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SPINACH CHLOROPLASTS AND ALGAE USING ELECTRON PARAMAGNETIC RESONANCE

M.D. Boska
(Ph.D. Thesis)

November 1985

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Probing the Donor Side of Photosystem II in Spinach Chloroplasts and Algae Using Electron Paramagnetic Resonance

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Ph.D. Thesis

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November 1985

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Probing the Donor Side of Photosystem II in Spinach Chloroplasts and Algae Using Electron Paramagnetic Resonance

by Michael Douglas Boska

ABSTRACT

The focus of this work concerns electron transfer reactions in photosystem II (PS II). The acceptor side consists of an intermediate electron carrier (I) which is a pheophytin molecule, a first stable electron acceptor \( Q_A \) which is a plastoquinone and a second plastoquinone molecule \( Q_B \). \( Q_B \) accepts two electrons in two sequential light reactions before exchanging the hydroquinone (fully reduced form of the quinone) for a fully oxidized quinone from the plastoquinone pool. The donor side of PS II is not so well understood. Various models of electron transfer are still being considered. However, the donors certainly involve an enzyme (S) which oxidizes water, at least one electron transfer intermediate (Z) and the reaction center (P680). In PS II there is also a one-time donor which remains oxidized after initial illumination and is re-reduced with a halftime of several hours (D). D also donates electrons to the reaction center. Cytochrome \( b_{559} \) (Cyt \( b_{559} \)) is also present and associated with PS II, but it does not donate electrons to the reaction center at room temperature. Neither D nor Cyt \( b_{559} \) are directly involved in passing electrons to the reaction center from water.

Investigations carried out in this work examine the redox reaction rates in PS II using EPR in a variety of samples. In Tris-washed PS II preparations from spinach, it is observed that the
oxidation kinetics of $S_{II}$, the EPR signal formed by $Z^+$ after deactivation of oxygen evolution, mirror the reduction kinetics of $P_{680}^+$ seen by EPR in samples poised at a variety of pH's. These data agree with data from Mathis and co-workers on the optically measured reduction kinetics of $P_{680}^+$. The oxidation kinetics of $S_{II}$, the EPR transient seen from $Z^+$ in samples active in $O_2$ evolving samples, were instrument limited ($t_{1/2}$ less than 4 ms) and thus could not be directly measured. These results taken together support a model where $Z$ donates electrons directly to $P_{680}^+$.

The examination of the oxidation and reduction kinetics of $S_{II}$ in monovalent and divalent salt-washed PS II preparations from spinach correlated most of the change of $Z$ oxidation and re-reduction kinetics seen upon Tris-treatment with the loss of a 33 kDa polypeptide associated with the donor side of PS II. These data coupled with observations of steady-state light-induced amplitude changes in $S_{II}$ give evidence for the existence of an electron carrier between the water-splitting enzyme ($S$) and $Z$.

Observation of $S_{II}$ amplitude and kinetics in highly resolved PS II protein complexes from *Synechoccus* sp., consisting of either a 5 polypeptide PS II core complex (E-1) or a 4 polypeptide PS II core complex (CP2b), localize $Z$ and $P_{680}$ within the 4 polypeptide complex.

Observation of the amplitude and kinetics of $S_{II}$ in whole cells of *Synechococcus* 6301 before and after $Ca^{2+}$ depletion show that $Ca^{2+}$ depletion either blocks electron transfer from $Z$ to $P_{680}^+$ or from $P_{680}$ to $Q_A$.

Thus, investigations carried out in this work support a model for
the donor side of PS II where at least one intermediate electron carrier (Y) exists between the water splitting complex (S) and Z, and Z donates directly to P680\. This study also localizes Z and P680 within a 4 polypeptide protein complex in cyanobacteria.

Kenneth Sawyer
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I would like to thank the Grateful Dead and the sport of hang-gliding for keeping my spirits high even when my experimental efficiency was low.

Last, but not least, I would like to thank my Mom for putting up with me as a child. Yay, Mom!
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Chl:</td>
<td>chlorophyll</td>
</tr>
<tr>
<td>CIDEP:</td>
<td>chemically induced dynamic electron polarization</td>
</tr>
<tr>
<td>DCBQ:</td>
<td>2,5 dichlorobenzoquinone</td>
</tr>
<tr>
<td>DCMU:</td>
<td>3-(3,4 dichlorophenyl)-1,1-dimethylurea</td>
</tr>
<tr>
<td>HEPES:</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>MES:</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>PS:</td>
<td>photosystem</td>
</tr>
<tr>
<td>P680:</td>
<td>primary electron donor of PS II</td>
</tr>
<tr>
<td>QA:</td>
<td>primary electron acceptor of PS II</td>
</tr>
<tr>
<td>QB:</td>
<td>secondary electron acceptor of PS II</td>
</tr>
<tr>
<td>S II:</td>
<td>EPR signal II</td>
</tr>
<tr>
<td>SDS:</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE:</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TRIS:</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Z:</td>
<td>electron donor to P680</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS

ACKNOWLEDGEMENTS......................................................... i

ABBREVIATIONS............................................................... ii

CHAPTER 1

INTRODUCTION

1.1 Introduction ........................................................................ 1
1.2 The Donor Side of Photosystem II........................................... 4
  1.2.A Primary Charge Separation.............................................. 4
  1.2.B The Water Splitting Complex.......................................... 5
  1.2.C Secondary Donation to the Photosystem II Reaction Center. 7
1.3 Focus of This Work............................................................. 9

CHAPTER 2

SIMILARITY OF EPR SIGNAL $r_{1}$ RISE AND P680$^{+}$ DECAY
KINETICS IN TRIS-WASHED CHLOROPLAST PHOTOSYSTEM II
PREPARATIONS AS A FUNCTION OF pH

2.1 Introduction ......................................................................... 15
2.2 Materials and Methods....................................................... 16
2.3 Results ................................................................................ 18
2.4 Discussion ........................................................................... 25

CHAPTER 3

KINETICS OF EPR SIGNAL $I_{\nu f}$ IN CHLOROPLAST PHOTOSYSTEM II

3.1 Introduction ......................................................................... 29
3.2 Materials and Methods....................................................... 30
3.3 Results ................................................................................ 32
CHAPTER 4
THE EFFECT OF MONO- AND DIVALENT SALTS ON THE RISE AND DECAY KINETICS OF EPR SIGNAL II IN PHOTOSYSTEM II PREPARATIONS FROM SPINACH

4.1 Introduction.................................................................43
4.2 Materials and Methods.........................................................44
4.3 Results..............................................................................46
4.3.A Peptide Content and O₂-Evolution Activity of the Salt Washed Preparations.........................................................46
4.3.B Signal II Rise Kinetics.........................................................46
4.3.C Signal II Decay Kinetics.........................................................52
4.3.D Steady State Spectra...............................................................57
4.3.E Flash Number Dependence of Signal II Decay Kinetics.....59
4.4 Discussion............................................................................59

CHAPTER 5
EPR SIGNAL II IN CYANOBACTERIAL PHOTOSYSTEM II REACTION CENTER COMPLEXES WITH AND WITHOUT THE 40 kDa CHLOROPHYLL-BINDING SUBUNIT

5.1 Introduction........................................................................68
5.2 Materials and Methods............................................................70
5.3 Results...............................................................................72
5.4 Discussion...........................................................................80
CHAPTER 6

THE EFFECT OF CALCIUM DEPLETION ON EPR SIGNAL II IN

ANACYSTIS NIDULANS

6.1 Introduction.............................................................93
6.2 Materials and Methods.................................................95
6.2.A Sample Preparation..................................................95
6.2.B Spectroscopic Measurements.......................................95
6.3 Results.........................................................................96
6.4 Discussion.....................................................................107

CHAPTER 7

CONCLUSIONS AND WILD SPECULATION

7.1 Conclusions.................................................................112
7.2 Wild Speculation..........................................................113
CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Photosynthesis is the process by which visible light reaching the earth is transformed into chemical energy and reducing equivalents. This storage process occurs in photosynthetic bacteria, algae and higher plants for subsequent use of the stored energy by themselves and other living organisms. The transduction of the photon into chemical energy proceeds through an electron transfer which is initiated at a reaction center involving bacteriochlorophyll molecules in bacteria and chlorophyll molecules in algae and higher plants. These reaction centers are in a special protein environment where they receive energy sometimes directly by absorption, but more often by energy transfer funneled from antenna pigments surrounding the reaction center. The electrons in the reaction center are subsequently excited, raising the reduction potential of the reaction center above that of a neighboring acceptor molecule. Thus, one reducing equivalent is sent down a chain of acceptor molecules.

Simultaneously, a chain of donor molecules sends the oxidizing equivalent now residing on the reaction center to a terminal electron donor, further stabilizing the charge separation and preparing the reaction center to receive the energy from another photon.

In algae and higher plants, there are two different photosystems which operate in series (1). One has a very oxidizing midpoint potential for the reaction center donor. This reaction center
oxidizes water and reduces a plastoquinone pool, both processes occurring through a series of electron transfer intermediates. This reaction center is referred to as photosystem II (PS II). A second reaction center, photosystem I (PS I), has a more reducing midpoint potential. PS I oxidizes the plastoquinone pool, which has been reduced by PS II, and reduces NADP to NADPH. These reactions also proceed through chains of electron carriers, although different ones from those of PS II. PS I, aside from conducting linear electron transport from plastoquinone to NADP, also undergoes cyclic electron transport through enzymes which use the energy stored from the photon to phosphorylate ADP to ATP. A model of light-driven electron transport in higher plants and algae, referred to as the Z scheme, is shown in Fig. 1-1.

The reactive intermediates on the acceptor side of PS I have not all been fully characterized, but it appears that there are at least two acceptors, labelled A₁ and A₂ before the first iron-sulfur protein acceptor (FeSₐ). A second iron-sulfur center (FeSₐ) may act in series with FeSₐ, although parallel paths are possible. The FeS proteins reduce water soluble two iron-two sulfur ferredoxin (Fd), which subsequently is used either for the reduction of NADP⁺ or in a cyclic path around PS I to phosphorylate ADP.

On the donor side of PS I plastocyanin, a copper containing protein, is responsible for the reduction of the reaction center of PS I (P700). The plastocyanin (PCy) is reduced by a protein complex containing cytochromes and an FeS center—the cytochrome b₆-f complex. The FeS center which seems to be responsible for accepting electrons
Adapted from:


Fig 1-1
from the plastoquinone pool has been called the Rieske iron-sulfur center.

The focus of this work concerns electron transfer reactions in photosystem II (PS II). The acceptor side consists of an intermediate electron carrier (I) which is a pheophytin molecule, a first stable electron acceptor (Q_A) which is a plastoquinone and a second plastoquinone molecule (Q_B) which accepts two electrons in two sequential light reactions before exchanging the hydroquinone (fully reduced form of the quinone) for a fully oxidized quinone from the plastoquinone pool (PQ pool). The donor side of PS II is not as well understood. Various models of electron transfer are still being considered (2). However, it certainly involves an enzyme (S) which oxidizes water, at least one electron transfer intermediate (Z) and the reaction center (P680). In PS II there is also a one-time donor which remains oxidized after initial illumination and is re-reduced with a halftime of several hours (D) (3, 4). D also donates electrons to the reaction center. Cytochrome b_{559} (Cyt b_{559}) is also present and associated with PS II, but it is not donating electrons to the reaction center at room temperature (5-7). Neither D nor Cyt b_{559} are directly involved in passing electrons to the reaction center from water.

1.2 The Donor Side of Photosystem II

1.2. A Primary Charge Separation

Primary charge separation occurs between P680 and Q_A through an intermediate electron carrier, I, which is a pheophytin molecule (8, 9). Once it has received the electron, Q_A transfers an electron
to \( Q_B \) in 0.5 ms at room temperature (10). This process is repeated to fully reduce \( Q_B \), then \( Q_B H_2 \) is exchanged for a fully oxidized quinone from the plastoquinone pool on the time scale of a few ms (2). \( P680^+ \) can back-react with \( Q_A^- \) in 200 \( \mu s \) (11-13), but this reaction is normally prevented by the rapid reduction of \( P680^+ \) by the donor side of PS II. This reduction rate depends upon whether the membrane has been dark-adapted, and the reaction time ranges from 35 to 200 ns in thylakoids competent in oxygen evolution (14,15). When oxygen evolution is deactivated by alkaline Tris-treatment, the reduction of \( P680^+ \) slows to 2-20 \( \mu s \) depending upon pH (11). It has been shown (16, chapter 2 of this thesis) that under these conditions \( Z \) donates electrons directly to \( P680^+ \).

1.2.2 The Water Splitting Complex

In 1969, experiments done in Joliot's lab which measured oxygen yield as a function of flash number (17) showed that dark adapted chloroplasts emit a large burst of oxygen on the third flash with a period four oscillation in the oxygen yield in subsequent flashes. These results were translated into a model by Kok et al (18,19) of an enzyme with 4 metastable redox states and one unstable redox state, labelled \( S_0, S_1, S_2, S_3 \) and \( S_4 \), respectively. Advancement from one \( S \)-state to the next is caused by extraction of one electron from the enzyme by an oxidizing equivalent which originates from the oxidized reaction center. Upon advancement of \( S_3 \) to \( S_4 \), oxygen is spontaneously released from the enzyme in about 1 ms, and the enzyme automatically resets to \( S_0 \). From the flash number dependence of the oxygen yield, it was determined that in dark adapted samples, 75% of
the centers were in the $S_1$ state and 25% were in the $S_0$ state (18-20),
although some more recent work supports the idea of 100% of $S_1$
residing in dark adapted samples, with some of the flashes initially
oxidizing a species on the donor side of PS II which does not lead to
oxidation of the water splitting enzyme (21).

The molecular nature of the water splitting complex has not been
worked out in detail; however, manganese has been shown to be
involved. Early experiments in which chloroplasts were Tris-washed to
inactivate the water-splitting complex showed the release of manganese
from the membranes (22,23). Subsequent reconstitution of the oxygen
evolution activity involved reincorporation of the manganese into the
membranes (24). Quantitation of manganese in thylakoid membranes (25)
and in samples in which PS II was separated from PS I by detergent
treatment with the retention of oxygen evolution capability (26-28)
showed that there may be as many as four manganese atoms per water
splitting complex. Additional evidence concerning the role of
manganese in the water splitting complex arises from an EPR visible
signal (multiline signal) first seen by Dismukes et al (29) and
associated with the $S_2$ state of the water splitting enzyme. Attempts
at simulation have led to the conclusion that this multiline signal is
due to two or four coupled manganese atoms (30). This conclusion is
also supported by analysis of EXAFS data on the manganese of PS II
preparations which show a manganese atom residing within 3 Ås
of another manganese (31,32). This does not exclude the possibility
of manganese atoms at longer distances or two pairs separated by over
5 Ås operating in parallel. More recently, optically detected
changes in the ultraviolet region with period four behavior have been reported (33,34). These transients have been attributed to changes in the oxidation state of the manganese in the water splitting complex.

1.2.C Secondary Electron Donation to the PS II Reaction Center

EPR signal II (S II) originates from the oxidized form of both Z and D in the Z-scheme (Fig. 1-1). The EPR spectrum of S II consists of a broad free radical ($\Delta H_p = 19 G$) with 5 or 6 hyperfine components visible. The radical is centered at $g = 2.0049\pm0.0004$. Over the years there have been various proposals concerning the molecular identity of Z$^+$ and D$^+$. In 1968, Ellen Weaver suggested that S II may be due to a quinone species, because algae low in plastoquinone did not display the signal (35). More direct evidence for the correlation of S II with some type of quinone moiety was presented by Kohl and Wood (36) in the form of plastoquinone extraction and reconstitution experiments. It was found that reincorporation of plastoquinone into thylakoids restored S II, and that reincorporation of deuterated plastoquinone narrowed the linewidth of S II by a factor of two. In an attempt to understand what form of quinone may give rise to S II, Hales and Das Gupta performed spectral simulations of plastoquinone in various environments and concluded that the only way that a quinone could have such a large linewidth was for the semiplastoquinone anion to be near a divalent cation with no d-orbitals, such as Ca$^{+2}$ or Mg$^{+2}$ (37). However, even with these constraints, the hyperfine splittings visible in S II were not seen in the simulation. More recently, O'Malley and Babcock provided extensive evidence for S II being a semiplastoquinone cation (38,39). This assignment is more reasonable,
because the immobilized solution spectrum, orientation dependence of
the spectra and midpoint potential of the model quinones are quite
similar to those of S II (39). Thus it seems most likely that S II is
due to a plastosemiquinone cation.

As is implied in the Z-scheme, there are two different functions
for the semiquinone cations on the donor side of PS II. The species
D, which appears partly as a stable paramagnetic species in
chloroplasts (designated S II_u) and partly as a slowly decaying
species formed upon illumination after prolonged darkness in
chloroplasts (designated S II_s) (3). S II_s is oxidized within the
first few flashes in chloroplasts or during about 1 sec at the onset
of continuous illumination and decays on the order of several hours
(4). The midpoint potential of D has been measured to be 760 mV at pH
8.5 (40). In Tris-washed PS II preparations, the distribution of D
between the dark stable S II_u and the slowly decaying S II_s is pH
dependent and ranges from 50% S II_u at pH 8.5 to almost 100% S II_u at
pH 6.0 (41). The amount of D as measured by the spin quantitation of
S II in the dark immediately after illumination has been shown to be
about one spin per PS II reaction center (42).

In thylakoids and PS II preparations competent in O_2-evolution,
the precise spectrum of Z^+ has not been determined, because the water-
splitting enzyme supplies Z^+ with electrons too fast to allow a
steady-state buildup of the radical in the light (43,44). However, a
rough field position dependence of the amplitude of the transient seen
from Z^+ under these conditions has been reported (43) and it is
similar to the spectrum of D^+. The transient produced by Z oxidation
and $Z^+$ reduction in PS II preparations with active $O_2$-evolving systems has been labeled $S \text{II}_{vf}$. The oxidation of $S \text{II}_{vf}$ has been measured to occur in less than 4 $\mu$s (45) followed by a biexponential reduction of $Z^+$ with halftimes of around 100 $\mu$s and 1 ms (45, 46).

Upon deactivation of the water-splitting enzyme, the oxidation (16, 46) and re-reduction (4, 46) of $Z$ becomes slower. The transient observed under these conditions has been labeled $S \text{II}_f$ (4). Due to the slower reduction kinetics of $Z^+$ under these conditions, a steady-state light-induced spectrum of $Z^+$ can be observed. When the water-splitting enzyme is deactivated by Tris, the microwave power saturation (44) and pH dependence (41) of the EPR signals from $Z$ and $D$ are identical. However, the midpoint potential of $D$ has been measured as 760 mV (40), about 200 mV lower than is estimated for $Z$. The appearance of the light-induced spectrum of $Z^+$ upon deactivation of the water-splitting complex has allowed the amount of both $D^+$ and $Z^+$ to be measured. Careful quantitation of $S \text{II}_s$ and $S \text{II}_f$ in Tris-washed PS II preparations (42) has shown that there is about one $D$ and one $Z$ per P680.

1.3 Focus of This Work

Investigations carried out in this work examine the redox reaction rates in PS II using EPR in a variety of samples. In Tris-washed PS II preparations from spinach, it is observed that the oxidation kinetics of $S \text{II}_f$ mirror the reduction kinetics of $P680^+$ seen by EPR in samples poised at a variety of pH's. The oxidation kinetics of $S \text{II}_{vf}$, however, were instrument limited ($t_{1/2}$ less than 4 $\mu$s) and thus could not be directly measured. These data coupled with
data from other labs on the optically measured reduction kinetics of P680+ (11) support a model where Z donates electrons directly to P680+.

The examination of the oxidation and reduction kinetics of S II in monovalent and divalent salt-washed PS II preparations from spinach correlated most of the change of Z oxidation and re-reduction kinetics seen upon Tris-treatment with the loss of a 33 kDa polypeptide associated with the donor side of PS II. These data coupled with observations of steady-state light-induced amplitude changes in S II give evidence for the existence of an electron carrier between the water-splitting enzyme and Z.

Observation of S IIf and S II<sub>s</sub> in highly resolved PS II protein complexes from Synechococcus sp., consisting of either a 5 polypeptide PS II core complex (E-1) (47) or a 4 polypeptide PS II core complex (CP2b) (47), localize Z and P680 within the 4 polypeptide complex.

Observation of the amplitude and kinetics of S II in whole cells of Synechococcus 6301 before and after Ca<sup>2+</sup> depletion show that Ca<sup>2+</sup> depletion either blocks electron transfer from Z to P680<sup>+</sup> or from P680 to Q<sub>A</sub>. 
REFERENCES


CHAPTER 2

Similarity of EPR Signal II_f Rise and P680+ Decay Kinetics in Tris-washed Chloroplast Photosystem II Preparations as a Function of pH

2.1 INTRODUCTION

An intermediate electron carrier on the oxidizing side of PS II, most likely a plastoquinone species (1), is usually designated as Z (2,3). In its oxidized form this species gives rise to EPR Signal II_f in chloroplasts inhibited at the O_2-evolving complex and to Signal II_vf in chloroplasts competent in water splitting (2,4,5). The difference in nomenclature for the two signals arises because the mode of reduction and the decay kinetics of Z^+ differ in these two types of chloroplasts. In O_2-evolving preparations Z^+ is reduced by the O_2-evolving complex in a reaction which occurs in the submillisecond time range (4,6). When O_2 evolution is inhibited, the reaction is blocked, and Z^+ is re-reduced by either endogenous reductants or by exogenous electron donors in a reaction which typically occurs in the 10 ms to 1 s time range (7,8).

In Tris-washed chloroplasts, Z was originally postulated as the immediate reductant of the oxidized reaction center chlorophyll, P660^+ (9). In this model the species Z would be identified with D_1, the donor to P680^+ which has been inferred from optical measurements (10,11). It is now possible to test this hypothesis directly by measuring the rise kinetics of S II_f under conditions where the decay of P680^+ is well characterized. In the work reported here we have
measured the rise kinetics of the EPR Signal II attributed to Z⁺ as well as the decay of an EPR signal that corresponds to P680⁺, as determined from the field profile of this kinetic component. A comparison of our results at different pH values with optical data reported for the decay of P680⁺ (10,11) shows good agreement and indicates that in Tris-washed chloroplasts Z reduces P680⁺ directly.

2.2 MATERIALS AND METHODS

Spinach chloroplasts were used to prepare O₂-evolving PS II fragments according to the method of Berthold et al (12). These fragments were subsequently Tris washed, centrifuged at 40,000xg for 20 min, washed in buffer, centrifuged again at 40,000xg for 20 min, and resuspended in the same buffer. The buffers contained 0.4 M sucrose, 10 mM NaCl, and 50 mM of buffer (Hepes at pH 6.9, MES at pH 6.0 and 5.6, and succinate-MES at pH 5.2). The pellet was resuspended to a total chlorophyll concentration of 4-8 mg/ml, as determined from chlorophyll absorbance at 645 and 663 nm (13). The sample was then frozen in two separate aliquots until the start of the experiment. Upon thawing, 10 mM ferrocyanide and 10 mM ferricyanide were added as exogenous electron carriers.

During the experiment the sample was flowed through a Scanlon EPR flat cell at a rate of 1 ml/min by a Gilson minipuls II peristaltic pump and then back into an ice-cooled reservoir. The temperature in the flat cell was 24.5±0.5°C as measured by a Fluke 2100 A digital thermometer using a copper-constantan thermocouple. The thermocouple was located inside the flow system at the exit of the flat cell.

Before kinetic measurements were initiated, an X-band field-swept
spectrum was taken to check the amplitude of the light-induced Signal II. This was also done to check for the absence of Signal I. The absence of Signal I is important, because in preliminary rise time experiments, we found that the CIDEP signal from PS I (14) obscures the rise time of S II\(_f\). Once the spectrum was taken, the field position was set to the maximum of the lowest-field hyperfine component of Signal II (g 2.010). The decay kinetics of S II\(_f\) were measured to determine an appropriate flash repetition rate in the rise time experiments. Typical values of this decay time were 30-150 ms and consequently a flash repetition rate of 2 Hz was used. During the Signal II\(_f\) rise time and P680\(^+\) decay measurements, the sample integrity was checked every 10,000 passes by measuring the reversible light-induced increase in Signal II upon continuous illumination at this field position.

One of the two aliquots was used for a Signal II\(_f\) rise time measurement at the maximum of the low-field hyperfine component of Signal II (g 2.010). The other aliquot, treated and monitored as previously described, was used to measure the decay kinetics of P680\(^+\) by increasing the magnetic field strength by 12 G (g 2.003) (see Fig. 2).

A Varian E-109 EPR spectrometer modified for 1 MHz field modulation was used for the measurements (15). The instrument response time was limited to 2 µs. When field-swept spectra were taken, an output low-pass RC filter of 1 s time constant was used on the 1 MHz receiver, and the output signal was fed into a chart recorder. When the kinetic traces were obtained, the output signal of
the receiver was digitized by a Nicolet Explorer IIIA oscilloscope and accumulated in a signal averager which was designed and constructed by Mr. Gary Smith in our laboratory. A photodiode detecting the laser flash was used to trigger the oscilloscope to eliminate errors due to delays in the firing circuit. For the kinetic traces, the output was filtered by a 0.5 μs low-pass filter and digitized at 200 ns/channel. In all cases 4096 points were collected.

The laser used is a modified flashlamp pumped dye laser (Phase-R Corp. DL-1400), which provided 0.3-0.5 μs nonsaturating light pulses at 640 nm with energies of 5-8 mJ/pulse at the sample in the EPR cavity. Rhodamine 640 (Exciton, Dayton, OH, U.S.A.) in methanol was the dye in the laser for all of the traces. The signal-averaged trace was sent upon completion to a VAX 11/780 computer for data analysis and display.

2.3 RESULTS

Examples of the kinetic traces and theoretical fits can be seen in Fig 1. The experimental conditions are as noted in the figure and in the figure legend. The fits of the data were performed by using ZXSSQ, a least-squares fitting routine in the IMSL library (16). The \( Z^+ \) rise times were calculated by fitting each experimental trace to a theoretical expression of the form:

If \( t < t_0 \), \( Y(t) = X(1) \)

If \( t \geq t_0 \), \( Y(t) = X(1) + X(2) \exp \left( \frac{(t-t_0)}{X(3)} \right) \)

where \( Y(t) \) is the calculated EPR signal amplitude at time \( t \), \( t_0 \) corresponds to the time at which the laser was fired, \( X(1) \) and \( X(2) \) are the initial and final EPR signal amplitudes, respectively, and
Fig 1

Transient kinetic traces of EPR signals photoinduced in Tris-washed PS II particles from spinach chloroplasts. Temperature, 24.5±0.5°C; samples suspended in 0.4 M sucrose, 10 mM NaCl, 50 mM buffer (MES at pH 6.0 and succinate/MES at pH 5.2), 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide. EPR instrument conditions: microwave power, 20 mW; modulation amplitude, 2.3 G; field modulation at 1 MHz; output time constant, 0.5 μs. (a) Rise kinetics and computer generated least-squares fit of Signal II, measured at the low-field hyperfine component (g 2.010); pH 5.2 trace, averages 40,000 passes; pH 6.0 trace, averages 50,000 passes. (b) Decay kinetics and fit of EPR signal attributed to P680+ measured at 12 G higher field (g 2.003); pH 5.2 trace, 10,000 passes; pH 6.0 trace, 20,000 passes.
a) $\frac{d}{dt}E_1$, Rise

b) $P680^o$, Decay

$\frac{d}{dt} = 9 \mu s$

$\frac{d}{dt} = 15 \mu s$

$\frac{d}{dt} = 8 \mu s$

$\frac{d}{dt} = 15 \mu s$
X(3) is the inverse first-order rate constant for the rise of Signal IIₚ.

The fast decay times of the signal attributable to P₆₈₀⁺ were obtained in a similar fashion. However, a fit of the entire curve (including the prerecorded baseline) was complicated by two factors. The first is the 2 μs response time of the instrument. The other factor is the contribution of S IIₚ to the P₆₈₀⁺ decay curve, leading to a different baseline after the flash than before. Hence, the kinetic information was extracted by fitting only the decay portion of the curve to either a single or double exponential decay, depending on the amplitude of the contribution from the back-reaction of P₆₈₀⁺Q⁻. The kinetics of this back-reaction have been found to have a t₁/₂ of 100-200 μs and to be pH independent (11,17). The amplitude of the contribution from the back-reaction increased with decreasing pH due to inactivation of the donation from Z to P₆₈₀⁺. This slow component is apparent in the P₆₈₀⁺ decay trace at pH 5.2 in Fig 1. A summary of the data analysis along with a comparison of P₆₈₀⁺ rereduction kinetics measured by Reinman et al (11) can be found in Table I.

The field profile of the fast decay measured at pH 5.5 can be seen in Fig 2. The amplitude of the decay at each field position was normalized to the amplitude seen at g 2.003 as described above taken before and after the kinetic trace taken at each field position. The error bars reflect the variation seen in amplitudes at the g 2.003 reference position. The initial amplitude at pH 5.5 reflects only P₆₈₀⁺, since Z has not yet been oxidized, allowing the P₆₈₀⁺ amplitude to be separated from that of S IIₚ where the spectra
TABLE I

PRIMARY ELECTRON-TRANSFER KINETICS OF PS II IN TRIS-WASHED PREPARATIONS FROM SPINACH CHLOROPLASTS

The precision of the kinetic measurements is estimated to be 2 μs.

<table>
<thead>
<tr>
<th>EPR signals (this work)</th>
<th>Optical signals (Ref 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>t₁/₂ (μs)</td>
</tr>
<tr>
<td>Signal II</td>
<td>P₆₈₀</td>
</tr>
<tr>
<td>5.2</td>
<td>15</td>
</tr>
<tr>
<td>5.6</td>
<td>10</td>
</tr>
<tr>
<td>6.0</td>
<td>8ᵃ</td>
</tr>
<tr>
<td>6.9</td>
<td>≤4</td>
</tr>
</tbody>
</table>

ᵃAverage of two experiments.
Fig 2

Field position profile of the photoinduced EPR transient attributed to P680⁺. Samples are suspended as noted in Fig 1, except that pH 5.6 (50 mM MES) buffer was used. Each field position is a sum of 10,000 passes normalized to the amplitude at g 2.003. Instrument conditions, except for the field position in most cases, were as in Fig 1. S II is overlayed for reference.
overlap. Each point is the sum of 10,000 passes. Within the signal-
to-noise of this measurement, the field profile matches the EPR
spectrum of P680+ (18,19).

2.4 DISCUSSION

The data of Table I show good agreement between the decay of the
reaction center chlorophyll absorbance change and the rise time of the
Z+ free radical EPR signal (Signal II_f), indicating that the two
processes are coupled; i.e., that in Tris-washed chloroplasts Z is
oxidized as P680+ is reduced. An interesting aspect of these data is
that the Z and P680 kinetics are largely independent of the
chloroplast or particle preparation after deactivation of the O_2-
evolving complex. This is consistent with a model having these two
reaction partners in the same thylakoid membrane complex.

Experimental results reported for small PS II particles which were
treated with urea (20) support this conjecture, as do recent results
for the behavior of Signal II_f in reaction centers prepared from
Chlamydomonas reinhardii (Babcock, G.T., Ghanotakis, D.F., Ke, B. and
Diner, B.A., unpublished data).

Our conclusions about the rise of Signal II_f and the coupled
reduction of P680+ in Tris-washed chloroplasts are difficult at
present to extrapolate to preparations with intact O_2 evolution.

Measurements of fluorescence yield changes in Chlorella vulgaris under
repetitive-flash conditions showed a 400 ns kinetic component
attributed to the decay of P680+ (21). The rise of Signal II_vf in
intact spinach chloroplasts has been measured at neutral pH, and a 20
μs halftime was reported (22). These results were obtained, however,
in chloroplasts under conditions where we have observed a weak contribution from the CIDEP signal of P700\(^+\), and it is quite possible that this phenomenon obscured the true rise of Signal II\(_{\text{vf}}\).

Experiments being developed to reexamine the Signal II\(_{\text{vf}}\) kinetics in \(\text{O}_2\)-evolving PS II particles (12) should resolve this uncertainty.

The results that we have obtained with Tris-washed PS II particles are summarized as follows:

Donor \(\rightarrow\) \(Z\) \(\rightarrow\) P680 \(\rightarrow\) I \(\rightarrow\) Q \(\rightarrow\) PS I.

In these Tris-washed preparations, the rate of photogeneration of \(Z^+\) matches the reduction rate of P680\(^+\). These rates are seen to match over a variety of pH values, showing that the oxidation of Z is directly coupled to the reduction of P680\(^+\).
REFERENCES


CHAPTER 3

Kinetics of EPR Signal II\textsubscript{vf} in Chloroplast Photosystem II

3.1 INTRODUCTION

Z is a plastoquinone species (1) which acts as an electron carrier on the oxidizing side of PS II. The oxidized form of Z, probably a semiquinone cation (2), gives rise to EPR Signal II (S II). A fast kinetic component of this signal, designated Signal II\textsuperscript{vf}, is involved in physiological electron transfer between the O\textsubscript{2}-evolving enzyme and the PS II reaction center (P680) (3-5). When O\textsubscript{2} evolution is blocked, the normal source of electrons for Z\textsuperscript{+} is destroyed, slowing the decay kinetics of S II. Under these conditions the kinetic component decaying in 10 ms to 1 s is referred to as S II\textsubscript{f} (3,4). S II\textsubscript{f} has been shown by detailed kinetic studies to reflect donation by Z directly to the oxidized primary donor P680\textsuperscript{+} (6).

In O\textsubscript{2}-evolving chloroplasts and in Chlorella pyrenoidosa, the re-reduction of P680\textsuperscript{+} occurs in the submicrosecond range (7,8). However, an induction time of S II\textsubscript{vf} in chloroplasts was previously measured to be 20±10 µs (9). We now find that a more sensitive measurement of the rise of this signal in O\textsubscript{2}-evolving PS II particles shows an instrument-limited rise (t\textsubscript{1/2}≤3 µs). Thus, there is no longer any discrepancy between the P680\textsuperscript{+} reduction kinetics and the S II\textsubscript{vf} rise, allowing Z to be a likely candidate for the immediate donor to P680\textsuperscript{+}. 

3.2 MATERIALS AND METHODS

Samples used were similar to other $O_2$-evolving PS II preparations reported in the literature (10,11). Spinach leaves were blended for 10 s in 0.4 M sucrose/50 mM MES (pH 6.0)/10 mM NaCl/5 mM MgCl$_2$ and filtered through 8 layers of cheesecloth. This sample was centrifuged at 7000 rpm for 10 min in a Sorvall SS34 rotor. The resulting pellet was resuspended in 50 mM MES (pH 6.0)/10 mM NaCl/5 mM MgCl$_2$/1 mM ascorbate. The suspension was accelerated in the centrifuge to 3000 rpm, which was immediately shut off, to pellet cellular debris. The supernatant was treated with 25 mg Triton X-100 per mg Chl at a sample concentration of 2 mg Chl per ml and immediately subjected to a low speed centrifugation at 3000 rpm for 4 min (SS34 rotor). The resulting supernatant was centrifuged at 18,500 rpm for 10 min. After discarding the supernatant the pellet was resuspended to 2 mg Chl per ml in 50 mM MES (pH 6.0)/10 mM NaCl/5 mM MgCl$_2$ treated with 5 mg Triton X-100 per mg Chl, spun at 3000 rpm for 5 min and the supernatant centrifuged at 18,500 rpm for 10 min. The resulting pellet was washed in 50 mM MES (pH 6.0) and 10 mM NaCl and pelleted at 18,500 rpm for 10 min. This final pellet was then resuspended in 50 mM MES (pH 6.0) and 10 mM NaCl to a Chl concentration of 3 mg per ml. Potassium ferricyanide (1 mM) and ferrocyanide (1 mM) were added as exogenous electron carriers and for redox buffering. The sample was kept on ice until the measurement began. The samples exhibited $O_2$ evolution rates of 200-300 $\mu$M $O_2$ per mg Chl per h initially, which decayed 10-25%
during the experiment. During the measurements the samples were flowed through a Scanlon EPR flat cell at a rate of 0.4 ml per min by a Gilson minipuls II peristaltic pump then back into an ice cooled reservoir. At a 2 Hz flash frequency this gives the sample 15 flashes while it is in the laser beam. The temperature in the flat cell was 10±2°C as measured by a Fluke 2100 A digital thermometer using a copper-constantan thermocouple. The thermocouple was located inside the flow system at the exit of the flat cell.

An X-band field-swept spectrum of the sample was taken before the experiment began. The field was then set to $g = 2.010$ (low-field hyperfine maximum of $S$ II), and a 1000 pass kinetic trace of the decay of $S_{IIv}$ was acquired. The sample was then used to collect $S_{IIv}$ risetime data, which were stored after 7500 and 15000 passes. After the risetime data were stored a 2000 pass decay trace at $g = 2.010$ was collected. These final decay data were taken to check for the appearance of $S_{II}$ resulting from deactivation of the samples. In all cases, the final decay trace was not less than 90% $S_{IIv}$.

A Varian E-109 EPR spectrometer modified for 1 MHz magnetic field modulation at a modulation amplitude of 4.2 G was used for the measurements. The instrument response time was about 2 μs (12). When field-swept spectra were taken, an output low-pass RC filter of 0.3 s was used, and the data were digitized at 36 ms per channel. When $S_{IIv}$ decay traces were collected, an output low-pass filter of 300 μs was used, and the data were digitized at 20
µs per channel. While collecting the risetime data, the output
was filtered by a 0.5 µs low-pass filter, and the data were
digitized at 200 ns per channel. In all cases the output was fed
into a Nicolet Explorer IIIA digital oscilloscope, and 4096
channels were collected. The data were accumulated in a
laboratory-built signal averager. The signal-averaged trace or
spectrum was sent upon completion to a VAX 11-780 computer for
data analysis and display.

Light pulses were provided by a modified flashlamp-pumped dye
laser (Phase-R Corp. DL-1400) which delivered pulse widths of 0.3-
0.5 µs FWHM. Energies of 3–5 mJ per pulse were delivered to the
sample. Rhodamine 640 (Exciton, Dayton, OH, U.S.A.) in methanol
was used as the dye. A repetition rate of 2 Hz was used for all
kinetic traces.

3.3 RESULTS

The sum of 67,500 traces can be seen in Fig 1. On the left
is the full kinetic trace and on the right is an expanded time-
scale for a clearer view of the rise kinetics. As can be seen in
the figure, the rise of $S_{II_vf}$ is instrument limited ($t_{1/2}$ ≤ 3 µs).
The decay of the signal was fit in the manner described in chapter
2 of this thesis (6). The fit is shown in Fig 2. A clearly
biexponential decay is observed with $t_{1/2} = 50 ± 25 \mu s$ and $0.9 ± 0.4 \text{ ms}$. The 50 µs decay component is $60 ± 15\%$ of the initial amplitude.
The other $40 ± 15\%$ is due to the $0.9 \text{ ms}$ component.

During our control experiments decay of $S_{II_vf}$ in
chloroplasts and PS II particles at pH 6.0 and T=10±2° C was
Fig 1

EPR S II_{vf} kinetics with 2μs time resolution. (A) Full scan, 820μs; (B) Expanded data, 200μs. Flowing sample contained 3 mg Chl per ml, 1 mM potassium ferri/ferrocyanide, 50 mM MES (pH 6.0), 10 mM NaCl; T = 8-12°C. Varian E 109 spectrometer: 1 MHz field modulation, amplitude 4.2 G; response time, 2μs; microwave power, 100 mW. Laser excitation intensity, 3-5 mJ per pulse at sample; 15-20 flashes per sample volume under flow; frequency, 2 Hz. Total flashes, 67,500; sample changes after each 15,000 flashes showed at least 90% S II_{vf} at g 2.010.
Fig 2

Least squares fit to the decay data from Fig 1. The biexponential decay shows $t_{1/2}=50\pm25 \ \mu$s and $0.9\pm0.4 \ ms$ with decays terminating at a prerecorded baseline. Instrumental and sample conditions given in Fig 1.
Fig 3

Model of electron transfer in PS II. $S_0$ through $S_4$ represent the $S$-states of the $O_2$-evolving enzyme according to the model by Kok et al (14). $Y$ is a proposed electron carrier between the $O_2$-evolving enzyme and $Z$. $Z$ is a semiplastoquinone which donates electrons directly to the PS II reaction center. $P680$ is the reaction center of PS II. $I$ is a pheophytin molecule which acts as an intermediate electron carrier on the acceptor side of PS II. $Q_A$, $Q_B$ and $PQ$ are plastoquinone molecules which accept electrons from PS II.
measured using 100 kHz field modulation. Both the particles and the chloroplasts had decay halftimes of 0.9±0.3 ms, in agreement with the data collected using the 1 MHz field modulation system. The 50 µs decay component was not visible in the control due to the 250 µs response of the 100 kHz system.

3.4 DISCUSSION

A submillisecond decay component of S II_vf was postulated in earlier work on the flash number dependence of S II_vf decay (5). In that study, decay halftimes of 400 µs following the second flash, 1 ms after the third flash and only small spikes on the first and fourth flashes were seen. If S₀ → S₁ and S₁ → S₂ feed electrons to Z⁺ with a halftime of 50 µs and S₂ → S₃ and S₃ → S₀ feed electrons to Z⁺ with a halftime of 1 ms, we expect to observe a biexponential decay where the sample is present in the flat cell during 15 flashes. The 400 µs halftime on the second flash in (5) could then be due to a combination of a 1 ms decay and an instrument limited spike. Experiments are being developed to test these assignments.

In our control experiments we find that our PS II particles are more stable in O₂-evolution than are chloroplasts. Even with this increased stability, cooling the sample and changing samples every 15,000 flashes was necessary. Because the S II_vf risetime reported in (9) was collected on a 5 ml sample for 20,000 kinetic traces at room temperature, it seems likely that much of the data collected was measuring the risetime of S II_vf. Although the chloroplasts were in a pH 7.5 buffer, the thylakiod membrane's proton gradient could set the pH inside of the thylakiod membrane.
where the $O_2$-evolution system is located, to between 5.5 and 6.0, where the risetime of $S_{II_f}$ is 20 µs (6,13).

The instrument-limited risetime of $S_{II_f}$ is consistent with a model where the plastoquinone species is the immediate electron donor to the reaction center, as it is in Tris-washed samples (6). All of these data combined give rise to a model of the donor side of PS II shown below, where the plastoquinone species giving rise to $S_{II_f}$ is the physiological electron transfer component $Z$ donating directly to P680. In this model, $S_0-S_3$ represent Kok's four-state model for the $O_2$-evolving enzyme (14). $Y$ represents an electron carrier between the enzyme and the quinone responsible for $S_{II_f}$.
REFERENCES


CHAPTER 4

The Effect of Mono- and Divalent Salts on the Rise and Decay Kinetics of EPR Signal II in Photosystem II Preparations from Spinach

4.1 INTRODUCTION

Recent advances (1,2) in the preparation of O₂-evolving PS II preparations have stimulated extensive research into the mechanism of the water splitting reactions and the organization of electron transport components on the donor side of PS II. The ability of high concentrations of salts to inhibit the steady-state O₂-evolution activity of these preparations has been well documented (3-6). This inhibition is attributed to the extraction of three extrinsic proteins, namely the 16 kDa (7), the 24 kDa (3,4) and the 33 kDa proteins (5,6). Washing with 0.8 to 2M NaCl depletes the preparations of the 24 and 16 kDa proteins (3,4) and produces 70 to 80% inhibition of O₂ evolution (3,4,6), while extraction with 0.8 to 1M CaCl₂ or MgCl₂ causes ≥95% inhibition of O₂-evolution and extracts the 33 kDa protein as well as the 16 and 24 kDa proteins (5,6). Reconstitution studies (3,6-9) indicate that both the 24 and 33 kDa proteins, and probably the 16 kDa protein (7) are needed for competent water splitting activity, although none of the three proteins appears to carry a transition metal or prosthetic group capable of electron transport (5,6,10). More recently, the 33 kDa protein has been suggested to stabilize Mn within its membrane binding site (11,12), while the 24 kDa protein has been implicated as a Ca²⁺ binding protein.
These proteins appear to maintain a proper conformation of electron transport components on the donor side of PS II so that facile electron transport from the oxygen evolving complex to the reaction center of PS II, P680, can occur.

We have previously observed that treatment of PS II preparations with NaCl or MgCl₂ blocks the formation of the low temperature multiline EPR signal attributed to Mn in the S₂ state of the O₂ evolving complex (6). To examine further the effects of salt inhibition on the electron transfer components of the donor side we have measured the rise and decay kinetics of EPR Signal II (S II) in untreated and salt inhibited samples. This signal arises from a semiplastoquinone cation (14) which passes oxidizing equivalents from the PS II reaction center (P680⁺) to the oxygen evolving complex (15,16). Inhibition of O₂-evolution activity by Tris has been shown to slow both the rise and decay kinetics of S II (17-19). In contrast, we find that extensive depletion of the 16 and 24 kDa proteins by NaCl or NaBr extraction causes the loss of 70-80% of the steady state O₂-evolution activity but leaves the rise and decay kinetics of S II substantially unaltered (within instrument resolution). However, after removal of the 33 kDa protein in addition to the 16 and 24 kDa proteins by CaCl₂ treatment, we find that the rise and decay kinetics are similar to those seen in Tris-treated preparations.

4.2 MATERIALS AND METHODS

Oxygen evolving PS II preparations were obtained and incubated in high concentrations of salt as previously described (6). The extracted preparations were then centrifuged (10 min, 34,000 g) and
washed free of the salt by suspension in 50 mM MES (pH 6.0), 10 mM NaCl followed by centrifugation. Steady-state \( \text{O}_2 \) evolution rates were measured polarographically, using a Yellow Springs Instrument 4004 electrode and a high sensitivity teflon membrane. Actinic light from a 200 W projector lamp was filtered through 2.5 cm of water and a cutoff filter (Corning C.S. 3-68) and focused onto the polarographic vessel of 4.3 ml capacity. Oxygen evolution was assayed at 21±2°C in 50 mM MES (pH 6.0) and 10 mM NaCl, which additionally contained 3 mM ferricyanide, 3 mM ferrocyanide and 500 \( \mu \)M 2,5-dichlorobenzoquinone (DCBQ) as an electron acceptor system. Chlorophyll concentrations in the assay were about 5 \( \mu \)g per ml. Chlorophyll concentrations were obtained by the method of Arnon (20). Conditions for electrophoresis are described in the legend to Fig. 1. Samples used for S II decay measurements were resuspended in 50 mM MES (pH 6.0) and 10 mM NaCl to a chlorophyll (Chl) concentration of 3 mg/ml. Samples used for risetime measurements were resuspended in the same buffer except that the pH was set at 5.5. Before the kinetic measurement, unless otherwise noted, 2.5 mM ferricyanide, 2.5 mM ferrocyanide and 500 \( \mu \)M DCBQ were added to the sample as redox buffers and external electron carriers. S II kinetic measurements were performed and analyzed as previously described (19,21). Sample excitation was acheived by pulsing the sample with a Phase-R DL-1400 pulsed dye laser using rhodamine 640 (Exciton) in methanol as the dye. Kinetic measurements in the ms and longer time regime were done on a Varian E-109 EPR spectrometer using 100 kHz magnetic field modulation and no filter on the output of the receiver. Faster kinetics (down to 2 \( \mu \)s resolution)
were obtained on the same spectrometer modified for 1MHz field
modulation. In all cases the laser repetition rate was 2 Hz and
kinetics were measured at g=2.010. Instrument settings are listed in
the figure and table legends.

4.3 RESULTS

4.3.A Peptide Content and O₂-Evolution Activity of the Salt Washed
Preparations

The effects of salt-washing on the O₂-evolution activity (Table
1) and peptide content (Fig 1, Table 1) of the PS II preparation are
reasonably consistent with previously published work (3,4,9). We find
that washing with 800 mM NaCl inhibits the O₂-evolution activity by
70% and depletes the preparations of only the 16 and 24 kDa proteins
(Fig 1B, Table 1), while washing with 800 mM CaCl₂ (or MgCl₂) produces
95% inhibition of O₂-evolution activity and completely removes the
16, 24 and 33 kDa proteins (Fig 1D, Table 1). In accord with our
previous work (6), Fig 1C and Table 1 show that extraction with 800 mM
NaBr results in greater inhibition of O₂-evolution than does washing
with 800 mM NaCl, and produces nearly complete release of the 24 and
16 kDa proteins, but only a small loss of the 33 kDa protein.

4.3.B S II Rise Kinetics

Previous work has established that untreated PS II preparations
exhibit a risetime of S II that is instrument limited (≈2 μs) (21),
while for Tris-inhibited samples the risetime is increased to 10 μs at
pH 5.5 (19). Fig. 2 reveals the effects of salt-washing on the
kinetics of S II appearance under comparable conditions. Preparations
treated with 800 mM CaCl₂ exhibit a risetime similar to that seen in
Fig. 1

Densitograms of SDS-PAGE gel illustrating the effects of salt extractions on the peptide content of PS II preparations. The procedure for salt extraction is described in the text and in (6). A) Control preparation, B) 800 mM NaCl, C) 800 mM NaBr, D) 800 mM CaCl₂. Prior to electrophoresis 100 μl of each sample (Chl concentration=1.5 mg per ml) was diluted into an equal volume of a solution containing 0.125 M Tris-HCl, pH 6.8, 4 M urea, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol. 25 μg of Chl was loaded in each case. Protein composition was analyzed by SDS-PAGE in the buffer system of Laemmli (22), additionally containing 2 M urea. The samples were electrophoresed under constant power (25 mW). The gel was stained with Coomassie brilliant blue R-250, and the densitograms were recorded on a Hoeffer Scientific Instruments GS 300 scanning densitometer. The residual band at the 32-34 kDa position in the CaCl₂-treated preparations apparently is due to the atrazine binding protein (23) and/or the protein described by Metz et.al. (24), because in other samples this band remained after extraction with 960 mM Tris-HCl (pH 9.3), a treatment known to release the 16, 24, and 33 kDa proteins completely (4).
TABLE I

The Effect of Mono- and Divalent Salts on the \( O_2 \)-Evolution Activity and Peptide Content of PS II Preparations

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>( O_2 ) EVOLUTION ACTIVITY(^a) (%)</th>
<th>RELEASE OF PEPTIDES(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>800 mM NaCl</td>
<td>69</td>
<td>16</td>
</tr>
<tr>
<td>500 mM NaBr</td>
<td>74</td>
<td>24</td>
</tr>
<tr>
<td>800 mM NaBr</td>
<td>82</td>
<td>33</td>
</tr>
<tr>
<td>800 mM CaCl(_2)</td>
<td>96</td>
<td>100</td>
</tr>
</tbody>
</table>

PS II preparations were salt treated as described in the text. \( O_2 \)-evolution activities were obtained as described in the text and in (6). The amount of the peptides was estimated from the peak height of the stained bands in densitograms (Fig. 1), using an untreated sample as reference for no release, and a sample treated with 960 mM Tris, pH 9.3 as reference for complete release(4). The peak heights of the 16, 24 and 33 kDa bands were normalized to the 43 kDa band to account for minor variations in the amount of Chl loaded in each lane. Decreasing the Chl load from 25 \( \mu \)g to 10 \( \mu \)g provided results which were within the indicated uncertainty.

\( ^a \)Control activity: 410 \( \mu \)mol \( O_2 \) (mg chl\(^{-1}\)) \( h^{-1} \). Estimated uncertainty, \( \pm 5\% \).

\( ^b \)Estimated uncertainty, \( \pm 10\% \).
Fig. 2

Formation kinetics of S II under different salt deactivation conditions. Top, 500 mM NaBr deactivated; bottom, 800 mM CaCl$_2$ deactivated. EPR S II kinetics measured using a Varian E-109 EPR spectrometer with 1 MHz field modulation, 2 μs time resolution, 4.3 G modulation amplitude, 55 mW microwave power, kinetics measured at g $= 2.010$. Flowing sample (8-12 flashes/turnover) contained 3 mg Chl per ml, 2.5 mM potassium ferricyanide, 2.5 mM ferrocyanide, 500 μM DCBQ, 10 mM NaCl and 50 mM MES (pH 5.5). Sample temperature was 17±1°C. Sample (volume=4-8 ml) was changed after each 20,000-30,000 flashes. Each kinetic trace represents the sum of 50,000 flashes. Gain settings were identical for each trace. Laser excitation, 5-10 mJ/pulse at the sample; flash frequency, 2 Hz.
\[ \frac{\delta x''}{\delta H} \]

500 mM NaBr

800 mM CaCl₂

Time(μsec)
Tris-inhibited samples (Fig. 1). In contrast, samples extensively depleted of the 16 and 24 kDa proteins by treatment with 500 mM NaBr show a risetime that is instrument limited (Fig. 2). Thus, it appears that the loss of the 33 kDa polypeptide is correlated with the slower donation of electrons from Z to P680⁺ in Tris- and CaCl₂-inhibited preparations.

4.3. C S II Decay Kinetics

Under the repetitive flash conditions (8-12 flashes before turnover), both untreated and salt-washed PS II preparations show multiphasic S II decay kinetics. Control and monovalent salt washed samples exhibited a fast phase in the µs time regime (Fig. 3A) and two slower phases in the ms regime (Fig. 3B). In contrast, the CaCl₂-treated preparations showed no µs decay component (Fig. 3A); instead, a biphasic decay on a ms time scale was observed (Fig. 3B).

The decay curves were fit by a non-linear least squares fitting routine to obtain the half-times (t) and amplitudes (A) of the various decay components. The slowest component in the millisecond time range (Fig. 3B) was fit using the pre-recorded baseline as the level at tₐ. Subsequently, the other decay component in the millisecond range was fit after fixing the slower component's time (tₜ) and amplitude (Aₜ). The amount of the fastest decay component which is visible in Fig. 3A could be determined from the data in the millisecond time range (Fig 3B) by measuring the difference between the CaCl₂-treated sample's amplitude and the amplitude of any other decay trace. These differences correlated well with the contribution of the fast phase to the total amplitude seen in Fig. 3A despite the difference in
Fig. 3

The effect of salt treatments on the decay kinetics of signal II.

3A. (submillisecond kinetics) From top to bottom are: control, 800 mM NaCl treated, 500 mM NaBr treated, 800 mM NaBr treated and 800 mM CaCl$_2$ treated. Sample and instrument conditions are as in Fig. 2, except that the sample was resuspended in MES buffer at pH 6.0. A 10 µs low pass R-C filter was used on the output of the 1 MHz receiver. Gain settings were identical for each trace. Each trace is the sum of 50,000 flashes.

3B. (millisecond kinetics) From top to bottom: Control, 800 mM NaCl treated, 800 mM NaBr treated and 800 mM CaCl$_2$ treated. SI kinetics measured at g 2.010. Varian E-109 settings: 10° gain, 100 kHz field modulation, 5 G modulation amplitude, 20 mW microwave power, halftime of instrument rise is 0.25 ms (time constant, out). Flowing sample contained 2.5 mM potassium ferricyanide, 2.5 mM potassium ferrocyanide, 500 µM DCBQ, 10 mM NaCl and 50 mM MES (pH 6.0). Sample temperature was kept at 17±1°C. Sample volume 2 ml. Each trace reflects the sum of 6000 flashes. Amplitudes are normalized for differences in chlorophyll concentration. Laser excitation, 5-10 mJ/pulse at the sample; laser flash frequency, 2 Hz; 8-12 flashes per sample turnover under flow.
microwave power used to collect the two sets of data (55 mW in Fig. 3A and 20 mW in Fig. 3B).

The results of the analysis are presented in Table II. For the untreated preparations, a rapid decay phase of about 140 µs ($t_r$) contributes 55% to the observed signal amplitude, while two slower phases of about 3 ms ($t_m$) and 100 ms ($t_s$) contribute 35% and 10% respectively. After CaCl$_2$ extraction of the 16, 24 and 33 kDa proteins the 140 µs decay disappears, leaving two decay components of 9 and 100 ms. In contrast, PS II preparations which were extensively depleted of the 16 and 24 kDa proteins by NaCl or NaBr extraction exhibited little or no loss of the 140 µs component, but they did show a 2-3 fold increase in $t_m$ and an increase in the percentage of the slower ms phase, $t_s$ (Table II).

In the above experiments, DCBQ was added so that there was a good electron acceptor for the samples. For the inhibited samples, the hydroquinone form of the molecule may act as an electron donor to S II. However, in the absence of quinone, we observed no increase in amplitude of the ms phases of the NaCl- or NaBr-inhibited samples, which indicates that the 140 µs component cannot be due to the rapid re-reduction of $Z^+$ by the exogenous hydroquinone (data not shown).

To investigate whether the 140 µs decay resulted from a forward donation reaction, as opposed to a back-reaction with the primary or secondary quinone acceptors, kinetic traces were collected on the control and salt-washed samples in the absence of added acceptor. The amplitude of the signal produced in control and 800 mM NaCl treated samples is only one-third that produced in CaCl$_2$ treated preparations.
TABLE II
The Effect of Mono- and Divalent Salts on the Decay Components of S II

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>A_f</th>
<th>t_f (μs)</th>
<th>A_m</th>
<th>t_m (ms)</th>
<th>A_s</th>
<th>t_s (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55±10</td>
<td>140±50</td>
<td>35±5</td>
<td>3.5±2</td>
<td>10±5</td>
<td>100±35</td>
</tr>
<tr>
<td>800 mM NaCl</td>
<td>55±10</td>
<td>140±50</td>
<td>25±5</td>
<td>9±2</td>
<td>20±5</td>
<td>100±35</td>
</tr>
<tr>
<td>500 mM NaBr</td>
<td>55±10</td>
<td>140±50</td>
<td>25±5</td>
<td>9±2</td>
<td>20±5</td>
<td>100±35</td>
</tr>
<tr>
<td>800 mM NaBr</td>
<td>50±10</td>
<td>140±50</td>
<td>35±5</td>
<td>9±2</td>
<td>15±5</td>
<td>100±35</td>
</tr>
<tr>
<td>800 mM CaCl₂</td>
<td>0</td>
<td>---</td>
<td>63±5</td>
<td>9±2</td>
<td>38±5</td>
<td>100±35</td>
</tr>
</tbody>
</table>

Halftimes (t) and relative amplitudes (A) of the slow (s) medium (m) and fast (f) components of S II were obtained from kinetic traces (Figs 3A and 3B) as described in Results under S II Decay Kinetics. Samples used were PS II subchloroplast preparations with 2.5 mM ferricyanide, 2.5 mM ferrocyanide and 500 μM DCBQ added. Instrument conditions are listed in Figs 3A and 3B.
(data not shown). Under multiple (8-12) flash conditions and in the absence of acceptor, little signal is expected if the rate of forward donation to Z⁺ is much greater than the rate of back reaction. The multiple flashes result in an accumulation of reducing equivalents on the acceptor side of PS II and in the reduction of QA, after which stable charge separation cannot occur. The diminution of S II amplitude in the control and monovalent salt washed samples implies that the fast 140 µs decay results from a forward donation, while the kinetics of S II reduction observed in the CaCl₂-treated samples appears to arise to a greater extent from a back reaction with the acceptor side. The conclusion that the 140 µs decay is due to a forward donation reaction is further supported by our observation that increasing the ferricyanide (acceptor) concentration from 2.5 mM to 10 mM did not increase the proportion of the ms phases in control and monovalent salt-washed samples.

4.3.D Steady State Spectra

When samples competent in oxygen evolution are given saturating, continuous illumination, there is very little buildup of light-induced S II due to rapid rereduction of the donor side of PS II by the intact oxygen evolving complex. In our control samples this increase consists of about 10% of the dark level of S II. At pH 6.0, the Tris-treated, NaCl-treated, NaBr-treated, and CaCl₂-treated preparations all show increases in the steady-state level of S II in the range of 45-65% of the dark level (Table III). Monovalent salt-washed samples display a light induced increase which is slightly less than that seen in divalent salt- or Tris-treated cases. This level of increase in S
TABLE III

The Effect of Mono- and Divalent Salts on the Steady State Light Induced Increase in S II.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>AMPLITUDE OF LIGHT INDUCED S IIa</th>
<th>AMPLITUDE OF S II IN THE DARK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>800 mM NaCl</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>800 mM CaCl₂</td>
<td>0.64</td>
<td></td>
</tr>
</tbody>
</table>

Amount of \( O_2 \) evolution and corresponding peptide release of these samples are described in Table I. PS II preparations were salt treated as described in the text. 1 mM ferricyanide and 1 mM ferrocyanide were added to each sample which were at a chlorophyll concentration of 3 mg ml\(^{-1}\) and buffered at pH 6.0.

Spectra were collected using a Varian E-109 EPR spectrometer with 100 kHz magnetic field modulation, 6.3 gauss modulation amplitude, 0.128 second time constant, 20 mW microwave power, gain of 50,000 and a scan rate of 25 gauss min\(^{-1}\). S II amplitudes were taken as the height from the low field maximum (g=2.010) to the high field minimum (g=1.999) in the derivative spectrum. The dark level was measured after illumination.

a Estimated uncertainty, ±0.05.
II during illumination of the samples indicates that the loss of either the 16 and 24 kDa proteins or additionally the 33 kDa protein substantially decreases the rate of electron transfer from the oxygen evolving complex to Z. However, the differences in the transient kinetics observed for the monovalent and CaCl₂ treated preparations indicate that the direct donor to Z is not disturbed by the removal of the 16 and 24 kDa proteins, but this donor is affected by the removal of the 33 kDa protein.

4.3. E Flash Number Dependence of S II Decay Kinetics

In Fig 4, the flash number dependence of the millisecond decay components in control, NaCl-treated and CaCl₂-treated PS II preparations is shown. Control traces are the same for PS II preparations at pH 6.0 (Fig 4) as for chloroplasts at pH 7.5 (26). The CaCl₂-treated sample is present for a full amplitude reference. The low amplitude of resolvable S II transient on each flash in NaCl-treated preparations shows that a constant, large (55%) amount of the S II transient is in the sub-millisecond time range on every flash. The lack of oscillation of either the millisecond or sub-millisecond range kinetics of S II in the NaCl-treated samples implies that the sub-millisecond component is not due to donation from S₀ and S₁ to Z⁺.

4.4 DISCUSSION

The results presented here can be interpreted within the framework of the mechanism presented in Scheme I. In this model, there is an intermediate donor Y between the oxygen evolving complex and Z. This donor equilibrates with Z⁺ during photochemical turnover with an apparent equilibrium constant of approximately 1 to 1.8. In
Fig. 4

Flash number dependence of the millisecond range kinetic components of S II under different salt deactivation conditions. Top, active sample, pH 6.0; center, 800 mM NaCl deactivated; bottom, 800 mM CaCl$_2$ deactivated. Sample and instrument conditions are as Fig 3B, except that a flow protocol of stopping the flow then separately recording each of 4 flashes separated by 500 ms which was repeated until all of the sample had passed into a second reservoir, and then dark adapting for 5 min was used. Sample volume was 20-30 ml. The timing circuitry was provided by laboratory-built switch programmed time-based oscillators. Each trace is the sum of 1500 flashes. Laser excitation, 50-70 mJ per pulse at the sample, laser flash frequency, 2 Hz. Microwave power, 5 mW.
Flash Number: 1

Untreated

800 mM NaCl

800 mM CaCl₂

\[ \frac{\delta x^*}{\delta H} \]

Time (msec)

500 msec
SCHEME I

A Extraction of 18, 24 kDa proteins
B Extraction of 33 kDa protein
control samples the 140 µs phase of Z⁺ reduction then corresponds to the rapid equilibration of Y with Z⁺, while the predominant ms phase (3 ms) results from rate-limiting steps of the reduction of Y⁺ by the oxygen evolving complex. Upon release of the 16 and 24 kDa proteins, electron donation from the oxygen evolving complex to Y⁺ is blocked or greatly diminished at the S₁ to S₂ transition (6). At the chloride levels in the final suspension buffers used in this study (10 mM), we find that, within experimental error, the loss of the multiline EPR signal is proportional to the loss of O₂ evolution activity. The multiline signal amplitude observed in samples depleted almost completely of the 16 and 24 kDa proteins (800 mM NaBr treated samples, Table I) may be due to the chloride effect described by Toyoshima et al. (29). The 140 µs phase of S II decay arise from the rapid equilibration of Z⁺ with Y, while the ms phases of S II decay arises from the slow, rate-limiting reduction of Y by an exogenous or endogenous donor, and/or a back reaction with the acceptor side. This accounts for the increase in the halftime of the middle phase of S II and the increase in the relative amplitude of the slow ms phase after monovalent salt washing. The model requires that Y be reduced during the dark cycle time (500 ms) in samples treated with monovalent salts. This condition appears to be met at pH 6.0, since we did not observe a significant difference between the amplitudes for the µs phase in the control and monovalent salt-washed samples under conditions in which they received 8 to 12 flashes before turnover. Within this model the additional release of the 33 kDa protein by CaCl₂ blocks or greatly decreases the rate of electron transfer between Y and Z⁺. The fact
that the rise of S II is also slower indicates that there is a major alteration of the electron transport chain on the donor side of PS II after extraction of the 33 kDa protein. The postulated electron transfer component (Y) may be identical to that giving rise to a g 4.1 EPR signal observed at 10 K after low temperature illumination (25). Experiments to test this possibility are currently in progress.

The model presented in Scheme I is also consistent with the flash number dependence of S II decay reported by Babcock et al. (26), if it is assumed that the 140 μs phase of equilibration between Y and Z⁺ was not resolved in those experiments. Thus, on the first flash, which reflects primarily the S₁→S₂ transition, no signal was observed, owing to the rapid reduction of Y⁺ by the oxygen evolving complex. On the two subsequent flashes, representing the S₂→S₃ and S₃→S₄ transitions, the observed amplitudes and half-times of S II decay reflects the rate limiting steps of the reduction of Y by the oxygen evolving complex. On the fourth flash (S₀→S₁ transition), again little signal would have been observed owing to the rapid reduction of Y. The flash number dependence shown here (Fig 4) agrees with this assignment. The amplitude of S II on the third flash in the control is only 70% of the amplitude seen in the CaCl₂ treated sample. The missing 30% is not likely to be due to misses and double hits due to the high intensity and short duration of the laser flash. These data argue for an intermediate donating on the sub-millisecond time range on every flash. In the NaCl-treated sample, the changes of resolvable S II with flash number seen in the control sample is nearly absent. A small amount of the flash number dependence seen in the control sample
is expected, since about 25% of the \( \text{O}_2 \)-evolution activity remains in this sample. Hence, in this case, 55% of the signal is missing on each flash in the millisecond time regime, omitting the possibility that the 140 \( \mu \text{s} \) decay is from donation of \( S_0 \) and \( S_1 \) to \( Z^+ \).

Although they did not resolve the 140 \( \mu \text{s} \) phase of S II decay, the recent results of Ghanotakis et. al. (27) reporting the effects of NaCl washing on the kinetics of S II decay are reasonably consistent with ours. However, they concluded that the unresolved rapid phase of S II decay was the result of rapid donation from Mn in the lower \( S^- \)-states of the oxygen evolving complex. We have previously shown that release of the 16 and 24 kDa proteins by salt treatment inhibits the formation of the low-temperature multiline EPR signal attributed to Mn in the \( S_2 \) state of the \( \text{O}_2 \)-evolving site (6). Assuming the model of Ghanotakis et.al. (27), this result would indicate either that release of the 16 and 24 kDa proteins blocks or greatly decreases the rate of electron donation from the Mn complex to \( Z \) decreasing the yield of \( S_2 \), or that the Mn complex donates to \( Z \) but, in the absence of these proteins, can no longer give rise to the multiline signal. Although we cannot exclude the latter possibility, such a perturbation of the oxygen evolving complex should also alter the rate of electron transfer from \( S_1 \) to \( Z^+ \). However, we have observed no significant difference between the halftimes for the \( \mu \text{s} \) phase in the control and monovalent salt-washed samples (Fig. 3, Table II). Moreover, the observation by Ghanotakis, et.al. that the addition of benzidine to NaCl-washed samples decreases the amplitude of the ms phases of S II reduction through conversion of higher oxidation states of Mn to lower
ones which rapidly donate to \( Z^+ \) can be equally well explained as due to benzidine donation to \( Y \) in our model (Scheme I).

Recently, Akerlund et al. (28) found that NaCl washing of inside-out thylakoids decreases the amplitude of the ns phases of chla\(_{II}\) reduction and increases the \( \mu s \) phases, indicating that removal of the 24 kDa protein decreases the rate of electron transfer from \( Z \) to P680\(^+\) (Scheme I). This result is difficult to reconcile with our observation that the risetime of S II (Z in Scheme I) remains \( \approx 2 \mu s \) after extensive depletion of the 16 and 24 kDa proteins. We are presently attempting to measure by EPR the decay kinetics of P680\(^+\) in NaBr- and CaCl\(_2\)-treated preparations to investigate this discrepancy.

Although a more complete understanding of the organization of electron transport components on the donor side of PS II awaits the \( \mu s \) time resolution of the flash number dependence of the S II decay kinetics, the results presented here in combination with those of Casey and Sauer (25), and Blough and Sauer (6) support the idea that there is at least one donor between the oxygen evolving complex and \( Z \).
REFERENCES

EPR Signal II in Cyanobacterial Photosystem II Reaction Center Complexes with and without the 40 kDa Chlorophyll-binding Subunit

5.1 INTRODUCTION

Photosystem II reaction center complexes have been isolated from several organisms including higher plants (1,2), Chlamydomonas (3) and Synechococcus (4). These photosystem II reaction center complexes have similar polypeptide compositions. They contain two large polypeptides with molecular weights of between 40 and 50 kDa that are chlorophyll-binding proteins in PS II (4,5). There are two or more proteins having a molecular weight around 30,000. One of the 30 kDa proteins is assigned as a herbicide binding protein in higher plants (1). The PS II complex also contains a small polypeptide which is thought to be the apoprotein of cytochrome $b_{559}$ (2).

The site of the primary reaction of PS II correlates with the presence of the large chlorophyll-binding protein: 47 kDa protein in higher plants (6-8) and Synechococcus (9,10) and 50 kDa protein in Chlamydomonas (5,11). The chlorophyll protein complex called CP2b, which has 47, 31, 28 and 9 kDa subunits but no 40 kDa subunit, was isolated from Synechococcus (4,9). This chlorophyll protein complex shows high electron transport activity from diphenylcarbazide to dichlorophenolindophenol (9), and it contains an appreciable amount of $Q_A(X320)$, photoreducible pheophytin and cytochrome $b_{559}$ (10).

EPR signal II ($S_{II}$) originates from a radical species on the
donor side of PS II (12,13), most likely a semiplastoquinone cation (14,15). There are at least two different pools of this species with different roles in PS II. Half of the S II is either stable in the dark or is formed upon first illumination and decays with a halftime of several hours in whole chloroplasts. The dark stable portion and the slowly decaying portion of the free radical species are designated S II_u and S II_s, respectively (12,16). The functions of the molecular species responsible for S II_u and S II_s are not clear, although S II_s is proposed to originate from D, a donor present at the oxidizing side of PS II which is not directly transmitting oxidizing equivalents to the oxygen evolving system. This conclusion is based largely on the kinetics, which is too slow for a direct role in physiological electron transfer. Quantitation of this persistent pool of S II in chloroplast thylakoid membranes shows one free radical formed per PS II reaction center (12,17). The other half of the pool of S II originates from Z^+, which is involved in the transfer of positive charge from the oxidized reaction center of PS II, P680^+, to the oxygen evolving enzyme. This portion of S II reflects the direct donation of electrons to P680^+ in Tris-treated PS II preparations (18) and possibly in O_2-evolving preparations from spinach (19). In samples competent in O_2 evolution the observed S II transient is referred to as S II_{vf} (20-22). In samples completely devoid of O_2 evolution capability, the rise and decay kinetics of S II are slower than in O_2 evolving samples (18,19), and the transient signal observed is referred to as S II_{f} (23). The lineshapes of S II_f, S II_s, and S II_u are identical (23). Due to the rapid reduction kinetics the
steady-state light-induced S II is not normally observed (20,21), although a rough field position profile has been reported (20).

Signal II has been observed in various PS II reaction center preparations (17,24) and in some cases S II amplitudes were measured. Babcock et. al. reported 1 Z⁺ per P680 in Tris-treated chloroplasts, but only 0.6 Z⁺ per P680 in several PS II particle preparations (17). Satoh et. al. reported that the ratio of S II to reaction centers is similar in PS II particles to that in thylakoids (24). Here we report the presence and quantitation of S II in CP2b and E-1 preparations and discuss the localization of Z and D.

5.2 MATERIALS AND METHODS

The thermophilic cyanobacterium Synechococcus sp. (a gift from S. Katoh, Department of Pure and Applied Sciences, Tokyo Univ., Tokyo Japan) was grown at 53°C for 3 d with continuous bubbling of 5% CO₂ in air in the light (25,26). Thylakoid membranes were prepared as in (4), except that 1 mM PMSF (phenylmethylsulfonylfluoride) was added to the medium during the thylakoid membrane preparation after lysozyme treatment.

The PS II reaction center complex (E-1) was prepared as in (9) with slight modification. The PS II reaction center complex was extracted from the thylakoid membranes with 0.8% β-octylglucoside. The crude extract of PS II was diluted by a factor of two with 50 mM Tris-HCl (pH 7.5)/10 mM NaCl medium and collected by centrifugation, 250,000 x g for 2 h, and stored at -80°C. The crude extract was suspended in the Tris-HCl (pH 7.5)/NaCl medium containing 0.8% β-octylglucoside and then purified by digitonin-polyacrylamide gel
electrophoresis (0.2% digitonin, 4.5% acrylamide), as described previously (4,9).

CP2b was prepared as in (9). Gels containing CP2b were stored at -80°C until use. Frozen gels were homogenized with a Teflon tissue homogenizer in 50 mM MES/NaOH, pH 6.0 buffer. Gel debris was separated from the homogenate by 30,000 x g, 30 min centrifugation, and the complex was recovered by 260,000 x g, 2 h centrifugation. The precipitate was suspended in MES buffer and used immediately for the measurement.

Steady state and kinetic EPR measurements were performed on a Varian E-109 EPR spectrometer using 100 kHz magnetic field modulation. Instrument settings for each measurement are listed in the figure captions. Data were averaged on a laboratory-built signal averager and sent to a VAX 11-780 for data analysis and display. The kinetics were analyzed using a nonlinear least-squares fitting routine as previously described (18,19). Double integration of steady-state spectra was done on the VAX using a laboratory-written program. Samples were suspended in 50 mM MES, pH 6.0 containing 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide, unless otherwise stated. The temperature was set at 0°C by a cooled N2 gas flow and monitored by a thermocouple. Continuous white light of saturating intensity was provided by a microscope illuminating system. Light pulses for kinetic measurements (640 nm, 50 mJ/flash, 0.3-0.5 μs FWHM) were provided by a dye laser (Phase-R DL-1400) using rhodamine 640 (Exciton) in methanol as laser dye. To estimate the concentration of radical species, S II spectra were collected using nonsaturating
microwave power (5 mW). Spectra were doubly integrated and compared with an optically calibrated solution of potassium nitrosodisulfonate (Aldrich) in a carbonate buffer according to Babcock et al (17), except that the light-induced S II spectra were taken at only one magnetic field modulation amplitude to prevent light-induced damage to the samples. Chlorophyll concentrations were obtained by the method of Arnon (27).

5.3 RESULTS

The EPR spectra of the photosystem II reaction center complex, E-1, are shown in Fig. 1a. The E-1 complex has 47, 40, 31, 28 and 9 kDa protein subunits (9,10), and has been found to contain 1 QA, 1 photoreducible pheophytin and 1 cytochrome b₅₅₉ per 32-46 Chl (10). The dark and light-induced spectra show the characteristic line shape of S II reported earlier for thylakoid membrane preparations (12,13,16,20) and PS II samples (17,24). Only a small amplitude of S II is present before initial illumination (D) following 1-2 h dark adaptation. During illumination (L) a large amplitude of S II is induced, half of which decays in the subsequent 5 min dark period before completion of the collection of the next spectrum (D(L)). The pool of S II observed in the dark after illumination has a decay halftime of 25-30 min under the measuring conditions, and could account for the small amplitude of S II seen before the initial illumination.

Fig. 1b shows the EPR spectra of CP2b, which has no 40 kDa chlorophyll-binding subunit (see Introduction). The EPR spectrum obtained before illumination is composed of mainly a small
Fig. 1. Dark and light-induced EPR spectra at 0°C in E-1(a) and CP2b(b) Photosystem II particles. Spectrum D was recorded in the dark, L during continuous saturating illumination and D(L) within 5 min following the end of continuous illumination. Microwave power, 5 mW; modulation frequency, 100 kHz; modulation amplitude, 2 G in (a), 4 G in (b). Signals were averaged for 1 and 5 passes in (a) and (b), respectively. Scan time of 8 min in (a) and 4 min in (b) with time constants of 0.25 s in (a) and 0.128 s in (b). Arrows indicate field position where kinetics in Figs. 2 and 3 were obtained.
structureless free radical signal centered at g 2.0025±0.0003 with an 8 G linewidth. This free radical is similar to P680+(28,29), and could be due to oxidized bulk chlorophyll. Illumination clearly causes the appearance of S II. There is no significant increase of the structureless free radical signal induced by continuous illumination; the light minus dark difference spectrum (not shown) displays the same lineshape as is seen in E-1 samples (Fig. 1a). The structureless free radical signal similar to that seen in CP2b samples in the dark was also observed in aged E-1 samples in the dark (data not shown). It can also be seen that there is very little of the slowly decaying portion of S II present in CP2b after illumination (Fig 1b, curve D(L)).

Spin concentrations of E-1 and CP2b were measured by double integration of the EPR spectra and comparing the amplitude to an optically calibrated spin standard as in (17). These spectra yielded spin concentrations shown in Table 1. The dark spectra and the spin standard were found to have double integrals which were linear with modulation amplitude between 0.5 G and 8 G, as in (17). Such measurements were not possible with the light-induced signals due to light-induced degradation of S II in the samples. However, double integration of S II in two different E-1 samples at two different modulation amplitudes yielded similar results (Table 1). The total amount of S II present in the E-1 sample under illumination is 1 equivalent per 17-20 Chl. Approximately half of this signal decays before the dark measurement 5 min later. However, in CP2b the total amount of free radicals visible in the light was 1 equivalent per 42 Chl, most of which was S II. As can be seen in Fig. 1b, almost all of
Table I.

SPIN CONCENTRATION IN PS II REACTION CENTER COMPLEXES

Spectra were taken of the spin standard (potassium nitrosodisulfonate) and S II in the dark after illumination, D(L), at a variety of modulation amplitudes according to the protocol of Babcock et al. (17). The light-induced spectrum, L, was taken either at 2 G (E-1(1)) or 4 G (E-1(2) and CP2b) magnetic field modulation amplitude. All spectra were taken at 5 mW microwave power on a Varian E-109 EPR spectrometer as described in Materials and Methods. Samples ranging from 0.5 to 0.7 mg Chl/ml were suspended in a 50 mM MES buffer (pH 5.5) containing 1 mM ferricyanide and 1 mM ferrocyanide. The temperature was set at 0°C. Estimated uncertainty of Chl/spin is ±20%.

<table>
<thead>
<tr>
<th>Sample</th>
<th>L</th>
<th>D(L)</th>
<th>L-D(L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-1 (1)</td>
<td>17</td>
<td>38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>E-1 (2)</td>
<td>20</td>
<td>57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CP2b</td>
<td>42</td>
<td>316</td>
<td>49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> S II<sub>S+u</sub>
<sup>b</sup> S II<sub>f</sub>
the S II decayed before the dark measurement after illumination.

The decay kinetics of S II in E-1 are shown in Fig. 2a and summarized in Table II. The signal was monitored at the position indicated by the arrows in Fig. 1 (g 2.010), where there is no contribution from any other free radical signals. As can be seen in Fig 2b, the amplitude of each of the 2 decay components in the biphasic decay of S II in CP2b is the same as in E-1 within the signal-to-noise of this measurement; however, the decay times decreased from 20 ms in E-1 to 7 ms in CP2b for the fast component and from 300 ms in E-1 to 200 ms in CP2b for the slow component. This indicates that not only the functional connection between P680 and Z which oxidizes Z, but also the components in the membrane which effect S II reduction are preserved in CP2b, although the geometry may be altered by the removal of the 40 kDa polypeptide.

Figure 2c shows the decay kinetics of E-1 measured on a sample flowing through the cavity and back into an ice cooled reservoir to allow extensive signal averaging while avoiding sample degradation. The kinetics of S II in E-1 (Fig 2c, Table II) shows biphasic decay behavior. One component (55% of the total amplitude) has a decay halftime of 300 ms. The other decay component (45% of the amplitude) has a decay halftime of 20 ms. A 10% decrease in the amount of the slow component is observed upon the addition of DCMU (100 μM) to an E-1 sample containing 1 mM ferricyanide and 1 mM ferrocyanide (Table II). DCMU at this concentration has been shown to inhibit electron transport rates in E-1 (4), indicating that the electron transport beyond QA has only a small effect on S II kinetics under this
Fig. 2. Flash illumination-induced EPR signal II in E-1(a and c) and CP2b(b) Photosystem II particles. Signals were recorded at the field position indicated by arrow in Fig. 1. Microwave power, 20 mW; modulation frequency, 100 kHz; time constant=0.01 (250 µs rise halftime); modulation amplitude, 4 G (a and b) or 5 G (c). Signals were averaged for 200 flashes (a and b) or 2000 flashes (c) at a repetition rate of 0.2 Hz. Temperature, 0°C (a and b) or 15°C (c). Sample was flowed as in (19) for (c).
condition. Results presented in Fig. 3 and Table II show the effect of added ferricyanide on S II kinetics. The addition of ferricyanide increased the amplitude of S II by 50%. The decay times of the two components remained similar, but the amplitude of the fast component increased by 10% while the amplitude of the slow component increased by 100%. Further addition of the lipophilic electron acceptor 2,5-dichloroparabenzoquinone had no additional effect (Table II). This is suggestive that the rate of electron transfer from $Q_A$ to exogenous acceptors has only a small effect the rate of S II reduction, but having ferricyanide present to allow $Q_A$ to be reoxidized between flashes is necessary for the subsequent photo-oxidation of P680 to be stabilized, allowing more complete oxidation of Z.

5.4 DISCUSSION

Both a rapidly decaying EPR signal (S II$_f$) and a slowly decaying component (S II$_{u+s}$) are clearly present in E-1, as they are in several types of PS II particles (17, 24). Signal II$_f$ arises from $Z^+$, the oxidized form of the direct electron donor to P680$^+$ (16, 18). S II$_s$ reflects a one time donor (D) on a side path of the electron transfer chain on the donor side of PS II (16, 23). The concentration of the EPR detectable $Z^+$ is about 1 equivalent/30 Chl and that of D$^+$ is about 1/40 Chl in E-1. The antenna size in E-1 has been estimated as 32 to 46 Chl per reaction center, based on the measurement of several redox components in E-1 (10). Accordingly, the amounts of $Z^+$ and D$^+$ estimated here correspond to about one of each species per reaction center. This corresponds well with the amount of D$^+$ measured in thylakoid membranes and some PS II preparations per reaction center.
Fig. 3 The effect of ferricyanide on EPR signal II kinetics of E-1 Photosystem II particles. Samples were suspended in MES-NaOH buffer (pH 5.5) and signals were measured in absence (a) or in the presence (b) of 1 mM potassium ferricyanide at 15 °C. Measuring conditions were as in Fig. 2c, including sample flow. No ferrocyanide was present during these measurements.
TABLE II

THE EFFECT OF SEVERAL REAGENTS ON THE DECAY COMPONENTS OF S II IN E-1 PHOTOSYSTEM II REACTION CENTER COMPLEXES

Experimental conditions are the same as in Fig. 3. Each set of measurements was carried out sequentially after addition of the components listed. S II decay kinetics was analyzed as the sum of two exponential decay components.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Amplitude</th>
<th>Halftime (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Fast Slow</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fast</td>
</tr>
<tr>
<td>1 mM ferricyanide/ferrocyanide</td>
<td>100</td>
<td>43</td>
</tr>
<tr>
<td>+100 μM DCMU</td>
<td>87</td>
<td>46</td>
</tr>
<tr>
<td>No Additions</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>+1 mM ferricyanide</td>
<td>152</td>
<td>59</td>
</tr>
<tr>
<td>+ 250 μM DCMQ</td>
<td>154</td>
<td>62</td>
</tr>
</tbody>
</table>
and of \( Z^+ \) measured in Tris-treated thylakoid membranes (17), although lower amounts of \( Z^+ \) were reported for some PS II preparations. These facts support a model where \( Z \) plays an essential role as a direct electron donor to P680 (18, 19). The presence of equimolar \( D^+ \) indicates the possible importance of this species in electron transfer on the donor side.

However, in CP2b almost all of the \( S \) II observed is rapidly reduced after illumination (about 1 equivalent/50 Chl), with only a small amount of mainly a structureless free radical seen in the dark (Table 1, Fig 1b). The amount of \( Z^+ \) observed (1/50 Chl) is the same as the amount of functional Q\(_A\) in CP2b (10). The amount of D\(^+\) is very small (less than 1/400 Chl), but some of this slowly decaying species is present in CP2b in addition to the structureless free radical seen before illumination (see the difference between D(L) and D at g 2.010, Fig 1b).

The species which produces \( S \) II was correlated with a quinone moiety through extraction and reconstitution experiments by Kohl and Wood (30). Kohl et al had previously suggested that \( S \) II arises from a plastochromanoxyl radical (31). Hales and Das Gupta proposed that a plastosemiquinone anion perturbed by a divalent cation produces the signal (32). More recently O'Malley and Babcock provided more extensive evidence for a plastoquinone cation radical as the species responsible for \( S \) II by showing that quinone cation model compounds have similar line shapes and orientation dependance as \( S \) II (14, 15).

Omata et al reported two plastoquinone molecules per reaction center in spinach PS II preparations (33). This seems to be the case
also in E-1 preparations (Y. Takahashi, Univ. of Tokyo, personal communication). There is one primary acceptor $Q_A$ present per reaction center in both E-1 (10) and spinach PS II preparations (34). So the remaining one plastoquinone per reaction center is not enough to explain two $S_{II}$ spins ($D^+$ and $Z^+$) per reaction center. Thus, either $Z$ or $D$ is a quinone other than a plastoquinone, is not extractable by organic solvents, or is not a quinone at all.

The decay kinetics of signal II have been studied in Tris-washed chloroplasts and Tris-washed PS II preparations. Babcock and coworkers (16,17,20,23,35-38) have studied the effects of midpoint potential, donor concentration, salt concentration, pH and DCMU on $S_{II}$ decay kinetics in Tris-washed chloroplasts. With no additions or with 10 mM ferricyanide added to Tris-washed chloroplasts a slow decay of 0.4 to 1.2 s has been observed (16,37,38). This decay was attributed to an unknown species on the donor side of PS II with a midpoint potential of $-80$ mV (16). A donor capable of reducing $P680^+$ (possibly through $Z$) at 77°K has been reported by Malkin and Bearden (28). To date, no spectroscopic signals have been found that correspond with the proposed donor. Upon addition of DCMU, the decay of $S_{II}$ speeds up to 300 ms due to back reaction of $Z^+$ with $Q_A^-$ (16,37). The back-reaction rate is pH independent from pH 5.5 to 6.5, but accelerates to 100 ms at pH 5.5 (37). The observation of this back-reaction was dependent upon having $Q_A^-$ reoxidized between flashes by ferricyanide in the presence of high salt to allow the oxidant to react with the quinone. Otherwise, under signal-averaging conditions, $Q_A^-$ would build up, stopping the light-driven oxidation of $Z$. Dekker
et al (39) studied Tris-washed PS II particles prepared according to
the protocol of Berthold et al (40) and observed a 50 ms decay of S II
in the presence of DCMU and ferricyanide. This component was
correlated with a 50 ms decay seen at 325 nm which is due to QA 
oxidation. However, at 325 nm only 40% of the signal decays with a 50
ms halftime while 60% decays with a 3 s halftime. The 3 s component
is presumably due to oxidation of QA by ferricyanide. Dekker et al
concluded that S II was being re-reduced by both QA and ferrocyanide
with halftimes of 120 ms and 80 ms, respectively, to give an overall
decay time for S II for 50 ms.

The addition of ferricyanide increases the slow component from
45% to 60% while increasing the total amplitude by 50%, presumably by
oxidizing QA in competition with the back-reaction. Similarly,
addition of 100 µM DCMU to an E-1 sample containing 1 mM ferricyanide
shifts the amount of the fast component from 43% to 53%. Thus we
conclude that the fast decay is probably due to back-reaction of Z
with QA and the slow decay component is due to donation from some
intrinsic donor as in the model of Babcock and coworkers (16,37). The
decay times are faster than in thylakoid membranes for toth reactions;
however, the combination of samples derived from a different organism
and manipulations for the purpose of achieving biochemical purity may
affect the geometry of the reactants, their midpoint potentials, or
both. Even isolating PS II from PS I by making BBY particles (40)
changes the apparent back reaction time at pH 6.0 to 50 ms (39) from
100ms-300 ms (37). In E-1, the back-reaction time is reduced to about
20 ms, with the slower forward donation decreased to about 300 ms at
pH 5.5. However, this model does not explain the change in the decay time of the slow component upon addition of ferricyanide to the E-1 preparation. Possibly the differences arise from the presence of multiple donors, such as ferrocyanide and an endogenous donor.

In CP2b, S II<sub>f</sub> is clearly observed. The line shape of the light-minus-dark signal is the same as that observed in E-1. The decay kinetics is similar, though slightly faster than in E-1 (see results). These results indicate that both the environment to produce the characteristic line shape of S II<sub>f</sub> and the components in E-1 which rereduce Z<sup>+</sup> are well preserved in CP2b, although an alteration of the geometry and thereby the kinetics may occur when the 40 kDa protein is removed.

In conclusion, we can state that the 40 kDa protein is not necessary for the function of Z<sup>+</sup> as a donor to P680; the 47, 32, 28 and 9 kDa proteins are sufficient to support these reactions. Z, which exists in a 1:1 ratio with P680, must be integral to the reaction center complex. These conclusions are supported by the retention of the amplitude, lineshape, and kinetics of S II<sub>f</sub> seen in both E-1 and CP2b. Although the amount is small, D seems also to be present in CP2b. Because the amount of D is very low in CP2b and the amount of D is less than that of Z in E-1, D may be more susceptible to the detergent or purification process used than is Z. From the kinetics observed in E-1 under various conditions, electron transfer must be similar to that of Tris-washed chloroplasts (16,36,37), where a slow donor, probably intrinsic to the protein complex, competes with the back reaction between Q<sub>A</sub><sup>-</sup> and Z<sup>+</sup>. This probably holds also in
CP2b, based on the similarity in the kinetics of S II.
REFERENCES

CHAPTER 6

The Effect of Calcium Depletion on EPR Signal II in Anacystis Nidulans

6.1 INTRODUCTION

Calcium has been shown to stabilize PS II in whole cells (1), isolated membrane preparations (2), and PS II fractions (3,4) from cyanobacteria. In Anacystis nidulans one site of Ca$^{2+}$ function appears to be near the reaction center of PS II. Electron transfer from diphenylcarbazide to silicomolybdate is blocked in the absence of Ca$^{2+}$ (5). Variable fluorescence remains near $F_0$ and delayed fluorescence is eliminated (6) as Ca$^{2+}$ is pumped out of the cells by some light driven process (7). Turnover rates of remaining active photosynthetic units do not decline as Ca$^{2+}$ depletion progresses, but the steady state rate decreases (5). These results indicate that Ca$^{2+}$ depletion causes a switching off of individual PS II units rather than gradually slowing down rates of electron transfer. These effects of Ca$^{2+}$ depletion on whole cells are fully reversed when the external medium is supplied with Ca$^{2+}$ and illuminated, at which time normal culture growth resumes within a few hours.

In this work, we have measured the amplitude and kinetics of EPR signal II (S II) in untreated and in calcium-depleted cells of Anacystis nidulans. This signal has been proposed to arise from a semiplastoquinone cation (8), usually designated $Z^+$, which probably is formed by electron transfer directly to the oxidized reaction center.
(P660$^+$) of PS II (9-11). This species has complex decay kinetics. There is a component not directly involved in electron transfer from water to P680 that gives rise to a constant dark level, usually designated S II$_u$, and there is a slowly decaying ($t_{1/2}=4$ h) portion of the signal which is usually formed on the first illumination. This slower decaying portion is usually called S II$_s$ (12,13). S II also has rapidly decaying components which reflect electrons passing from water to the reaction center under physiological conditions. These kinetic components have been designated S II$_{vf}$ (14). These kinetics also show a dependence upon flash number (15) which appears to be correlated with the states of the O$_2$ evolving complex, commonly designated as the S-states of the model of Kok(16,17). The kinetics of the decay of Z$^+$ changes with the condition of the donor side of PS II. When O$_2$ evolution is deactivated by removal of the 16 and 23 kDa polypeptides in PS II preparations (18,19) using sodium salt extractions (20-23), the kinetics of S II remains substantially unaltered from the preparations competent in O$_2$ evolution (24). However, when a treatment which removes the 16, 24 and 33 kDa proteins is used, such as Ca$^{2+}$ salt extractions, Mg$^{2+}$ salt extractions, Tris treatment, or high pH treatment (20-23), the oxidation of Z slows to the microsecond range (10,24) as is seen by the reduction kinetics of P660$^+$ observed optically (25). The subsequent reduction of Z$^+$ also slows to the millisecond range (10,12,24). This behavior has traditionally been referred to as S II$_r$ (12). Heat treatment of chloroplasts also induces S II$_r$ decay kinetics (14), although the biochemical characterization of the effects of heat treatment has not
been reported.

6.2 MATERIALS AND METHODS

6.2.A Sample Preparation

*Anacystis nidulans* was grown as previously described (1). Calcium depletion was carried out in Cg-10 medium as described previously (26). Cells in depletion medium were illuminated until they reached the desired level of O₂ evolution activity. Reconstitution was done by adding 0.35 mM Ca(NO₃)₂ to the depleted cells in the modified Cg-10 medium, then continuing illumination.

Control, depleted and reconstituted cells were pelleted and resuspended to a chlorophyll concentration of 1 mg/ml in the same medium that they were incubated in. These concentrated cells were maintained in darkness prior to EPR measurements. Heat treatment of cells was accomplished by warming to 55°C for 10 min, followed by immediate cooling to room temperature and measurement of light induced S II transients. Steady state O₂ evolution rates were measured polarographically using a Yellow Springs Instrument 4004 electrode and a high sensitivity teflon membrane. Actinic light from a 200 W projector lamp was filtered through 2.5 cm of water and a cutoff filter (Corning C.S. 3-66) and focussed onto a polarographic cell of 4.3 ml capacity. Oxygen evolution of cells was assayed at 35°C in growth medium at 3-6 µg chl/ml. Chlorophyll concentrations were obtained by the method of Arnon (27).

6.2.B Spectroscopic Measurements

Steady state and kinetic measurements in the ms time range were performed on a Varian E-109 EPR spectrometer using 100 kHz magnetic
field modulation and no filter on the output of the receiver for kinetic measurements (halftime of the instrument limited rise is 250 μs), or with a .128 s low pass R-C filter on the output for steady state measurements. Faster kinetics on samples which were not heat treated were measured using the same spectrometer modified for 1 MHz magnetic field modulation (28). The output of the 1 MHz receiver was filtered by a 30 μs low pass R-C filter. The data were averaged on a laboratory built signal averager and sent to a VAX 11-780 for data analysis and display. The kinetics were analyzed using a nonlinear least-squares fitting routine as previously described (10,11). In all cases, kinetics were measured at a low-field hyperfine component of S II (g 2.010). The sample was flowed at a rate which exposed a sample to 4-8 flashes per transit volume unless otherwise noted. With the 2-4 ml sample volumes used, this flow rate provided a dark adaptation time of 3-6 min before the transit volume was exposed to another series of flashes. Sample excitation was achieved by illuminating the sample with a Phase-R DL-1400 pulsed dye laser at a repetition rate of 2 Hz. The dye used was rhodamine 640 (Exciton) in methanol. A flash energy of 10-20 mJ/pulse was delivered to the sample with FWHM of 0.3-0.5 μs.

For the power saturation curve, the microwave power at the cavity was measured by coupling out -24.1 db (measured) into a Hewlett Packard 432A power meter with a Hewlett Packard 8478B thermistor.

6.3 RESULTS

The effect of Ca²⁺ depletion and reconstitution on S II kinetics in heat-treated cells can be seen in Fig. 1. The kinetics remain
Fig. 1

Signal II kinetics in control and in calcium-depleted and reactivated *Anacystis nidulans* after 10 min of heat treatment. Kinetics were measured at g 2.010 using a Varian E-109 EPR spectrometer with 100 kHz field modulation, 5 G field modulation, 250 \( \mu \)s halftime for the instrument rise (time constant=1 out), gain of 10^4 at a microwave power of 55 mW. Flowing sample received 4-8 flashes per transit volume with a dark period of 3-6 min before subsequent flashing. The sample of *Anacystis* was concentrated to 1 mg chlorophyll/ml in growth medium and heated to 55 °C for 10 min before cooling to 25 °C for the measurement. From top to bottom are: control, calcium depletion to 75% of control \( O_2 \) evolution, calcium depletion to 25% of control \( O_2 \) evolution, calcium depletion to 10% of control \( O_2 \) evolution, and cells reactivated to 85% of control \( O_2 \) evolution from 25% \( O_2 \) evolution by adding 0.35 mM Ca(NO\(_3\))^2 to the cell medium and illuminating. Each kinetic trace is the sum of 5000 laser flashes at 2 Hz.
unaltered, but the amplitude decreases with decreasing O₂ evolution caused by Ca²⁺ depletion. Upon reconstitution the signal amplitude returns nearly to control levels (Table I).

To check for the appearance of a block beyond pheophytin on the acceptor side, the dependence of S II amplitude on the number of flashes during the sample transit was measured. By increasing the flow rate and decreasing the laser flash frequency to 0.5 Hz, S II amplitude and kinetics on the first flash after darkness were obtained. As can be seen in Fig. 2, there is essentially no difference between the amplitudes of the first flash and multiple flash data.

The spectrum of S II in the dark after illumination (S II_u + S II_s) is similar to that seen in spinach chloroplasts (12,14,29) (Fig. 3). Also, the signal amplitude of S II in the dark after illumination in Anacystis at 1 mg chl/ml is lower than S II in the dark after illumination of chloroplasts at 3 mg chl/ml.

Fig. 4 shows the microwave power saturation curve of the S II transient in Anacystis nidulans after 10 min of heat treatment. The microwave power saturation curve is similar to that reported for Tris-treated spinach chloroplasts (14).

Attempts to measure the kinetics of S II in untreated cells of Anacystis nidulans yielded only a small amplitude (=10% of the total) of a signal with a decay halftime of 0.7 ms (data not shown). Presumably the bulk of the signal decays more rapidly and is suppressed by the 30 μs instrument time constant. Upon reaching 90% inhibition of O₂ evolution by depletion of Ca²⁺ measurements with 500
Table I

The Effect of Calcium Depletion and Reconstitution on $O_2$ Evolution and S II Transient Amplitude of *Anacystis nidulans*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$O_2$ evolution$^a$ (% of control)</th>
<th>S II Transient$^a$ (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control$^b$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$-Ca^{2+}$</td>
<td>75</td>
<td>85</td>
</tr>
<tr>
<td>$-Ca^{2+}$</td>
<td>25</td>
<td>46</td>
</tr>
<tr>
<td>$-Ca^{2+}$</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>reconstituted</td>
<td>85</td>
<td>85</td>
</tr>
</tbody>
</table>

Calcium depletion and $O_2$ evolution before heat treatment of the cells was as described in Materials and Methods. Cells were heated for 10 min at 55 °C and the S II transient was measured at 25°C as in Fig 1.

a) estimated uncertainty ±5%

b) control activity = 350 μM $O_2$ (mg chl)$^{-1}$ h$^{-1}$
Fig. 2

Signal II in control and in calcium depleted cells with either 1 or 4-8 flashes during transit through the sample volume in a flowing, heat treated sample. Kinetics were collected as in Fig. 1 except that for the first flash data the flow rate of the cells was doubled and the laser repetition rate was slowed to 0.5 Hz. From top to bottom are: control cells, 4-8 flashes per sample volume; calcium depleted to 10% of control O$_2$ evolution, 4-8 flashes per sample volume; control cells, 1 flash per sample volume; calcium depleted to 10% of control O$_2$ evolution, 1 flash per sample volume.
Fig. 3

Spectrum of S II in the dark after illumination. Microwave frequency, 9.459 GHz; microwave power, 10 mW; magnetic field modulation frequency, 100 kHz; modulation amplitude, 2 G; gain, 80,000. Flowing sample (total volume of 2 ml) was illuminated for 15 min before observing the spectrum. No heat was applied to these untreated cells.
Fig. 4

Microwave power dependence of the amplitude of the millisecond decay components of S II in heat-treated *Anacystis*. Sample was flowed and kinetics were measured as in Fig. 1 except that the microwave power was varied. Each kinetic trace is the sum of 4000 events at a laser repetition rate of 2 Hz.
Microwave Power $^{1/2}$ (mW $^{1/2}$)

(Arbitrary Units)

Transient Amplitude
μs time resolution yielded a small amplitude of the S II transient in
the ms range comparable to that seen in untreated cells. However,
upon heat treatment of the cells, a large amplitude of reversible
light-induced S II is resolvable in the millisecond range in both
control and Ca\(^{2+}\) depleted samples. The light-induced S II was
measured as a function of heating time from 1 to 15 min in untreated
cells at 55 °C and was found to have invariant amplitude and decay
times between 3 and 15 min (data not shown).

6.4 DISCUSSION

As calcium is pumped out of Anacystis by a light driven process
(7) the amplitude of the S II transient decreases (Figs. 1 and 2).
This decrease parallels the decrease in O\(_2\) evolution, but it is less
pronounced than the decrease in O\(_2\) evolution (Table I). The decrease
in amplitude of S II is the same for cells subjected to a single flash
and those subjected to multiple flashes after darkness (Fig. 2).

Fig 2 shows that the decrease in amplitude of the S II transient
upon Ca\(^{2+}\) depletion is not due to a shift of the S II decay kinetics
to a component slower than 0.5 s which would be filtered out by the
repetition rate, since the amplitude after a single flash is the same
as that after 4-8 flashes at 2 Hz. The data in Fig 2 also indicate
that the decrease in amplitude is not due to a block of electron
transport beyond the first stable acceptor, Q\(_A\), since a back reaction
from Q\(_A^-\) to Z\(^+\) should be visible as a slower decay of S II. In
spinach PS II this reaction has a halftime of 300 ms (30). The data
on first-flash kinetics also show that the decreased amplitude upon
Ca\(^{2+}\) depletion is not due to a block in the donation of electrons to
Z⁺, because such a block would not cause a decreased amplitude of S II on the first flash. Thus, the results presented here, along with the previous demonstration of diminished delayed fluorescence and variable fluorescence in Ca²⁺ depleted cells (6), show that one block aused by Ca⁺² depletion is either between Z and P₆₈₀ or between P₆₈₀ and QA.

The depletion of Ca²⁺ from the cells also appears to affect electron transport from the O₂ evolving enzyme itself, because O₂ evolution drops faster than the amplitude of the S II transient (Table I). This additional effect may involve the site of the 16 and 24 kDa polypeptides released in PS II preparations from spinach by monovalent salt washing (20-23), because Ca²⁺ has been shown to play a structural role in that site (31-33). This likelihood is also reinforced by the lack of resolvable S II transient in the millisecond time regime in Ca²⁺ depleted cells which have not been heat-treated, because removal of these two proteins inhibit O₂ evolution in PS II preparations from spinach but leave the S II amplitude and kinetics largely unaltered (23).
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CHAPTER 7

Conclusions and Speculation

7.1 Conclusions

In this thesis work, the main message in terms of the role of Z in electron transport is that the semiplastoquinone cation is donating directly to P680+, and there is an intermediate electron carrier between the water splitting complex and Z. Most of the evidence for an intermediate, Y, between the water splitting complex and Z comes from information about the kinetics and amplitude of the fast phase of Z+ reduction upon treatment of PS II with 0.8 M NaCl. Under these conditions the S1 -> S2 transition is changed somehow as is evidenced by the lack of a multiline EPR signal from S2. 75% of the S4 -> S0 transition is blocked, or the overall kinetics of water-splitting is reduced by a factor of 4, since the treatment of PS II preparations with NaCl decreases the steady state O2 evolution rates to 25% of control levels. Under these conditions it would be very unusual to see no alteration in the amplitude or kinetics of the fast phase of S II reduction while the sample gets 8-12 flashes if the fast phase were directly reflecting the donation of electrons from S0 and S1 to Z. The amplitude of the slow (millisecond range) kinetics of S II as a function of flash number (chapter 4) shows that a fast phase of reduction of Z occurs on every flash. This could be due to inhomogeneity in the PS II preparations. However, the S II kinetics in Anacystis nidulans also point to an intermediate electron carrier
which reduces Z fast, even after deactivation of the O₂-evolving enzyme by calcium depletion. In this case, there are no millisecond range kinetics to monitor. All of the reduction is quite fast, faster than 30 μs in untreated cells. However, the lack of signal in deactivated cells is relieved by heat-treatment, which deactivates or severely slows donation from the proposed intermediate. Taken together, these data lead to the model proposed in chapter 4 of at least one intermediate existing between the water splitting complex and Z.

Another message acquired during these studies is that Z is integral to the reaction center of PS II. The coupling of S II rise and P680⁺ decay show direct donation in Tris-washed chloroplasts. In addition, the quantitation of S II in CP2b preparations, where there is only a four polypeptide complex, shows that Z is present in near a 1:1 ratio with the reaction center.

7.2 Wild Speculation

Although these data contribute to the understanding of the organization of the donor side of PS II, many questions remain unresolved. The major question raised here is what is the nature of the electron transfer intermediate between the water splitting complex and Z. Is it a separate molecule from the O₂-evolving enzyme or possibly a ligand between the active site and Z? This question would be best answered by searching for spectroscopic signals corresponding to this electron transfer intermediate. One possibility is the g 4.1 signal observed at low temperature (1) which appears to be a precursor to the multiline EPR signal associated with the S₂ state of the water
splitting complex. Experiments are in progress which will address this question. However, a search for EPR or optical signals generated by such an intermediate would probably serve to uncover more information about this species. Under conditions where the $O_2$-evolving enzyme is deactivated, but the intermediate donor is still operating normally, such as with monovalent salt treatments, difference spectra at a variety of illumination temperatures using a given number of saturating laser flashes should uncover signals associated with this intermediate. However, such a systematic search for signals associated with intermediates in the electron transfer chain and the subsequent interpretation of those signals could take several years to accomplish. Another possibility for gaining information about the magnetic environment, and hence the nature of species which could be donating electrons to $Z$, would be to look at S II using double resonance techniques such as ENDOR of ELDOR on samples poised in various S-states.

Another question raised by these data on S II kinetics is what contributes to the shift seen in S II$_{vf}$ kinetics under various conditions and in different preparations. In chapter 3, a biexponential decay was observed and seen to have halftimes of 50 $\mu$s and 0.9 ms. However in a similar preparation used in chapter 4, the fast decay shifted to 140 $\mu$s and the slow component shifted to 3 ms. In control experiments done while searching for conditions where PS II preparations were light stable for signal averaging purposes, leading to the information in chapter 3, I observed that ethylene glycol shifted the fast component to several hundred $\mu$s and became resolvable
by the 100 kHz field modulation system. At the same time, the slower component slowed to 10 ms. Could these conditions be systematically studied and used to produce conditions where signals due to Y are visible? What happens to the kinetics of the O₂-evolving enzyme under these conditions? Answering these questions could lead to a more complete understanding of electron transfer on the donor side of PS II.

With the development of biochemical preparations which have simple polypeptide compositions and contain the reaction center of PS II such as E-1 and CP2b, physical measurements could be performed analogous to those done on bacterial reaction centers to obtain primary data on P680. Is the reaction center a dimer? Could ENDOR give specific information regarding the oxidized reaction center's delocalization of the positive charge, as has been done in bacteria? Can analogous measurements be done on the triplet of the reaction center to look for similar symmetry to that seen in bacteria?

One other obvious question is raised in these studies when comparing the kinetics of P680⁺ reduction seen by Akerlund et al upon NaCl treatment of PS II preparations (2). The study sited sees an increase in the amount of τS phase reduction kinetics of P680⁺ upon NaCl treatment, conditions under which the risetime of S II has been measured to be instrument limited (chapter 4). Is this a difference in sample preparation? Is there another species donating electrons to P660⁺ besides S II under these conditions? It would be useful to measure S II rise and P680⁺ decay (as in chapter 2) in the same sample preparation which has been NaCl treated to resolve this discrepancy.

The resolution of these questions, coupled with the recent
explosion of both biochemical and physical experimental activity in
the study of PS II should lead to a complete understanding of the
structure and function of the donor side of PS II within a few years.
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