^-$ while that for NaCl-washed PSII was only 35.7 $\mu$mol O$_2$/mg Chl/hr at 0.7 mM NO$_2^-$. The former is about 10 times as high as the latter. $V_{\text{max}}$ for intact Cl$^-$ depleted PSII is about 1.65 fold as much as that for NaCl-washed PSII. The Michaelis constant for activation ($K_m$) of NaCl-washed PSII is 6.4 fold as high as that for intact Cl$^-$ depleted PSII. These indicate that the NO$_2^-$ dependent
oxygen evolution activity in intact Cl⁻ depleted PSII is much higher than that in NaCl-washed PSII. The $K_i$ value for NaCl-washed PSII is much lower than that for intact Cl⁻ depleted PSII, which means that NO₂⁻ inhibition is much more significant in NaCl-washed PSII than in intact Cl⁻ depleted PSII.

Although about 6.5 mM of Ca²⁺ was present during the assays, the O₂ evolution activity of NO₂⁻ in NaCl-washed PSII was still very low, indicating that the addition of Ca²⁺ did not enhance the activity much. However, under the same conditions, the activity in NaCl-washed PSII can be increased to about 400 µmol O₂/mg Chl/hr by the addition of Cl⁻. This can be verified from Figure 21.
Figure 20 Comparison of NO$_2^-$ dependence of O$_2$ evolution rate in intact Cl$^-$ depleted PSII vs. in NaCl-washed PSII
Figure 21 Comparison of Cl⁻ dependence of O₂ evolution rate in intact Cl⁻ depleted PSII vs. in NaCl-washed PSII
NO\textsubscript{2}\textsuperscript{-} and Cl\textsuperscript{-} Dependence of O\textsubscript{2} Evolution in the PSII for the Study

Since Cl\textsuperscript{-} activation of O\textsubscript{2} evolution has been well studied and reported in many publications before, the comparison of Cl\textsuperscript{-} activation in PSII used in this research is helpful in understanding the differences between intact Cl\textsuperscript{-} depleted PSII (pretreated with Br\textsuperscript{-}) and NaCl-washed PSII. The Cl\textsuperscript{-} activation kinetic model for both NaCl-washed PSII and intact Cl\textsuperscript{-} depleted PSII can be written as

\[ v = V_0 + \frac{V_{\text{max}}[\text{Cl}^-]}{K_m + [\text{Cl}^-]} \]  

Equation 20

in which \( V_0 \) is the O\textsubscript{2} evolution activity in the absence of added Cl\textsuperscript{-}. Figure 21 shows the plots for the two types of PSII for Equation 20. The \( V_{\text{max}} \) and \( K_m \) values for Cl\textsuperscript{-} dependence of O\textsubscript{2} evolution in intact Cl\textsuperscript{-} depleted and NaCl-washed PSII are shown in Table 7. As for the NO\textsubscript{2}\textsuperscript{-} activation experiments, the initial activity \( V_0 \) in intact Cl\textsuperscript{-} depleted PSII was about 330 µmol O\textsubscript{2}/mg Chl/hr, which is very high, and the initial activity \( V_0 \) in NaCl-washed PSII was 20.5 µmol O\textsubscript{2}/mg Chl/hr, which is quite low. For the Cl\textsuperscript{-} activation of O\textsubscript{2} evolution in PSII, \( V_{\text{max}} \) for NaCl-washed PSII was about 6.8 fold of that for intact Cl\textsuperscript{-} depleted PSII while \( K_m \) for NaCl-washed PSII was about 9.3 fold of that for intact Cl\textsuperscript{-} depleted PSII. At high concentrations of
chloride, the activities for both types of PSII reached around 400 µmol O₂/mg Chl/hr. This indicates that the removal of PsbP and PsbQ from intact PSII did not have a significant effect on the level of full activity.

Table 7  Vₘₐₓ and Kₘ values of NO₃⁻ and Cl⁻ Dependence of O₂ Evolution in the PSII

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Cl⁻</th>
<th>NO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vₘₐₓ (µmol O₂/mg Chl/hr)</td>
<td>Kₘ (mM)</td>
</tr>
<tr>
<td>NaCl-washed PSII (with substrate inhibition)</td>
<td>438 ± 19</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>NaCl-washed PSII (No substrate inhibition)</td>
<td>438 ± 19</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>Intact Cl⁻ depleted PSII</td>
<td>64.6 ± 2.2</td>
<td>0.54 ± 0.10</td>
</tr>
</tbody>
</table>

Due to the similarity of NO₃⁻ and NO₂⁻, the effect of NO₃⁻ on these two types of PSII was also investigated to facilitate the comparison between these two anions. The same kinetic models for the NO₂⁻ dependence (Equations 18 and 19) were applied to describe NO₃⁻ dependence with
substrate inhibition in NaCl-washed PSII and intact Cl⁻ depleted PSII, respectively. The NO₃⁻ dependence plots with substrate inhibition are given in Figure 22. The data for NO₃⁻ dependence were normalized to the data for Cl⁻ dependence (used as the control data) so that they have the same initial rate with 0 mM of added anions in each type of PSII. The Vₘₐₓ and Kₘ values for NO₃⁻ dependence of O₂ evolution in intact Cl⁻ depleted and NaCl-washed PSII with substrate inhibition are shown in Table 7.

However, the data for NaCl-washed PSII showed little substrate inhibition. The curve fitting using Equation 18 resulted in very high Vₘₐₓ and Kₘ values, and the Vₘₐₓ value was even higher than that of Cl⁻, which was unreasonable. This may result from the lack of data for substrate inhibition, which takes place at high NO₃⁻ concentrations. Based on this observation, the curve fitting on NO₃⁻ dependence in NaCl-washed PSII without substrate inhibition was carried out. This fit only involved the activation part of NO₃⁻ dependence. Using the equation for Cl⁻ activation (Equation 20), the equation for NO₃⁻ dependence of O₂ evolution in NaCl-washed PSII without consideration of substrate inhibition became

\[
v = V₀ + \frac{Vₘₐₓ[NO₃⁻]}{Kₘ + [NO₃⁻]}
\]

Equation 21
Figure 22 Comparison of NO$_3^-$ dependence of O$_2$ evolution rate in intact Cl$^-$ depleted PSII vs. in NaCl-washed PSII with substrate inhibition
Its constant values can be found in Table 7 and the plot is shown in Figure 23.

The \( V_{\text{max}} \) values were 639 and 72.8 µmol O\(_2\)/mg Chl/hr for NaCl-washed PSII with substrate inhibition and intact Cl\(^-\) depleted PSII, respectively; the former is about 8.8 times as much as the latter. The \( K_m \) value for NaCl-washed PSII with substrate inhibition was 196 fold of that for intact Cl\(^-\) depleted PSII. The \( V_{\text{max}} \) and \( K_m \) values for NaCl-washed PSII without substrate inhibition were 183 µmol O\(_2\)/mg Chl/hr and 5.5 mM, which is about 2.5 and 33 times as high as those of intact Cl\(^-\) depleted PSII, respectively. Based on the \( R^2 \) values obtained from curve fitting (Table 8), the kinetic expression for NaCl-washed PSII with substrate inhibition (Equation 18) fits the NO\(_3^-\) dependence data better than the NO\(_2^-\) dependence data, but the expression for intact Cl\(^-\) depleted PSII (Equation 19) fits the NO\(_2^-\) dependence data better. However, these differences could be due to random errors in the data.
Figure 23  Comparison of NO$_3^-$ dependence of O$_2$ evolution rate in intact Cl$^-$ depleted PSII vs. in NaCl-washed PSII without substrate inhibition
Table 8  $R^2$ from regression in fitting the data for $\text{NO}_2^-$ and $\text{NO}_3^-$ dependence of $O_2$ evolution rates

<table>
<thead>
<tr>
<th>Anion</th>
<th>NaCl-washed PSII</th>
<th>Intact Cl$^-$ Depleted PSII</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{NO}_2^-$</td>
<td>0.9204</td>
<td>0.9991</td>
</tr>
<tr>
<td>$\text{NO}_3^-$ (Substrate inhibition)</td>
<td>0.9941</td>
<td>0.9273</td>
</tr>
<tr>
<td>$\text{NO}_3^-$ (No substrate inhibition)</td>
<td>0.9488</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Comparison of $\text{NO}_2^-$, $\text{Cl}^-$ and $\text{NO}_3^-$ for Dependence of $O_2$ Evolution**

It is interesting to compare the effect of $\text{NO}_2^-$ on the $O_2$ evolution rate with the effects of $\text{Cl}^-$ and $\text{NO}_3^-$. $\text{Cl}^-$ is well known as the natural activating anion in the process of oxygen evolution while $\text{NO}_3^-$ is an anion close to $\text{NO}_2^-$ in chemical characteristics.

In NaCl-washed PSII, the apparent maximum $O_2$ evolution rates due to $\text{NO}_2^-$, $\text{Cl}^-$ and $\text{NO}_3^-$ (with substrate inhibition) were 36, 419 and 190 µmol $O_2$/mg Chl/hr (Figure 24). The highest activity in the presence of $\text{NO}_2^-$ was only 8.6% of that due to $\text{Cl}^-$ and about 19% of that due to $\text{NO}_3^-$. Chloride activated the oxygen evolution process at all
concentrations tested. When we only consider NO$_3^-$ dependence without substrate inhibition, the mode is similar to that of chloride. The apparent maximum O$_2$ evolution rate due to NO$_3^-$ was about 190 µmol O$_2$/mg Chl/hr. As in intact Cl$^-$ depleted PSII, NO$_2^-$ also activated the oxygen evolution process at low concentrations and inhibited at high concentrations in NaCl-washed PSII. However, the activating ability of NO$_2^-$ was much lower than that of NO$_3^-$ and its inhibiting effect was stronger than that of NO$_3^-$.

This can be seen from comparison of the $V_{\text{max}}$, $K_m$ and $K_i$ values in Table 9.

For oxygen evolution in intact Cl$^-$ depleted PSII (see Figure 25), the maximum rates that were reached in the presence of NO$_2^-$, Cl$^-$ and NO$_3^-$ were 355, 393 and 384 µmol O$_2$/mg Chl/hr, respectively. The apparent maximum activity due to Cl$^-$ was higher than that due to NO$_3^-$; by comparison, the apparent maximum activity of NO$_2^-$ was equal to about 90.3% and 92.4% of those for Cl$^-$ and NO$_3^-$.

Unlike Cl$^-$ which only activated the oxygen evolution process, NO$_2^-$ activated at low concentrations and inhibited at high concentrations. This effect was similar to that of NO$_3^-$, but its activating ability was a little weaker than NO$_3^-$ while its inhibition effect was much stronger.

For oxygen evolution in intact Cl$^-$ depleted PSII (see Figure 25), the maximum rates that were reached in the presence of NO$_2^-$, Cl$^-$ and NO$_3^-$ were 355, 393 and 384 µmol O$_2$/mg Chl/hr, respectively. The apparent maximum activity due to Cl$^-$ was higher than that due to NO$_3^-$; by comparison, the apparent maximum activity of NO$_2^-$ was equal to about 90.3% and 92.4% of those for Cl$^-$ and NO$_3^-$.

Unlike Cl$^-$ which only activated the oxygen evolution process, NO$_2^-$ activated at low concentrations and inhibited at high concentrations. This effect was similar to that of NO$_3^-$, but its activating ability was a little weaker than NO$_3^-$ while its inhibition effect was much stronger.

All relevant constants are summarized in Table 9. For $K_m$ values, the order from low to high in terms of anions is
NO$_3^-$ < NO$_2^-$ < Cl$^-$ in intact Cl$^-$ Depleted PSII while that in NaCl-washed PSII is NO$_2^-$ < Cl$^-$ < NO$_3^-$ (without substrate inhibition) < NO$_3^-$ (with substrate inhibition). However, the $K_m$ values probably are within error of each other. The $V_{max}$ values for Cl$^-$, NO$_2^-$ and NO$_3^-$ in NaCl-washed PSII are in the order of NO$_3^-$ (with substrate inhibition) > Cl$^-$ > NO$_3^-$ (without substrate inhibition) > NO$_2^-$. Those in intact Cl$^-$ Depleted PSII are NO$_3^-$ > Cl$^-$ > NO$_2^-$, but the differences are small.
Figure 24  Comparison of anion dependence of O₂ evolution in NaCl-washed PSII
Figure 25 Comparison of anion dependence of O$_2$ evolution in intact Cl$^-$ depleted PSII
Table 9 Comparison of NO$_2^-$ with NO$_3^-$ and Cl$^-$ for activation and inhibition characteristics

<table>
<thead>
<tr>
<th>Type of PSII</th>
<th>Constant</th>
<th>Anion Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NO$_2^-$</td>
</tr>
<tr>
<td>V$_{max}$ (µmol O$_2$/mg Chl/hr)</td>
<td>62.2</td>
<td>72.8</td>
</tr>
<tr>
<td>V$_0$ (µmol O$_2$/mg Chl/hr)</td>
<td>329</td>
<td>329</td>
</tr>
<tr>
<td>K$_m$ (mM)</td>
<td>0.33</td>
<td>0.16</td>
</tr>
<tr>
<td>K$_i$ (mM)</td>
<td>14.5</td>
<td>97.8</td>
</tr>
<tr>
<td>V$_{max}$+V$_0$ (µmol O$_2$/mg Chl/hr)</td>
<td>391.2</td>
<td>401.8</td>
</tr>
<tr>
<td>V$<em>0$/(V$</em>{max}$+V$_0$)</td>
<td>0.841</td>
<td>0.819</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact Cl$^-$ Depleted PSII (Prepared by Dialysis with Br$^-$ Pretreatment)</td>
<td>V$_{max}$ (µmol O$_2$/mg Chl/hr)</td>
<td>38.0</td>
</tr>
<tr>
<td></td>
<td>V$_0$ (µmol O$_2$/mg Chl/hr)</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>K$_m$ (mM)</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>K$_i$ (mM)</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>V$_{max}$+V$_0$ (µmol O$_2$/mg Chl/hr)</td>
<td>58.5</td>
</tr>
<tr>
<td></td>
<td>V$<em>0$/(V$</em>{max}$+V$_0$)</td>
<td>0.350</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl-washed PSII (With substrate inhibition)</td>
<td>V$_{max}$ (µmol O$_2$/mg Chl/hr)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>V$_0$ (µmol O$_2$/mg Chl/hr)</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>K$_m$ (mM)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>K$_i$ (mM)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>V$_{max}$+V$_0$ (µmol O$_2$/mg Chl/hr)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>V$<em>0$/(V$</em>{max}$+V$_0$)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl-washed PSII (No substrate inhibition)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Kinetic Models for pH Dependence of O₂ Evolution under F⁻ Inhibition in Intact PSII

Two mathematical models were established for the pH dependence of the O₂ evolution rate under F⁻ inhibition in intact PSII. The simplified model is actually a preliminary model for the comprehensive one. Because a number of constants needed to be determined for different fluoride concentrations by nonlinear regression, some problems were encountered in curve fitting due to high interdependence of the parameters and other reasons such as the selection of initial guesses for parameters. The two-step modeling method was designed to reduce the number of parameters and thereby turn a more difficult task into an easier one.

The comprehensive model, as it is named, covers all necessary factors and the essential equilibria between protonation and deprotonation as well as fluoride binding to E, EH, and EH₂. As we have explained, the OEC (E), protons, fluoride and their complexes are involved in equilibria with EH⁺ being the active complex for oxygen evolution. In addition to the equilibria for protonation and inhibition as of Reaction 9, we must also consider the reaction scheme from substrate (H₂O) to product (O₂).[^50]
where P is O₂ and EH⁺S is the complex of the active center and the substrate. From these two reaction schemes, the mathematical expression for the comprehensive model can be derived (refer to Appendix A), which is Equation 14.

From Figures 12 and 13, we know that the peak of O₂ evolution activity changes with F⁻ concentration. The peak or the optimal pH shifts to higher pH side when F⁻ concentration increases. This can be explained by the equilibrium shifting in the scheme of Reactions 8 and 9. In the comprehensive model, $K_{i1}$ has the highest value (380 mM), compared to $K_{i0}$ (16 mM) and $K_{i2}$ (2.0 mM). The dominant species is determined by the concentrations of H⁺ and F⁻ in addition to $K_i$s. $K_{i2}$ is the most important inhibition constant for both the simplified and comprehensive models, because its value is the lowest among those $K_i$s for both cases and it is therefore most sensitive to [F⁻] and pH changes. There is no equilibrium for the binding of F⁻ to E considered in the simplified model, and $K_{i1}$ is the least important in the comprehensive model since it has the
highest value and is least sensitive to the $[F^-]$ and pH changes. $K_{i2}$ represents the low pH side on the plots, and therefore, this part of curve is affected most when $F^-$ concentration increases. Meanwhile, $K_{i0}$ is also significant and represents the high pH side on the plots. The high pH side is also affected some as the concentration of $F^-$ increases. On the other hand, the equilibrium for $K_{i2}$ favors $E(H^+)_2F^-$ formation, causing the equilibrium for $K_1$ to shift to $E(H^+)_2$ and decreasing the concentration of active $EH^+$ as the $F^-$ concentration increases. This results in the fluoride inhibition.

A higher concentration of $F^-$ results in increased formation of HF. HF is a weak acid and HCl is a strong acid, and therefore, $F^-$ does not function properly in the H-bonding network if $F^-$ takes the place of Cl$. As we know, for HF, $K_a = 7.2 \times 10^{-4}$ or $pK_a = 3.14$. Therefore, the concentrations of $F^-$ in the medium of PSII for various additions of $F^-$ can be easily calculated for different pH values. The following table shows the results for pH 5 and 7.
Table 10 The real concentrations of F⁻ in PSII solutions after the addition of F⁻

<table>
<thead>
<tr>
<th>Added F⁻ (mM)</th>
<th>pH = 5</th>
<th>pH = 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.9 mM</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>10</td>
<td>9.9 mM</td>
<td>10.0 mM</td>
</tr>
<tr>
<td>15</td>
<td>14.8 mM</td>
<td>15.0 mM</td>
</tr>
<tr>
<td>20</td>
<td>19.7 mM</td>
<td>20.0 mM</td>
</tr>
</tbody>
</table>

From the table, we find that the actual concentrations of F⁻ in PSII are very close to the added concentrations, and fluoride still keeps in the form of F⁻ ions. At pH 5, the concentrations of F⁻ are a little lower than the concentrations of added F⁻ concentrations. At pH 7, the concentrations of F⁻ have almost no changes from the concentrations of added F⁻. This may be related to the observation that F⁻ inhibition is greater at the lower pH side than at the higher pH side.

Chloride is thought to be a part of hydrogen bond network in PSII, and may participate in protonation stabilizing events through a hydrogen-bonding network during catalysis. The exact binding mode of chloride in PSII is still unclear. However, the OEC functions in a manner similar to other Cl⁻ activated enzymes. The studies on Cl⁻ binding to
enzymes may be helpful for us to study Cl\(^-\) binding to PSII. For example, Cl\(^-\) binding to \(\alpha\)-amylase was investigated by Qian et al. using crystallography.\(^{[51]}\) The coordination number was found to be 6 for the binding of Cl\(^-\), which changed H-bonding network and caused activation of the enzyme. The pH dependence of \(\alpha\)-amylase was also investigated by Numao and coworkers.\(^{[52]}\) They found that the pH dependence of \(\alpha\)-amylase showed a changing pattern with Cl\(^-\) addition similar to the mode for the pH dependence of O\(_2\) evolution under F\(^-\) inhibition. Recently, other anion substitutions for Cl\(^-\) were also studied on human pancreatic \(\alpha\)-amylase.\(^{[53]}\) The study showed that many anions, such as Br\(^-\), I\(^-\), NO\(_2\)^\(-\), NO\(_3\)^\(-\), N\(_3\)^\(-\) etc., have an activating effect on \(\alpha\)-amylase. In PSII, fluoride displaces Cl\(^-\) in PSII. Also, F\(^-\) can form strong hydrogen bonds that may affect pK\(_1\) or the acidic pK\(_a\) value. These factors cause a shift in the optimal pH when the concentration of F\(^-\) increases.

In Sandusky’s study, the inhibition constants for F\(^-\), K\(_i\) and K\(_i\)' were about 4 mM and 60-70 mM at pH 7.5;\(^{[38]}\) while in Thomas Kuntzleman’s research, they were 1.8 mM and 78.7 mM at pH 6.3.\(^{[44]}\) In both of the studies, K\(_i\) and K\(_i\)' were found using Dixon plot and Cornish-Bowden plot, respectively. These K\(_i\) values compare well with the K\(_{i2}\) value of 2.0 mM F\(^-\) found in this study using the comprehensive model. Therefore, we can conclude that K\(_{i2}\) is a competitive
inhibition constant. $K_{i0}$ and $K_{i1}$ may be weaker competitive inhibition constants.

The comprehensive model matches the experimental data fairly well, but does not quite encompass the points at high pH accurately. However, the situation was greatly improved compared to the simplified model. Several other more advanced models had been tried to resolve the problem, but all of them required the introduction of more equilibria for $E$ and its complexes with $H^+$ and $F^-$, resulting in the use of more parameters. This, in turn, made the modeling more complicated and less likely to fit the data unambiguously. Some of the advanced models can predict the data for higher pH range quite well, but these models were rather difficult to apply using Sigma Plot because too many parameters were introduced. The more complex models may be fitted by holding some parameters constant to test them, but this will need a further study.

**Nitrite Inhibition and Activation of O$_2$ Evolution in PSII**

One of the subjects that had been studied was the inhibition mode of nitrite in intact and NaCl-washed PSII. From Figures 14 and 15 which represent intact PSII, it has been noticed that the values of the competitive inhibition constant $K_i$ and the uncompetitive inhibition constant $K_i'$ are fairly closed (29 mM and 14 mM respectively) except
that the error of $K_i$ was very high (243 mM). This means that the inhibition may be actually in the uncompetitive mode because of the large error of $K_i$. In the case of NaCl-washed PSII as shown in Figures 16 and 17, where $K_i = 12$ mM (with very high error) and $K_i' = 0.6$ mM, the inhibition is primarily uncompetitive since $K_i$ is over 10 folds greater than $K_i'$. That is to say, NO$_2^-$ may function as an uncompetitive inhibitor with respect to chloride activation in both intact and NaCl-washed PSII. It indicates that NO$_2^-$ mainly binds at an uncompetitive site in PSII after Cl$^-$ has bound to PSII. The reason for the high errors of $K_i$ obtained by Kumar is probably that the data don’t really show Cl$^-$ activation. The lines for the Dixon plots should be separated better and they would be if the measurements for various Cl$^-$ concentrations at 0 mM of NO$_2^-$ had showed variation in activity.

If we compare the results for intact PSII and NaCl-washed PSII in the study of NO$_2^-$ inhibition modes (Table 5), it can be seen that $K_i$ and $K_i'$ decrease after the loss of 17 and 23 kDa polypeptides. This indicates that removal of the extrinsic subunits affects both constants, but it affects the constant $K_i'$ much more. From this observation, we can conclude that the uncompetitive site in PSII must be revealed more by the removal of the subunits. It also
implies that the site is near the OEC rather than at some other location of PSII.

Because NO$_2^-$ inhibition is uncompetitive in NaCl-washed PSII with activation by Cl$^-$, the binding of inhibitory NO$_2^-$ will occur after the binding of an activator such as Cl$^-$ or NO$_2^-$. This implies that NO$_2^-$ inhibition is substrate inhibition in NaCl-washed PSII since uncompetitive inhibition correlates well with substrate inhibition. In the oxygen evolution process, the binding of Cl$^-$ takes place during the S$_2$ state or an earlier state.$^{[25]}$ So we can conclude that the binding of NO$_2^-$ as an inhibitor should occur after the S$_2$ state in PsbQ and PsbP depleted PSII membrane with chloride bound. The exact mechanism of this effect needs other detection tools, such as EPR spectroscopy, to be revealed.

Another subject under investigation was the nitrite dependence of the oxygen evolution rate in NaCl-washed PSII and intact Cl$^-$ depleted PSII. NO$_2^-$ was known to both activate and inhibit oxygen evolution process in these two types of PSII membranes. It activates the evolution process at low concentrations and inhibits the process at higher concentrations, similar to some other monovalent anions such as I$^-$ and NO$_3^-$.\textsuperscript{[3, 4]} Like I$^-$ and NO$_3^-$, NO$_2^-$ activates oxygen evolution in both intact Cl$^-$ depleted PSII and NaCl-wash PSII, which indicates that the 17 and 23 kDa extrinsic
subunits (or PsbQ and PsbP) do not prevent NO$_2^-$ access to the activating site on PSII membrane.\textsuperscript{[54, 55]} Its inhibitory effect on O$_2$ evolution is also much larger than I$^-$ and NO$_3^-$. The reaction scheme of the kinetic models for both types of PSII was determined to be like that in Reaction 11. Since NO$_2^-$ activates (at low concentrations) and inhibits (at higher concentrations) O$_2$ evolving activity, either NO$_2^-$ or Cl$^-$ bind to the activating site of OEC as a first step, then NO$_2^-$ binds to the enzyme complex at the inhibiting site. There is a $K_m$ for activation promoted by each anion, NO$_2^-$ or Cl$^-$. For both NaCl-washed PSII and intact Cl$^-$ depleted PSII, NO$_2^-$ acts as an activator binding on E at first and then as an inhibitor by binding on ENO$_2^-$. The derivation of the mathematical expressions is presented in Appendix B.

Equations 18 and 19 are the mathematical models describing NO$_2^-$ activation and inhibition in the PSII. The results show that these models predict the experimental data quite well except the fit for NO$_3^-$ in NaCl-washed PSII with substrate inhibition that has an unreasonably high $V_{max}$ value (Table 9). All PSII membranes used in the studies were Cl$^-$ deficient because they were either Cl$^-$ depleted and/or were washed with chloride-free buffer.

Thus, a conclusion can be made that, in the absence of chloride, the dependence of O$_2$ evolution on NO$_2^-$ is
activation followed by inhibition in both types of PSII and that there are two activators (Cl\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−}) and one inhibitor (NO\textsubscript{2}\textsuperscript{−}). The NO\textsubscript{2}\textsuperscript{−} inhibition mode is uncompetitive in NaCl-washed PSII, and is probably uncompetitive in intact PSII also, given the high error of the data. It would be very helpful to fully understand the inhibition mode of NO\textsubscript{2}\textsuperscript{−} in intact Cl\textsuperscript{−} depleted PSII if a study of the topic is performed.

A lower K\textsubscript{m} (0.33 mM) for NO\textsubscript{2}\textsuperscript{−} activation in intact PSII, compared to a higher K\textsubscript{m} (2.1 mM) for NO\textsubscript{2}\textsuperscript{−} activation in NaCl-washed PSII, indicates that the extrinsic subunits (PsbP and PsbQ) have the function of holding NO\textsubscript{2}\textsuperscript{−} that activates the oxygen evolution process. In addition, K\textsubscript{i} for intact Cl\textsuperscript{−} depleted PSII (14.5 mM) is over 3 times higher than K\textsubscript{i} for NaCl-washed PSII (4.5 mM). This indicates that the removal of PsbP and PsbQ polypeptides results in a stronger inhibiting effect, which implies that the inhibition site is closer to the OEC and the OEC was protected from NO\textsubscript{2}\textsuperscript{−} inhibition by these two extrinsic subunits.\textsuperscript{[4]}

The R\textsuperscript{2} for oxygen evolution dependence on NO\textsubscript{2}\textsuperscript{−} in intact Cl\textsuperscript{−} depleted PSII and NO\textsubscript{3}\textsuperscript{−} in NaCl-washed PSII with substrate inhibition are 0.9991 and 0.9941, respectively. They are close to one, which indicates that the equation for substrate inhibition in NaCl-washed PSII (Equation 18)
predicts the experimental data for NO$_3^-$ dependence accurately; also, the equation for substrate inhibition in intact Cl$^-$ depleted PSII (Equation 19) fits the related data on NO$_2^-$ dependence excellently. However, since the fit for NO$_3^-$ in NaCl-washed PSII with substrate inhibition resulted in an unreasonably high $V_{\text{max}}$ value, the model without substrate inhibition was used. The $R^2$ is 0.9488, which is not very high, but it was reasonable given the problem with the fit including substrate inhibition. $R^2$ for the dependence on NO$_2^-$ in NaCl-washed PSII was only 0.9204. This fit was probably subject to systematic errors in the measuring process because of the extremely low activity of NO$_2^-$ in NaCl-washed PSII, even though the PSII was incubated and kept in chloride-free buffer containing about 6.5 mM of Ca$^{2+}$. At such low activities, O$_2$ evolution rates were very difficult to be determined accurately. An interesting observation is that Equation 19 also did not predict the data for the dependence on NO$_3^-$ in intact Cl$^-$ depleted PSII accurately, as we noted that $R^2$ was only 0.9273 for the regression. This observation can be easily seen from Figure 22, where the peak area is not well modeled. The meaning of this is probably that NO$_3^-$ and NO$_2^-$ may have different modes or mechanisms of activation and inhibition in intact Cl$^-$ depleted PSII (prepared by dialysis with pretreatment of Br$^-$). Such observation was also found in other studies.[5] One
possible reason for the lower $R^2$ of some fits may be that a portion of the $V_0$ activity is not able to be inhibited, moving the effective baseline up from zero.

So far, we have seen that NaCl-washed PSII has much lower $O_2$ evolution activity than intact PSII, and that NaCl-washed PSII needs Ca$^{2+}$ to work properly. The reason that we use NaCl-washed PSII so often is that the studies on NaCl-washed PSII are more informative than intact PSII because of much less interference from residual Cl$^-$. Also, Cl$^-$ is easier to remove from NaCl-washed PSII than from intact PSII.

NO$_2^-$ versus NO$_3^-$ and Cl$^-$ on Activation and/or Inhibition

$V_{max}$, $K_m$ and $K_i$ data for each anion are summarized in Table 9.

1. In intact Cl$^-$ depleted PSII.

As we note the maximal activities $V_{max}$ of all three anions are 64.6, 62.2 and 72.8 µmol O$_2$/mg Chl/hr for Cl$^-$, NO$_2^-$ and NO$_3^-$ in intact Cl$^-$ depleted PSII, respectively. The values are closer to each other if the errors are considered. $K_m$ values for them are fairly close because they are all within the error range. Therefore, it is difficult to compare the activating effects of these anions from the data. This may be because $V_0$ is so high, resulting in higher errors in the activation portions. The $K_i$ value of
NO$_2^-$ is much lower than that of NO$_3^-$ with substrate inhibition, indicating that NO$_2^-$ has much stronger inhibiting effect on O$_2$ evolution.

2. In NaCl-washed PSII.

The $V_{\text{max}}$ of NO$_2^-$, Cl$^-$, NO$_3^-$ with substrate inhibition and NO$_3^-$ without substrate inhibition for NaCl-washed PSII are 38.0, 438, 639 and 183 µmol O$_2$/mg Chl/hr, respectively. The order of $V_{\text{max}}$ from high to low is: NO$_3^-$ with substrate inhibition $>$ Cl$^-$ $>$ NO$_3^-$ without substrate inhibition $>$ NO$_2^-$, which means that NO$_2^-$ has a very weak activating effect in NaCl-washed PSII. The pure activating effect of Cl$^-$ is higher than that of NO$_3^-$ without substrate inhibition. The $V_{\text{max}}$ for NO$_3^-$ with substrate inhibition was found when there were not enough data at higher concentrations obtained in the study, so the value cannot be count for the comparison. Meanwhile, NO$_3^-$ has some inhibiting effect and this may be the part of the reason why the apparent maximum activity is lower than that of Cl$^-$. 

On the other hand, $K_m$ values of NO$_2^-$, Cl$^-$, NO$_3^-$ with substrate inhibition and NO$_3^-$ without substrate inhibition for NaCl-washed PSII are 2.1, 5.0, 32.3 and 5.5 mM. The values for NO$_2^-$, Cl$^-$, and NO$_3^-$ without substrate inhibition are fairly close and all are within the error range except for that of NO$_3^-$ with substrate inhibition. The $V_{\text{max}}$ value of Cl$^-$ is higher than that of NO$_3^-$ without substrate inhibition.
and the $K_m$ value is lower than that of $\text{NO}_3^-$ without substrate inhibition, which indicates that $\text{Cl}^-$ has the highest activating effect among the anions. This also means that $\text{NO}_3^-$ promotes oxygen evolution activity at a higher concentration than $\text{Cl}^-$ does in NaCl-washed PSII.

3. Inhibition effects.

The lower $K_i$ values of $\text{NO}_2^-$ represent that it is a very strong inhibitor as compared to $\text{NO}_3^-$. The reason may be that $\text{NO}_2^-$ has strong ligand field strength while the strength for $\text{NO}_3^-$ is weak, and therefore, the $\text{NO}_2^-$ binding at the inhibiting sites is stronger than that of $\text{NO}_3^-$. Also, it may be related to the fact that $\text{HNO}_2$ is a weak acid while $\text{HNO}_3$ is a very strong acid. The $K_a$ of $\text{HNO}_2$ is $4.5 \times 10^{-4}$. $\text{NO}_2^-$ is likely to affect H-bonding more than $\text{NO}_3^-$. The $K_i$ value for intact $\text{Cl}^-$ depleted PSII in the study of nitrite dependence was 14.5 mM which is very close to the $K_i$' value for intact PSII in the study of nitrite inhibition (14.0 mM). The result implies that substrate inhibition and uncompetitive inhibition are well correlated in intact PSII. In Stemler’s study on bicarbonate-reversible and irreversible inhibition of photosystem II by $\text{NO}_2^-$ using a thylakoid preparation, the $K_d$ ($K_i$) values were found to be 0.264 mM and 0.481 mM, respectively, when the concentration of $\text{NO}_2^-$ was 5 and 10 mM. The large differences of the $K_i$ values are because the PSII used in this research was from
spinach thylakoids and the one in Stemler’s study was from maize thylakoids. The most important factor was that Stemler’s study consisted of two inhibitors, nitrite and bicarbonate at pH 7.6, and therefore, showed much lower inhibition constants. Since substrate inhibition correlates well with uncompetitive inhibition, it implies that nitrite is an uncompetitive inhibitor and binds at a site other than the active site in intact PSII. Moreover, Cl− depletion has no significant effect on the nitrite inhibition mode in intact PSII.

Activators or inhibitors of oxygen evolution bind at different sites, including active, competitive inhibition and uncompetitive inhibition sites of Cl−. Figure 26 represents a schematic diagram of the binding sites of Cl− and other anions competing with Cl− discussed in this research. In the figure, the active (or competitive inhibition) site is depicted to be close to the Mn4-Ca cluster. It can be bound by activators such as Cl−, NO3−, NO2− and I−, and by competitive inhibitors like F−. The uncompetitive inhibition site is depicted to be close to the extrinsic subunits, and can be bound by the uncompetitive inhibitors such as NO3−, NO2−, I−, etc. The uncompetitive site might be able to bind Cl− in a nonfunctional way.
Figure 26 Schematic diagram of the possible binding sites of activators and inhibitors
CHAPTER VI
CONCLUSION

In the first part of the studies, a comprehensive kinetic model for the pH dependence of oxygen evolution rate with fluoride inhibition in intact PSII was established, and in general, the kinetic constants determined for the mathematical expression can predict the experimental data quite well. There is no research on such models that has been published before. However, although fairly good, the model did not predict the oxygen evolution rates at higher pH very accurately; for instance, the plots for pH >7.5 do not represent the experimental data well. This is probably due to some reason(s) that has not been discovered or the model itself may need to be improved further. Additional efforts had been focused on solving this problem, but all efforts required models with more parameters, which introduced problems in finding unique solutions. Further studies should be carried out to address this topic by performing more experiments.

Nitrite activation and inhibition effects on oxygen evolution were also investigated in the research. Based on the data obtained by Kumar et al, the NO$_2^-$ inhibition modes in intact PSII and NaCl-washed PSII were found, which is that NO$_2^-$ may be an uncompetitive inhibitor for both types
of PSII. However, this conclusion is made based on the large error of the competitive inhibition constant in intact PSII. The reason for the large errors is probably that the data obtained by Kumar did not really show Cl⁻ activation. This should be studied in the future.

The research on NO₂⁻ dependence of oxygen evolution revealed that NO₂⁻ activated the oxygen evolution process at lower concentrations but inhibited the process at high concentrations in intact Cl⁻ depleted PSII and NaCl-washed PSII. In the experiments for both types of PSII, the kinetic models are similar to those for iodide.^[4^]
REFERENCES


APPENDIX A

DERIVATION OF KINETIC EXPRESSIONS OF pH DEPENDENCE OF OXYGEN EVOLUTION UNDER FLUORIDE INHIBITION IN INTACT PSII

Kinetic Expression for pH Dependence of $O_2$ Evolution without $F^-$

The equilibria for the pH dependence of oxygen evolution without inhibition can be expressed as

$$E(H^+)_2 \overset{K_1}{\longrightarrow} E + 2H^+$$

Reaction A1

where $E$, $EH^+$ and $E(H^+)_2$ are the oxygen evolving center and its protonated complexes, in which $EH^+$ is the active center. The reaction to produce $O_2$ is \(^{[45]}\)

$$E \overset{k_2}{\longrightarrow} EH^+ + S \overset{k_3}{\longrightarrow} EH^+ + P$$

Reaction A2

where $P$ is the product, which is $O_2$ here. Since the rate-determining step in the above scheme is the one involving $k_3$ and the substrate $S$ (which is water) is in an extremely excess amount, the reaction velocity can be expressed as
\[
v = \frac{d[P]}{dt} = k[EH^+]
\]

Equation A1

Here, \( k \) is the constant that includes \( k_2, k_{-2} \) and \( k_3 \). The protonation equilibria are given by

\[
K_1 = \frac{[H^+][EH^+]}{[E(H^+)_2]}
\]

Equation A2

\[
K_2 = \frac{[H^+][E]}{[EH^+]}
\]

Equation A3

The total concentration of \( E \) and its complexes is

\[
E_{total} = [E] + [EH^+] + [E(H^+)_2]
\]

Equation A4

By converting Equations A2 and A3 into the expressions for \( [E] \) and \( [E(H^+)_2] \) in terms of \( [EH^+] \), followed by insertion into Equation A4, we have

\[
E_{total} = [EH^+] \left[ 1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]^2} \right]
\]

Equation A5

Observing that

\[
\frac{A}{A_{\text{max}}} = \frac{[EH^+]}{E_{total}}
\]

Equation A6
the kinetic expression for the model of the pH dependence of oxygen evolution without inhibition in intact PSII can be found to be

$$A = \frac{A_{\text{max}}}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}}$$

Equation A7

**Kinetic Expression for the Simplified Model of pH Dependence of Oxygen Evolution under F⁻ Inhibition**

Because F⁻ inhibition equilibria for EH⁺ and E(H⁺)₂ are more significant than that for E, we can establish a simplified model of the pH dependence of oxygen evolution with F⁻ inhibition in intact PSII. This model has the equilibrium schemes shown as Reactions A2 and A3.

$${\text{Reaction A3}}$$

$${\text{where EH}^+\text{F}^-} \text{ and } E(H^+)\text{,}_2\text{F}^- \text{ are inhibited complexes. The oxygen evolution activity can still be expressed using Equation A1. Equations A2 and A3 are the expressions for } K_1 \text{ and } K_2. \text{ From Reaction A3, we also have}}$$
The total concentration of E and its complexes is

\[ E_{\text{total}} = [E] + [EH^+] + [E(H^+)_2] + [EH^+F^-] + [E(H^+)_2F^-] \]  

Equation A10

If we convert Equations A2, A3, A8 and A9 into the expressions for \([E], [E(H^+)_2], [EH^+F] \) and \([E(H^+)_2F] \) in terms of \([EH^+] \), and then insert them into Equation A10, the following equation can be obtained.

\[ E_{\text{total}} = [EH^+] \left\{ 1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} + [F^-] \frac{1}{K_{i1}} + \frac{[H^+]}{K_{i1}K_{i2}} \right\} \]  

Equation A11

Using Equations A6 and A11, we obtain the mathematical expression of the simplified model for the pH dependence of O₂ evolution rate on F⁻ inhibition in intact PSII, which is

\[ A = \frac{A_{\text{max}}}{1 + \frac{[H]}{K_1} + \frac{K_2}{[H]} + [F^-] \frac{1}{K_{i1}} + \frac{[H]}{K_{i1}K_{i2}}} \]  

Equation A12
Here $[F]$ is the concentration of $F^-$. $K_{i1}$ and $K_{i2}$ are the dissociation constants of fluoride inhibition for $EH^+F^-$ and $E(H^+)_2F^-$ respectively.

**Kinetic Expression for the Comprehensive Model of pH Dependence of Oxygen Evolution under $F^-$ Inhibition**

For a comprehensive model, the reaction scheme is

$$
\begin{align*}
E + h^+ & \rightleftharpoons EH + h^+ \rightleftharpoons E(H^+)_2
\end{align*}
$$

At the same time, Reaction A2 presents the reaction to produce the final product, P or O. Equation A1 can still be applied here, and also, $k$ is the constant that includes $k_2$, $k_{-2}$, and $k_3$. From Reaction A4, we have

$$
K_{i0} = \frac{[F^-][E]}{[EF^-]}
$$

Equation A13

The total concentration of $E$ and its complexes is

$$
E_{total} = [E] + [EH^+] + [E(H^+)_2] + [EF^-] + [EH^+F^-] + [E(H^+)_2F^-]
$$

Equation A14

Upon converting Equations A2, A3, A8, A9 and A13 into the expressions for $[E]$, $[E(H^+)_2]$, $[EF^-]$, $[EH^+]$ and $[E(H^+)_2F^-]$ in
terms of \([EH^+]\), followed by insertion into Equation A14; we have

\[
E_{\text{total}} = [EH^+] \left\{ 1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} + [F^-] \left( \frac{1}{K_{i1}} + \frac{[H^+]}{K_{i2}} \right) + \frac{K_2}{K_{i0}[H^+]} \right\}
\]

Equation A15

Given Equation A6, the rate becomes

\[
A = \frac{A_{\text{max}}}{1 + \frac{[H]}{K_1} + \frac{K_2}{[H]} + [F^-] \left( \frac{1}{K_{i1}} + \frac{[H]}{K_{i2}} + \frac{K_2}{K_{i0}[H]} \right) + \frac{K_2}{K_{i0}[H^+]}}
\]

Equation A16

which is the mathematical expression of the comprehensive model for the pH dependence of \(O_2\) evolution rate on \(F^-\) inhibition in intact PSII.
Both NO$_2^-$ and NO$_3^-$ activate and then inhibit the oxygen evolution, and therefore, the same model can be used for both. Let us use NO$_2^-$ for the derivation. Since Cl$^-$ also activates PSII, there is a $K_m$ for activation promoted by each anion, NO$_2^-$ or Cl$^-$. In both NaCl-washed PSII and intact Cl$^-$ depleted PSII, NO$_2^-$ acts as an activator binding on E at first and then as an inhibitor by binding on ENO$_2^-$. The equilibrium scheme is
We assume that the reactions are at the steady state. The kinetic expressions may be derived as follows. From Reaction B1, we have

\[
\frac{d[E]}{dt} = -k_1[E][NO_2^-] + (k_1 + k_2)[ENO_2^-] = 0 \quad \text{Equation B1}
\]

and

\[
\frac{d[E(NO_2^-)_2]}{dt} = k_i[ENO_2^-][NO_2^-] - k_1[E(NO_2^-)_2] = 0 \quad \text{Equation B2}
\]
Equations B1 and B2 can be rearranged into

\[ [E] = \frac{K_m[ENO_2^-]}{[NO_2^-]} \]  \hspace{1cm} \text{Equation B3} \]

\[ [E(NO_2^-)_2] = \frac{[ENO_2^-][NO_2^-]}{K_i} \]  \hspace{1cm} \text{Equation B4} \]

in which \( K_m = \frac{(k_{-1}+k_2)/k_1}{k_{-i}} \) and \( K_i = \frac{k_{-i}}{k_i} \). The total concentration of \( E \) and \( E \) complexes would be

\[ E_{total} = [E] + [ENO_2^-] + [E(NO_2^-)_2] = [ENO_2^-](1 + \frac{K_m}{[NO_2^-]} + \frac{[NO_2^-]}{K_i}) \]

\hspace{1cm} \text{Equation B5} \]

Since \( v = k_2[ENO_2^-] \), we have

\[ v = \frac{k_2E_{total}}{1 + \frac{K_m}{[NO_2^-]} + \frac{[NO_2^-]}{K_i}} = \frac{V_{max}[NO_2^-]}{K_m + [NO_2^-] + \frac{[NO_2^-]^2}{K_i}} \]

\hspace{1cm} \text{Equation B6} \]

where \( V_{max} = k_2E_{total} \). For NaCl-washed PSII, the residual activity was very low, so an additional term \( V_0 \) was added to the kinetic model to express it and the inhibition effect on \( V_0 \) was neglected. Therefore, the kinetic expression is
In the case of intact Cl\textsuperscript{−} depleted PSII dialyzed with Br\textsuperscript{−} pretreatment, the residue activity from Cl\textsuperscript{−} and/or Br\textsuperscript{−} was high, and therefore, the inhibition effect on $V_0$ was included. Then,

$$v = \frac{V_{\text{max}}[NO_2^-]}{K_m + [NO_2^-] + \frac{[NO_2^-]^2}{K_i}} + V_0$$  \hspace{1cm} \text{Equation B7}$$

This is the mathematical equation for the kinetic model of NO\textsubscript{2}\textsuperscript{−} dependence of oxygen evolution in intact Cl\textsuperscript{−} depleted PSII.\textsuperscript{[4]}

Since NO\textsubscript{3}\textsuperscript{−} has a similar behavior as NO\textsubscript{2}\textsuperscript{−} in oxygen evolution, Equations B7 and B8 can be applied to express the NO\textsubscript{3}\textsuperscript{−} dependence of oxygen evolution in NaCl-washed PSII with substrate inhibition and intact Cl\textsuperscript{−} depleted PSII, respectively.