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LOW TEMPERATURE ELECTRON PARAMAGNETIC RESONANCE
OF THE PHOTOSYNTHETIC OXYGEN EVOLVING SYSTEM

J.L. Casey
(Ph.D. Thesis)

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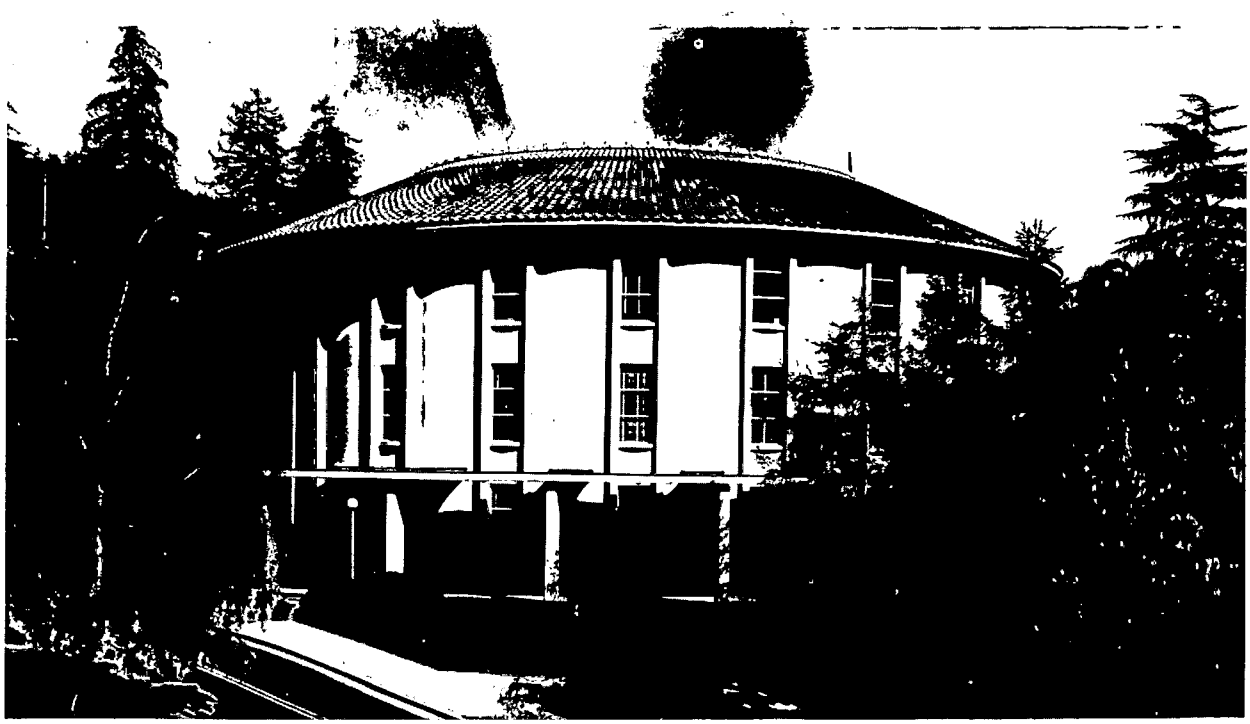

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Ph.D. Thesis

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August 1984

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Low Temperature Electron Paramagnetic Resonance

of the Photosynthetic Oxygen Evolving System

by John L. Casey

ABSTRACT

The oxidation of water to molecular oxygen by green plants requires the generation of four oxidizing equivalents per molecule of oxygen evolved. These oxidizing equivalents are stored in the photosystem 2 oxygen evolving complex, which can exist in five oxidation states, denoted S_i (i=0-4). A promising recent advance toward determining the molecular nature of the S-states was the observation of a multiline EPR which was assigned to a manganese complex and was suggested to arise from the S_2 state.

The initial studies in this thesis confirm the assignment of the multiline signal to the S_2 state by determining the temperature dependence of the formation and decay of the multiline signal and by comparing these properties with those reported for the S_2 state. The confirmation of the assignment of the multiline signal to the S_2 state allowed the use of the signal as a direct probe of the low temperature reactions of the oxygen evolving complex involving the S_2 state; threshold temperatures of 170 and 210 K are established for the light-induced advancements of S_1 to S_2 and of S_2 to S_3, respectively.

Further exploration of the S_1 to S_2 advancement threshold temperature revealed that illumination at 150 K of samples poised in the S_1 state produces a trapped intermediate state which gives a broad EPR signal at about g 4.1.; warming in the dark to 190 K results in

the disappearance of the g 4.1 signal and the appearance of the multiline signal. Low temperature illumination of samples poised in the S_2 state does not produce the g 4.1 signal; it thus appears that the species which is responsible for the g 4.1 signal is involved in the advancement of S_1 to S_2, but not of S_2 to S_3.

Further studies of the the low temperature reactions of photosystem 2 have shown that high salt and hydroxylamine, which inhibit oxygen evolution, also inhibit the formation of the g 4.1 signal; however, treatment with fluoride, which also inhibits oxygen evolution, does not inhibit the formation of the intermediate state, but does prevent the advancement of the intermediate state to S_2.

It is proposed that the species which gives the g 4.1 signal acts as an electron carrier between the oxygen evolving complex and the direct donor to the reaction center. The g-value of the signal is suggestive that non-heme ferric iron is involved in the reactions leading to the photosynthetic oxidation of water.

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ABREVIATIONS

ATP	adenosine triphosphate
chl	chlorophyll
cyt	cytochrome
EDTA	ethylenediamine tetraacetiic acid
ENDOR	electron nuclear double resonance
EPR	electron paramagenetic resonance
ESE	electron spin echo
EXAFS	extended x-ray absorption fine structure
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
G	gauss
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MES	2-(N-morpholino) ethanesulfonic acid
NADP	nicotinamide adenine dinucleotide phosphate
PS	photosystem
Tris	tris (hydroxymethyl) aminomethane

CHAPTER 1

INTRODUCTION

1.1 The Transduction Of Sunlight Energy By Living Organisms

The sustenance and growth of life require two fundamental elements; energy and building material. These needs have unquestionably been the primary driving forces in the evolution of life on earth. As the most abundant source of energy on the earth's surface is sunlight, which annually delivers some 10^{20} kcal of energy, it is not surprising that nature has devised several means of converting the electromagnetic energy of sunlight into chemical energy which can be used by living organisms. The development of the ability of life forms to use sunlight was critical in evolution, for it made available a virtually unlimited supply of energy for living systems, and ultimately provided an oxygen-rich atmosphere which fostered the development of higher forms of life.

The transduction of sunlight into forms of energy which can be used by living organisms requires first, means of interacting with the sunlight, and second, means of converting the interaction into chemical energy. The interaction, of course, is the absorption of light by chromophores in the organism. The absorption spectrum of the pigment must lie in a region of the solar spectrum which provides

sufficient intensity for the power needs of the organism. For optimal efficiency, the excitation energy of the pigment must be used rapidly, or the excited state of the pigment will decay with no trapping of useful energy by the organism. The machinery for converting the excitation is a molecular complex which includes the pigment and proteins; this complex interacts with the excited state of the chromophore, producing a chemical effect. The protein-pigment complexes are usually imbedded in membranes which provide a mechanism for the conversion of the light-produced chemical changes into a form of energy which can be used by the organism. The identical orientation of the individual complexes with respect to the plane of the membrane provides for the addition of the trans-membrane effects produced by the individual complexes.

1.2 Light-driven Proton Transport - The Bacteriorhodopsin Cycle

The bacterium Halobacterium halobium uses perhaps the simplest means of transducing solar energy (for review see Refs 1,2). At low oxygen concentrations this bacterium produces the light-harvesting protein bacteriorhodopsin, which is clustered in the outer membrane; it is the purple coloration of bacteriorhodopsin which gives the purple membrane its common designation. Associated with the protein is a single retinal chromophore, which is in the all-trans configuration in a light-adapted state. The absorption of green light by this chromophore initiates a series of events which results in the transfer of up to two protons across the membrane. The initial events in the transduction of the light energy, which include the isomerization of a double bond and proton transfer reactions, occur in

approximately 10 ps; the quantum yield is thus favorable (ca. 0.2 - 0.3). Because the entire cycle requires just 10 ms, each bacteriorhodopsin is capable of transporting many protons across the membrane in a short period of time.

All of the bacteriorhodopsin molecules have the same orientation with respect to the plane of the membrane, thus the absorption of light by many bacteriorhodopsin molecules results in the formation of an electrochemical gradient across the membrane. This light-generated proton gradient is used to form ATP, a universal source of energy for endergonic reactions in living organisms. The light energy which is absorbed by the halo bacteria does not participate directly in any reactions beyond the isomerization of the pigment. Thus, these organisms have developed a plentiful source of energy, namely sunlight, but must rely on their immediate environment for a source of carbon.

1.3 Light-driven Electron Transport

Photosynthetic organisms (see reviews in Refs. 3-5), which include the non-oxygen-evolving photosynthetic bacteria, the cyanobacteria, and plants, transduce light energy into chemical energy by an entirely different means from that used by the halo bacteria. In the former organisms, light initiates a series of oxidation-reduction reactions which leads to the formation of proton gradients for ATP formation, and, in some cases, to the creation of stored strongly oxidizing and reducing compounds. A wide variety of chromophores, absorbing throughout the visible and near infrared regions of the spectrum, is observed in photosynthetic organisms;

these pigments include chlorophylls, bacteriochlorophylls, carotenoids, and phycobilins. Most of these are so-called light-harvesting, or antenna, pigments which absorb the sunlight and transfer their excitation energy to a special pigment in a reaction center. The number of accessory pigments differs between 50 and 1000 per reaction center, and is usually responsive to the intensity of light received by the organism. The large variety of pigments in the antenna systems enable photosynthetic organsims to use a wide range of the solar spectrum and the variable size of the antenna provide the ability to grow under high or low intensity light.

The initial light-energy transduction events occur in the pigment-protein complex of the reaction center [6,7]. Upon excitation, a special pigment in the reaction center, which is either a type of chlorophyll or bacteriochlorophyll, rapidly transfers an electron to an acceptor which is in the same protein. Thus, the light excitation leads to the formation of an oxidant-reductant pair. The immediate recombination of this pair, with a resultant loss of useful energy in the form of heat or fluorescence, is prevented by a series of electron transfer reactions which separate the oxidizing and reducing equivalents across the photosynthetic membrane. The difference in the reduction potentials of the relatively stable oxidation and reduction products formed as a result of the electron transfer reactions is approximatley 40% of the excited state energy of the reaction center pigment, which is approximately 1.8 eV for chlorophyll and 1.45 eV for bacteriochlorophyll; however, the loss of energy is compensated by the storage of the charges with a high

quantum efficiency (ca. 98%). As in the case of the non-sulfur purple bacteria, all of the neighboringphotosynthetic units have the same orientation with respect to the plane of the membrane; thus, the individual photosynthetic units cooperate in generating the trans-membrane electrochemical gradient.

1.3.a Cyclic Electron Tranport

In many photosynthetic bacteria the oxidizing and reducing equivalents produced by excitation of the reaction center pigment are eventually allowed to recombine, with two protons transported across the membrane for each cycle of electron transport [8,9]. Thus, the net result of light excitation in these organisms is the same as that in the non-sulfur purple bacteria - generation of a trans-membrane electrochemical gradient which is used to drive the formation of ATP [10]. What distinguishes the photosynthetic bacteria from the non-sulfur purple bacteria, and leads to their designation as photosynthetic, is their ability to use CO_2 as their sole source of carbon. This ability is brought about by the reduction of CO_2 to simple sugars. The reducing agent, NADH, is generated by the oxidation of compounds such as H_2S, H_2, and succinate in a thermodynamically unfavorable reaction which is driven by ATP [11]. Thus, in the presence of suitable mild reductants, the photosynthetic bacteria are capable of photoautotrophic growth.

1.3.b Linear Electron Transport

While the purple photosynthetic bacteria such as Rps. sphaeroides produce the reducing equivalents for carbon fixation by the ATP-driven reduction of NAD, green photosynthetic bacteria

reduce NAD with reducing equivalents formed directly in the electron transfer reactions initiated by the absorption of light [11]. The reduction potential achived by the green bacteria is thus below that of the NAD/NADH couple (E_m = ca. -350 mV), while that of the purple bacteria is just below the reduction potential of succinate (E_m = ca. 0 mV); the chain of electron acceptors which stabilize the light-generated reducing equivalents is therefore completely different for these two types of photosynthetic bacteria [12]. Although the means differ, the net result of light excitation is the same for the green and purple photosynthetic bacteria - CO_2 is reduced at the expense of compounds such as thiosulfate, H_2S, H_2, NH_2OH, and succinate. Both types of organism thus share the requirement of a mild reductant for photosynthetic growth.

The cyanobacteria and green plants are similar to the green photosynthetic bacteria in that they contain a reaction center which provides reducing equivalents for the fixation of CO_2 directly from light-driven electron transport. These organisms do not require a source of reductants for growth, however, because they posess a second light-driven reaction center which provides the neccessary reducing equivalents by the oxidation of water to oxygen [13,14]. This provides a nearly ubiquitous source of reducing equivalents for the fixation of CO_2 for growth. The development of the ability to oxidize water was a major breakthrough not only in the evolution of photosynthetic organisms but also in the evolution of all life on earth, for the energy stored in the light-driven reduction of CO_2 by water is used by non-plant life in the process of respiration, and is

the source of the fossil-fuel generated power which is used to maintain and develop civilization. While human efforts to duplicate the ability of plants to store sunlight energy by the splitting of water have been successful for the trapping of the reducing equivalents in the form of hydrogen, the ability to store the oxidizing equivalents as molecular oxygen remains difficult.

1.4 Photosynthesis in Plants

As discussed above, green plants possess two light-transducing reaction centers. These reaction centers are contained in two distinct molecular complexes, designated photosystem 1 and photosystem 2 [14]. Photosystem 1 provides the reducing equivalents for carbon fixation in the form of NADPH while photosystem 2 oxidizes water and provides reducing equivalents for reducing the oxidizing equivalents created by photosystem 1. Each photosystem has its own array of light-harvesting antenna pigments and electron transport components.

In higher plants the photosynthetic processes occur in the chloroplast, a subcellular organelle. The light-driven reactions can be further localized to a membrane structure within the chloroplast called the thylakoid membrane. Photosystem 2 appears to be concentrated largely in the stacked, or granal, regions of the thylakoid, while photosystem 1 appears to be located in the unstacked, or stromal, regions [15]. This morphological separation allows for the relatively facile separation of the two photosystems by fractionation of grana and stroma with detergents [see e.g. 16] or with a pressure cell [17]. Both photosystems are oriented

specifically in the thylakoid membrane; electrons are transported from the inner side of the thylakoid to the outer side [18]. Thus, water oxidation occurs on the inner side of the thylakoid membrane, and NADP is reduced on the outside. Coupled with the electron transport, and as a result of water oxidation, protons are pumped in the opposite direction.

1.5 Mechanism of the Photosynthetic Oxidation of Water

1.5.a The Kok Scheme

In the oxidation of water to molecular oxygen, four electrons are removed (Eq. 1-2). The oxidation could conceivably occur in a

$$2\ H_2O \longrightarrow 4\ H^+ + 4\ e^- + O_2 \qquad E_m\ 7.0 = +800\ mV \qquad \text{Eq. 1-2}$$

single four-electron step, or as a series of two- or one-electron steps (see Table 1-1). Because each photosynthetic reaction center effects the transfer of one electron upon each excitation with light, oxygen could either be evolved after four excitations of one center or after fewer excitations of cooperating centers. Joliot et.al. [19] adressed the question of cooperativity by measuring the amount of oxygen evolved after each flash in a series of flashes; the duration and intensity of the flashes were adjusted so that each center received one excitation per flash. The results of such an experiment are shown in Fig. 1-1. Several general observations can be made on the results in this figure; 1) Oxygen is evolved with a periodicity of four in flashing light. 2) The maximum yield of oxygen occurs on the third flash. 3) After several cycles, the pattern becomes damped and all flashes yield the same amount of

Table 1-1

Reduction potentials for the reduction of oxygen to water at 25°C, pH 7.
From Blankenship, R.E. (1975) Ph.D. Thesis, University of California, Berkeley, California. (LBL report 3679)

	Via Hydrogen Peroxide		Via Oxygen Atom	
	Reaction	E_0' Volts	Reaction	E_0' Volts
One e^- steps	$O_2 + e^- \rightleftharpoons O_2^-$	-0.45	$O_2 + e^- \rightleftharpoons O_2^-$	-0.45
	$O_2^- + 2H^+ + e^- \rightleftharpoons H_2O_2$	+0.98	$O_2^- + 2H^+ + e^- \rightleftharpoons H_2O + O$	-0.57
	$H_2O_2 + H^+ + e^- \rightleftharpoons HO + H_2O$	+0.38	$O + H^+ + e^- \rightleftharpoons HO$	+1.92
	$HO + H^+ + e^- \rightleftharpoons H_2O$	+2.33	$HO + H^+ + e^- \rightleftharpoons H_2O$	+2.33
Two e^- steps	$O_2 + 2H^+ + 2e^- \rightleftharpoons H_2O_2$	+0.27	$O_2 + 2H^+ + 2e^- \rightleftharpoons H_2O + O$	-0.51
	$H_2O_2 + 2H^+ + 2e^- \rightleftharpoons 2H_2O$	+1.35	$O + 2H^+ + 2e^- \rightleftharpoons H_2O$	+2.12

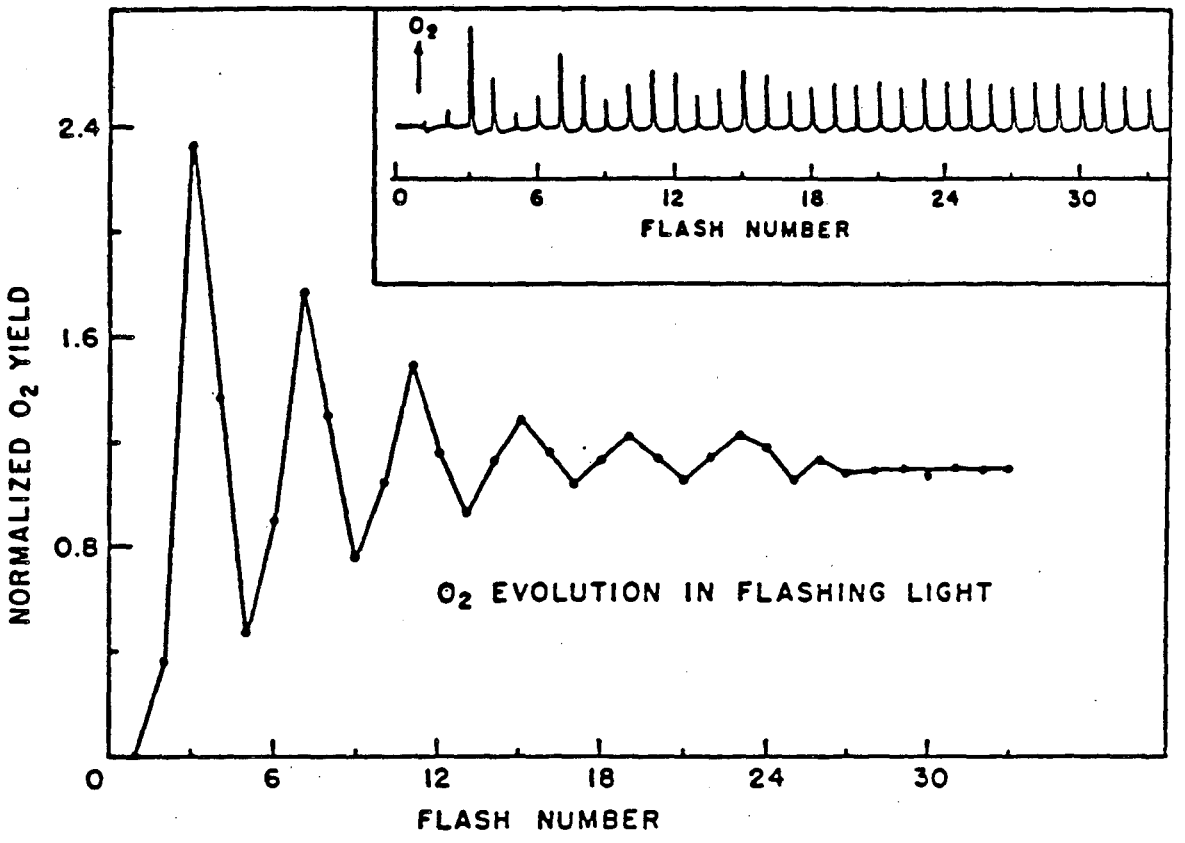


Figure 1-1

Pattern of oxygen evolution from chloroplasts in flashing light. Inset: trace of polarographic current. Main: Normalized yields. From Babcock, G.T. (1973) Ph.D. Thesis, University of California, Berkeley, CA. (LBL report 2172)

O_2. The observed periodicity indicated that the oxygen-evolving photosystem 2 centers operate independently of one another, i.e., in each photosystem 2 center, four excitations are required to oxidize two water molecules to molecular oxygen. The observed periodicity also provided evidence that the individual centers are initially in the same phase of the water-oxidizing cycle.

Kok and co-workers [20,21] proposed a model which accounted for most of the results observed in flashing light; a version of this model is shown in Fig. 1-2. The model postulates the existence of five oxidation states for the oxygen-evolving complex; these states are denoted S_i (i=0...4). Excitation of a photosystem 2 center results in the formation of an excited state which abstracts an electron from the complex and advances the S-state from S_i to S_{i+1}. Once advanced to the S_4 state, the system rapidly releases oxygen and cycles back to the S_0 state. The states S_2 and S_3 are only moderately stable, decaying to the S_1 state in approximately 60 s in the dark, with the consequent loss of the photogenerated oxidizing equivalents. That the third flash produces approximately three times the yield of the fourth flash is taken as evidence that the state S_1 is the stable state in the dark and that the ratio of S_1 to S_0 in the dark is about 3 to 1. The observed damping of the oscillating oxygen yield is explained by misses and double hits; between 10 and 20 % of the centers do not convert their excitation energy, while another 5 to 10 % receive two excitations during a flash.

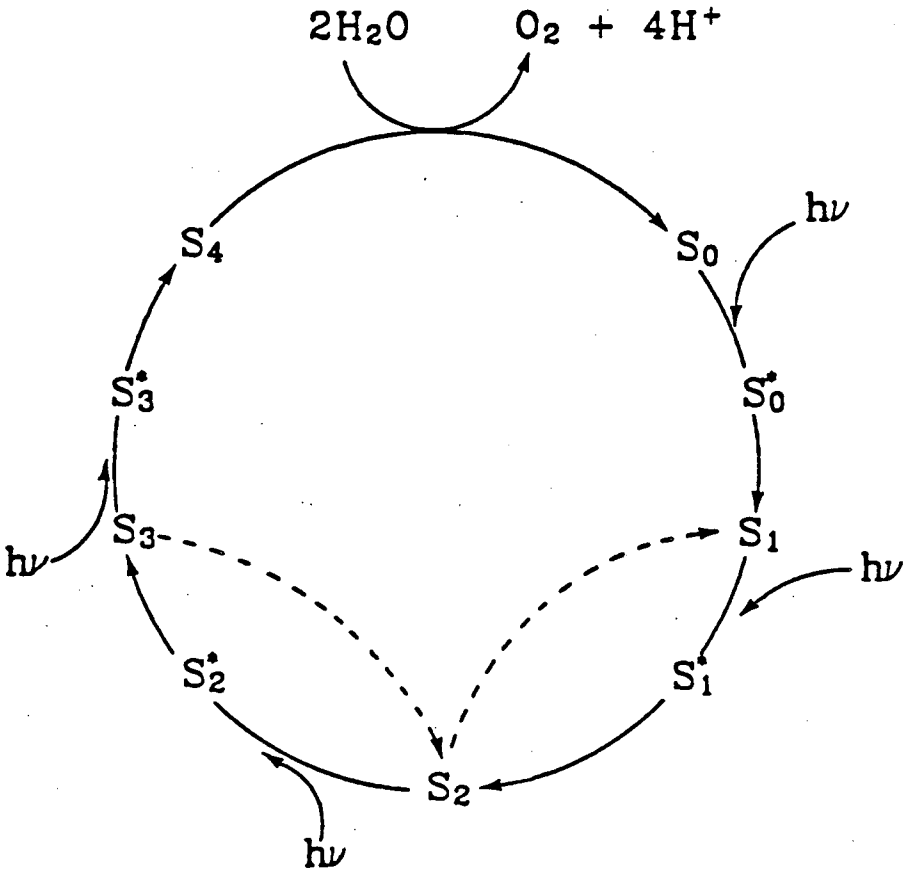


Figure 1-2

A version of the Kok model for the storage of oxidizing equivalents by the oxygen evolving complex of photosystem 2.

In the model shown in Fig. 1-2 the nature of the intermediate states S_i^* is unspecified. S_i^* may represent the system in any of the states between the initial excitation by light and the advancement of the oxygen-evolving complex to the state S_{i+1} in Eq. 1-3. P and Q represent the primary donor and acceptor, respectively, of photosystem 2; the intermediated donors represented by a subscripted D are hypothetical.

$$
\begin{array}{lllll}
S_i & D_n & D_2 & D_1 & P \quad Q \\
 & & & & \downarrow h\nu \\
S_i & D_n & D_2 & D_1 & P^* \quad Q \\
 & & & & \downarrow \\
S_i & D_n & D_2 & D_1 & P^+ \quad Q_A^- \\
 & & & & \downarrow \\
S_i & D_n & D_2 & D_1^+ & P \quad Q_A^- \\
 & & & & \downarrow \\
S_i & D_n & D_2^+ & D_1 & P \quad Q_A^- \\
 & & & & \downarrow \\
S_i & D_n^+ & D_2 & D_1 & P \quad Q_A^- \\
 & & & & \downarrow \\
S_{i+1} & D_n & D_2 & D_1 & P \quad Q_A^-
\end{array}
\qquad \text{(Eqs. 1-3)}
$$

1.5.b The Chemical Nature of the Oxygen-evolving Complex

Although the highly schematic S-state model for photosynthetic water oxidation was presented by Kok and co-workers nearly 15 years ago, relatively little information has been obtained since then on the molecular nature of the oxygen-evolving system. As a result of this lack of information, the mechanism of water oxidation remains one of the least understood aspects of photosynthesis. Although there is a lack of information on the detailed molecular structure, much has been

learned about the components involved and their behavior under certain conditions.

The actual steps which are involved in photosynthetic water oxidation must be quite different from those indicated for the four one-electron step process in Table 1-1, because the reduction potentials in some steps (e.g. +2.33 V) pose formidable barriers for a system with a one-electron oxidizing capability of about +1.0 to 1.2 V. Transition metal ions, which can bind the various species, could change the reduction potentials of the individual steps, or combine two one-electron steps into one two-electron step, thus making all of the steps in the oxidation of water accessible to the oxidizing capability of the system. Furthermore, transition metals could help stabilize the stored oxidizing equivalents. It is thus not surprising that there is evidence for the involvement of transition metals in oxygen evolution.

It has been known for some time that manganese is a necessary nutrient for plants to carry out oxygen evolution [22], and the involvement of Mn has been extensively investigated (see Refs. 23 and 24 for recent reviews). Numerous studies have attempted to determine the number of Mn in the oxygen-evolving complex. Cheniae and Martin [25] demonstrated that treatment of chloroplasts with Tris buffer released Mn; when two-thirds of the six Mn associated with their chloroplast preparation was released, no oxygen evolution activity remained. Many subsequent reports have substantiated the assignment of four Mn to each water-splitting complex (see Ref. 26); however, assignments based on the inhibition of oxygen evolution by the removal

of Mn may be difficult to assess, particularly if complexes release Mn in a cooperative or semi-cooperative manner. Nevertheless, there is general agreement that the number of Mn is between two and four.

The nature of the involvement of Mn has also been the subject of very close scrutiny. The approaches have included: NMR studies of the effect on the relaxation of water protons of the oxidation state of Mn in the different S-states [27]; EPR quantitation of Mn^{2+}, released as $Mn(H_2O)_6{}^{2+}$ by heat treatment, after a given number of flashes [28]; determination of the effects of the Mn which remains after different Mn-release treatments on the electron spin relaxation properties of electron transport components near the water-splitting site [29]; direct observation of the functional Mn in the S_2 state by EPR [30]; and observation of the X-ray absorption edge of Mn [31-33]. The EPR [30] and x-ray absorption edge data [31] support the suggestion [34], based on chemical and energetic arguments, that the Mn of the oxygen-evolving site are clustered in a complex which binds the oxidized intermediates of water. An attractive model for the system is a μ-oxo bridged complex, which can yield an EPR spectrum similar to the S_2 state [30] and exhibit extended x-ray absorption edge fine structure (EXAFS) [31] similar to dark-adapted choroplasts and photosystem 2 preparations.

The oxidation state of Mn in the different S-states is not yet completely determined. The X-ray absorption edge of Mn in dark-adapted samples falls between that of Mn(II) and Mn(III) in a variety of model compounds [32], leading to the suggestion that the Mn exists as a mixture of Mn(II) and Mn(III) in the S_1 state; this

assignment is in agreement with the proposal based on the results of quantitation of Mn after release by heat treatment [28]. Similations of the EPR spectrum of the S_2 state have led to the proposal that S_2 consists of a Mn(III)Mn(IV) dimer or a 3Mn(III)Mn(IV) tetramer [30,35]. The x-ray absorption edge data support this assignment, as the advancement of S_1 to S_2 is accompanied by a shift in the edge position which may be too large to be accounted for by a single electron transfer [33]. Analysis of the other S-states is incomplete, but there is evidence that the oxidation state of Mn in the S_3 state is the same as or lower than that in the S_2 state [28,33].

Many attempts have been made to isolate a protein containing Mn from oxygen-evolving systems. The report of a 65 kDa Mn-containing protein which could reconstitute oxygen evolution appeared to be very promising [36]; yet optimism turned to skepticism as attempts to duplicate the result failed. Further doubt was cast on that assignment by the preparation of oxygen-evolving PS 2 preparations which contained no peptides with such a high apparent molecular weight [37]. More recent investigations have shown that Tris, high salt concentrations, and high pH induce the release of peptides of about 18, 23, and 33 kDa with concomitant loss of oxygen evolution [38]; however, no Mn appears to be associated with any of these peptides [39]. Efforts have continued toward isolating a Mn-containing protein from oxygen-evolving systems, but as yet, these have borne no fruit.

While it is known that manganese has a functional role in photosynthetic oxygen evolution, no clear functional role for iron in the oxidizing photochemistry of photosystem 2 has been conclusively

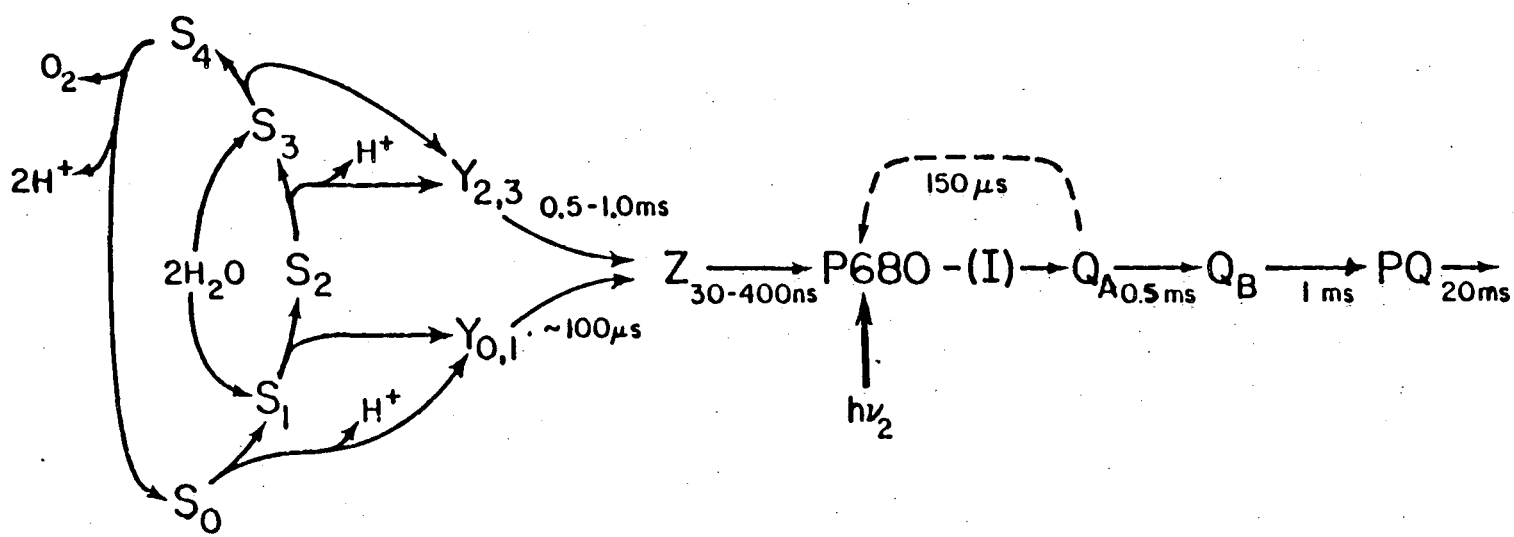
demonstrated, even though as many as 10 - 12 Fe are associated with each photosystem 2 unit in oxygen-evolving photosystem 2 preparations. Cytochrome b_{559}, which is a 10 kDa heme protein present in a 2:1 ratio to the reaction center, accounts for two of these iron atoms. However, the involvement of the cytochrome in oxygen evolution is uncertain because of the absence of evidence of direct involvement and because the reduction potential of the cytochrome, which is +350 mV, is too low to be involved in water oxidation, which requires potentials of at least +800 mV. Nevertheless, there is evidence that cyt b_{559} is closely associated with the oxidizing side of PS 2; at low temperature the cytochrome competes with the oxygen-evolving complex as a donor to the photosystem 2 reaction center [41], and a number of treatments which inhibit oxygen evolution cause the conversion of the cytochrome to a form with a lower reduction midpoint potential [42]. Aside from cytochrome b_{559}, the isolation of a different heme-containing catalase-type protein of high molecular weight which enhances oxygen evolution upon reconstitution has been reported [43], but the role, if any, of this protein in physiological oxygen evolution is undetermined.

1.5.c Species in the Path to Water Oxidation

A model for the photosystem 2 photochemistry is presented in Figure 1-3. Most of the experimental observations of photosystem 2 photochemistry can be incorporated into this model, but other models have been proposed, (see Ref. 44 for a summary).

Figure 1-3

Schematic diagram of the photosystem 2 photochemistry. PQ, plastoquinone pool; Q_B, secondary quinone acceptor; Q_A, primary quinone acceptor; I, intermediate acceptor (pheophytin); P680, primary chlorophyll a donor; Z primary donor to P680; $Y_{0,1}$, hypothetical donor to Z from the S_0 and S_1 states of the oxygen evolving system; $Y_{2,3}$, hypothetical donor to Z from the S_2 and S_3 states; S_i, states of the oxygen evolving complex which have accumulated i oxidizing equivalents.



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Upon excitation with blue or red light, the photosystem 2 primary donor, which is chlorophyll a, rapidly transfers an electron to a neighboring pheophytin, designated I for intermediate acceptor, in the reaction center. The primary donor is designated P_{680} because of the optical bleaching which is observed at 680 nm upon photooxidation [45]. The acceptor I [46,47], which can be observed under reducing conditions either optically or by EPR, is oxidized in a few ps by the first stable acceptor, the quinone Q_A. The quinone reduction is directly observable either optically at 320 nm [48], or by EPR [49] at liquid helium temperatures. The unusual EPR spectrum of Q_A^-, which has a g-value of approximately 1.82, as opposed to 2.00 for most organic radicals, is due to an interaction with a neighboring iron atom. In about 0.5 ms [50], Q_A^- transfers the electron to a second quinone acceptor, Q_B, which acts as a two-electron gate for the transfer of electrons to a pool of plastoquinone [51].

On the donor side the events are much less clear, but progress is being made. The oxidized primary donor, P^+_{680}, is reduced in times ranging from 30 to 400 ns [52,53], depending on the number of flashes given. Although an EPR signal at g 2 due to oxidized P_{680} is expected, the decay is much too rapid for observation with conventional EPR instruments. The rapid decay of the optical transient due to P^+_{680} was once thought to be too rapid for the assignment of an EPR observable species, designated Signal II_{vf} [54], as the direct donor to P^+_{680}; however, revised measurements of the kinetics of the formation of Signal II_{vf} have shown that it is formed in less than 2 μs [55], rather than the previously determined value of

approximately 20 μs [56]. Thus, Signal II_{vf} may represent the direct donor to P^{+}_{680}. The species which gives rise to this signal is designated $Z^{\cdot +}$. Although most investigations of Z have relied on EPR measurements, optical absorbance changes in the near UV have been assigned to the difference between Z and $Z^{\cdot +}$ [57].

Signal II_{vf} is similar in g-value and lineshape to a signal, denoted Signal II [58], which is associated with photosystem 2, but is of unknown function. Signal II exhibits much slower kinetics than Signal II_{vf}; it is formed in the light in a few seconds, and decays in the dark in hours. Because of its relative stability, the lineshape of Signal II has afforded careful investigation. The signal has a g-value of 2.0048, is 16 G wide, and has an anisotropic lineshape, which is orientation dependent in aligned samples. Signal II and Signal II_{vf} have been suggested to arise from a positively charged plastosemiquinone species, either as a protonated semiquinone [59], or as a semiquinone associated with a divalent metal cation [60]. One of the most unusual features of Signal II_{vf} is its rapid spin relaxation [61]; it has been suggested that this property arises from the interaction of $Z^{\cdot +}$ with neighboring paramagnetic Mn ions in the water splitting complex.

The kinetics of the reduction of $Z^{\cdot +}$ were determined Babcock et.al. [62]. The rate of decay of Signal II_{vf} following a flash was observed to depend on the number of flashes received; the decay is approximately 100 μs or faster after the first and fourth flashes, 500 μs after the second, and 1 ms after the third. The state of the oxygen-evolving complex thus determines the rate of reduction of $Z^{\cdot +}$.

The states S_0 and S_1 donate rapidly to $Z^{\bullet+}$, while the more highly oxidized states S_2 and S_3 donate more slowly. The rate of reduction of $Z^{\bullet+}$ by the oxygen-evolving system in the S_3 state matches that for the rate-limiting step in oxygen evolution determined by measurements of oxygen yields in a series of closely spaced flashes [63]. The identity, or identities, of the species which directly reduce $Z^{\bullet+}$ have not yet been determined; the model in Fig. 1-3 includes the possibility of unspecified intermediate electron carriers between the oxygen-evolving complex and Z, but it may be that the water-splitting complex is oxidized by $Z^{\bullet+}$ directly.

The S-states of the oxygen-evolving site eluded direct spectroscopic detection for ten years after the Kok scheme was first proposed; however, EPR and optical signals have recently been observed and assigned to species in the water-splitting complex. The most significant report was the observation at 10 K of a multiline EPR signal in chloroplasts which had been given one flash before being rapidly frozen [30]. The signal intensity was observed to decrease, relative to that observed after a single flash, when the sample received two, three, or four flashes, but appeared to increase when a fifth flash was given. The observed periodicity of four in the dependence of the signal intensity on flash number, and the observed maxima on the first and fifth flashes, led to the suggestion that the signal arises from the S_2 state of the Kok scheme [20] for oxygen evolution. The complex multiline structure of the signal is evidence that the species which gives rise to the signal contains Mn, which, because of its 5/2 nuclear spin, often gives spectra containing many

lines. Simulations of the spectrum have led to suggestions that the species is an antiferromagnetically coupled Mn(III)Mn(IV) dimer or 3Mn(III)Mn(IV) tetramer [30,35]. With the exception of an isolated report of an EPR signal due to the oxygen-evolving complex in the S_1 state [64], the S-states other than S_2 have not been detected by EPR.

Attempts to measure optical changes associated with the oxygen-evolving system have been complicated by absorption changes due to the acceptor reactions; however, subtraction methods and treatments which prevent acceptor reactions have been used in reports of absorbance changes in the ultraviolet which are assigned to the oxidizing photochemistry of photosystem 2 [65-67]. One of these absorption changes, which occurs on the first flash of a dark-adapted sample, and does not reverse until a third flash is given, is assigned to the oxidation of a species M. It has been suggested that the oxidized state M+ is present in both the S_2 and S_3 states, and becomes reduced as the system releases oxygen in advancing from S_3 to S_0. The proposed behavior of M is at variance with the multline signal behavior, which suggests that the species which stabilizes the oxidizing equivalents in the S_2 state is changed upon advancement to the S_3 state. Perhaps the optical difference is not directly related to the S-state advancements, but reflects the fact that the donor $Y_{0,1}$ is somehow changed upon the advancement of the system from S_1 to S_2.

1.5.d Photosystem 2 Photochemistry at Low Temperatures

While the initial charge separation occurs even near liquid helium temperatures, the secondary photochemistry on both the donor and acceptor sides is affected by decreased temperature (reviewed in

Refs. 68 and 69). A number of investigations have attempted to characterize the oxygen-evolving system by monitoring the photochemistry in photosystem 2 at low temperature; these have included measurements of variable fluorescence [70-72], absorbance changes associated with cyt b_{559} [71-74], EPR Signal II [75], and thermoluminescence [76,77]. The acceptor Q_A can be photoreduced at very low temperatures, but the reoxidaion of Q_A^- by Q_B appears to become retarded at 238 K, and is practically prevented at 213 K [78]. If the oxidized primary donor is not rapidly reduced, a charge recombination between P^+_{680} and Q_A^- will occur with a half time of about 4 ms [79].

A decrease in the rates of the oxidizing reactions of photosystem 2 at low temperature is evident from the decreased rate of the rise of photosystem 2 variable fluorescence, which, at low temperature, is a measure of the rate of the reduction of the oxidized primary donor. Parallelling the slower fluorescence rise is the photooxidation of cyt b_{559} at low temperature [71,72]. As the cytochrome is not observed to undergo any photooxidation under physiological conditions, it is believed that the photooxidation of the cytochrome is a competitive reaction which becomes favored at low temperatures due to the decreased rate of donation from the water-splitting complex. Interestingly, the threshold temperature for the onset of the observed effects of low temperature is dependent on the number of preilluminating flashes given before cooling [70,73,74]. The threshold is about 170 K for PS 2 centers in the S_0 and S_1 states, and, although no specific threshold is reported for the S_2 and S_3

states, it must be at least as high as 220 K.

Measurements of the ability to charge thermoluminescence bands at low temperature have provided another probe of the low temperature photochemistry of the oxygen-evolving system [76,77]. The thermoluminescence band associated with the S_2 state can be charged with continuous light at temperatures as low as 210 K [76]; unfortunately, however, no low temperature threshold is reported for the charging of this band. There is an illumination temperature threshold at 170 K for an effect on the thermoluminescence behavior [77], but the origin of the effect and its relation to the S-states is uncertain. With regard to the higher S-states, it has been shown that the charging of the thermoluminescence band associated with S_3 by two flashes has a temperature threshold of about 240 K [76]. These threshold temperatures are consistent with those reported for the effects of temperature on the fluorescence rise kinetics and on cytochrome b_{559} photooxidation.

None of the methods discussed above directly monitors the S-states. The observed effects of temperature on the fluorescence rise kinetics and on cytochrome photooxidation most likely reflect the decreased rate of reduction of the oxidized primary donor, P^+_{680}, at low temperature. The charging of thermoluminescence bands, which result from the temperature-induced charge recombination through P^+_{680} and Q_A^-, is only sensitive to the stabilization of the light-generated donor-acceptor pair; it is thus not possible to distinguish between the complete advancement of S_1 to S_2 and the advancement of S_1 to an intermediate state S_1*. Therefore, although the state of the

oxygen-evolving system determines the threshold for the effects of low temperature, the effect of temperature on the reactions at the water-splitting site itself is unknown.

1.6 Focus of This Work

The investigations reported in this work examine the oxidizing reactions of photosystem 2 by the use of EPR. Initial studies confirm the association of the multiline EPR signal with the S_2 state of the oxygen-evolving complex and investigate directly the effects of low temperature on the reactions of the oxygen-evolving system. By the use of low temperature illumination, it is demonstrated that an intermediate state S_1*, between the S_1 and S_2 states, can be trapped, and an EPR signal which appears at g 4.1 is assigned to the intermediate. The characteristics of the signal lead to the suggestion that non-heme ferric iron is involved in the oxidizing reactions of photosystem 2. The intermediate state is further characterized by investigations of its behavior at low temperature, after preillumination, and under several conditions for which oxygen evolution is inhibited.

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CHAPTER 2

CORRELATIONS BETWEEN THE MULTILINE EPR SIGNAL AND THE S_2 STATE

2.1 Introduction

Investigations into the mechanisms of the photosynthetic oxidation of water have been hampered by the lack of spectroscopic probes of the S-states. Yet without such probes it is impossible to identify the chemical species involved and to fully investigate the reactions of the water-splitting complex. A significant advance was the observation of the multiline EPR signal [1-3]. While analysis of the lineshape of the signal does not allow a conclusive determination of the molecular species, it is evident that a manganese complex is the source of the signal, and that between two and four manganese are associated in this complex, in such a way that they are magnetically coupled [2,4].

The observed dependence of the amplitude of the multiline signal on the number of flashes given the sample [1,2], and the ability to generate the signal in the presence of DCMU [1-3], which prevents the transfer of more than one electron to the acceptor [5], led to suggestions that the signal arises from the S_2 state of the oxygen-evolving complex. However, in previous reports, sample preparations involved rapid freezing of samples during or immediately following illumination. Thus, it is not possible to rule out the

assignment of the multiline signal to an intermediate state S_1*, which describes the system in an undefined early stage of the photochemistry following excitation, prior to the advancement of the water-splitting site to the state S_2.

A more specific correlation between the multiline signal and the oxygen-evolving system might be obtained by a comparison of the properties of the S_2 state of the oxygen-evolving system with the conditions required for generating the signal. In particular, an investigation of the kinetics of the decay of the species which gives the multiline signal will allow a determination of whether the signal is due to an intermediate S_1*, or to the S_2 state. The lifetime of the S_2 state is between 40 and 90 s at room temperature [6-8]; do the kinetics for the decay of the multiline signal match those for the S_2 state?

Additional evidence regarding the assignment of the multiline signal to the S_2 state might be obtained by an investigation of the temperature dependence of the light-induced formation of the signal. The donor reactions of photosystem 2 at low temperature are not completely determined, but the existing data place limits on the low temperature behavior of species associated with the oxygen-evolving system (see Chapter 1). A determination of the low temperature behavior of the multiline signal species could lead to a more complete understanding of the low temperature reactions of the photosystem 2 donors.

The experiments discussed in this chapter confirm the assignment of the multiline signal to S_2, and expand our understanding of the decay kinetics of the S_2 state and of the photosystem 2 donor reactions at low temperature.

2.2 Materials and Methods

2.2.a Chloroplast Preparation

Broken chloroplasts were prepared from market spinach. Destemmed leaves were ground at 4°C in a Waring blendor for 10 s in a medium containing 0.15 M NaCl, 4 mM $MgCl_2$, 5 mM EDTA, and 0.05 M HEPES at pH 7.5. The homogenate was strained through three layers of cheesecloth and centrifuged at 5000×g for 8 min. The pellet was resuspended in the same buffer and spun for 5 s at 1000×g, and the supernatant from this low-speed spin was centrifuged at 5000×g for 8 min. The final pellet was resuspended in buffer containing no EDTA and centrifuged again at 5000×g for 8 min. Samples were kept on ice in the dark for 15 - 75 min before use.

2.2.b Preparation of Oxygen-evolving Photosystem 2 Samples

Photosystem 2 preparations devoid of PS 1 and cyt b_6/cyt f complex components [9] were obtained by modifications of two Triton X-100 fractionation procedures [10,11]. Broken chloroplasts were prepared from market spinach in a manner similar to that described above, with the exception that the buffer for grinding the spinach contained 0.2 M sucrose, 5 mM $MgCl_2$, 15 mM NaCl, and 0.5 M HEPES at pH 7.5. The chloroplast preparation was suspended in buffer containing 15 mM NaCl, 5 mM $MgCl_2$, and 0.5 M MES at pH 6.0 and spun at 5000×g for 8 min. The pellet was suspended in the same buffer, with

5 mM sodium ascorbate and 25:1 (w:w) Triton X-100 per chlorophyll added. The pellet from a 2 min spin at 1000×g was discarded, and the supernatant was spun at 35000×g for 10 min. The pellet was taken up in the MES suspension medium and spun again at 35000×g for 10 min after the pellet from a 1000×g spin was discarded. Samples were either kept on ice in the dark and used within 90 min or were stored in buffer containing 50% glycerol in liquid nitrogen for no more than two days before use. Storage in liquid nitrogen was found to have no effect on rates of oxygen evolution, which were typically between 300 and 500 μmol O_2 (mg chl)$^{-1}$h^{-1} when measured in the presence of suspension buffer containing 2,5-dichloro-p-benzoquinone.

2.2.c Electron Paramagnetic Resonance Spectra

For EPR measurements, samples were suspended in the final wash buffer and placed in Suprasil quartz tubes (3 mm inner diameter). Except where indicated, samples contained 50% glycerol and had chlorophyll concentrations of between 3 and 5 mg/ml as determined by the method of Arnon [12]. EPR spectra were recorded at approximately 9.2 GHz with a Varian E 109 spectrometer equipped with a Liquid Transfer Heli-Tran Refrigerator (Air Products, Model LTR). Spectra were recorded either directly on the spectrometer chart recorder or on a digital oscilloscope (Nicolet Explorer III) and stored in a signal averager. Signal averaged spectra were transferred to a VAX 11/780 computer for manipulation and display. Spectra were typically obtained by averaging four or more sweeps of the magnetic field.

2.2.d Illumination of Samples.

All samples were illuminated in 3 mm inner diameter quartz

tubes. Flash illumination was performed with short (10 μs), intense (14 J/flash) flashes from a xenon flash lamp; the oblong image of the lamp was tightly focused on the sample. Continuous illumination was for 30 s with a 300 W tungsten source; to diminish heating effects, 10 cm of water were placed between the source and the sample. Sample temperature was maintained with a Varian V 6040 NMR Variable Temperature Controller or with a dry ice/methanol bath; 3 min equilibration was allowed before illumination. Except where noted, samples were dark-adapted on ice for at least 15 min before illumination. Sometimes, in order to prepare the system completely in the S_1 state, samples were dark adapted, given two flashes, and dark adapted again. Except where indicated, samples were placed in liquid nitrogen immediately after illumination.

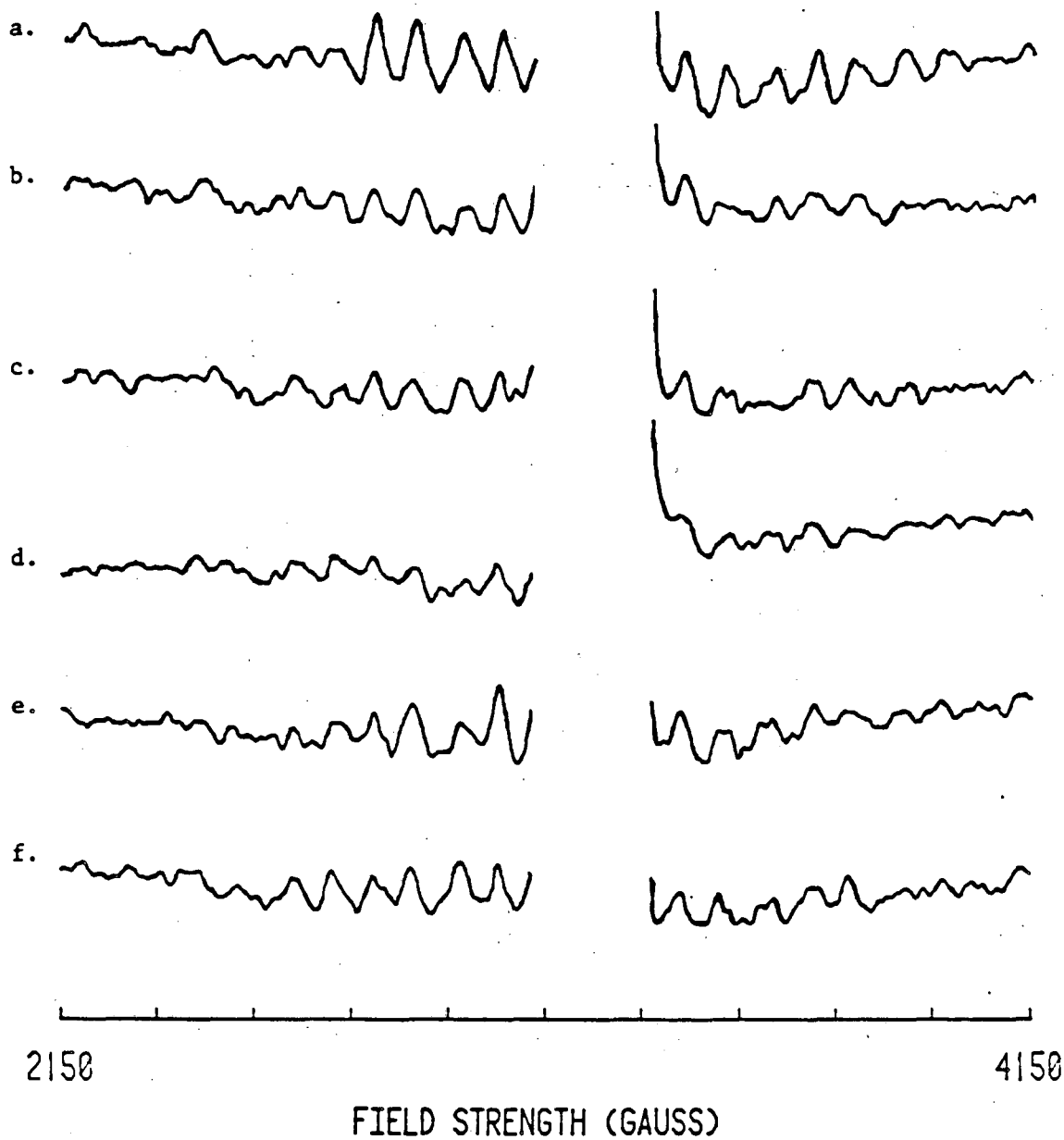
2.3 Results

2.3.a Flash Number Dependence of the Multiline Signal Amplitude at 273 K

A single intense flash of light at 273 K followed by freezing in liquid nitrogen generates and traps a species which gives a multiline EPR signal when observed at 10 K (Fig. 2-1a); the observed signal, which contains over 16 lines spaced nearly evenly across 1500 G, is identical to that reported and assigned by other workers to the S_2 state [1-3]. It was found that, for flash illumination at or above 273 K and for continuous illumination at lower temperatures, the presence of 50% glycerol greatly enhances the observed signal intensity.

Figure 2-1

Dependence of the multiline signal amplitude on the number of illuminating flashes. Photosystem 2 preparations suspended at 0.75 mg(Chl)/ml were given a flash at 0°C, equilibrated in the dark for 15 min, then given a variable number of flashes at 0°C followed by rapid freezing in liquid nitrogen. Number of flashes: a) 1, b) 2, c) 3, d) 4, e) 5, f) 6. Spectrometer conditions: microwave power, 50mW; microwave frequency, 9.2 GHz; modulation amplitude, 32 G; modulation frequency, 100 kHz; temperature, 10 K; scan rate, 1000 G/min (10 scans); time constant, 0.128 s. The same dark-adapted spectrum was subtracted from all samples.


a.
b.
c.
d.
e.
f.
2150
4150
FIELD STRENGTH (GAUSS)

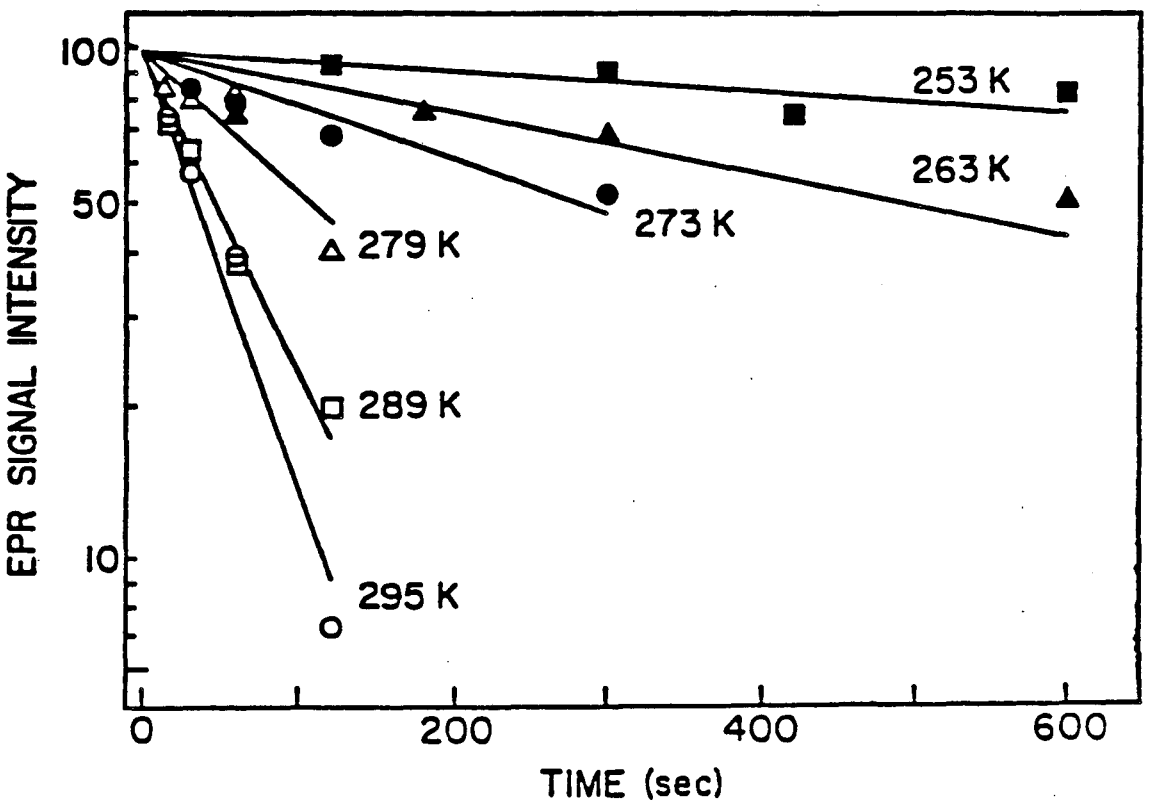
The dependence of the multiline signal amplitude on flash number is shown in Fig. 2-1 for an oxygen-evolving photosystem 2 preparation. The results in this figure are in qualitative agreement with those reported for chloroplasts [1,2]: the signal intensity is maximal after one flash, decreases progressively on the second, third and fourth flashes, and appears to increase on the fifth flash. Whereas it is reported [1,2] that the intensity decreases slightly on the sixth flash, the data in Fig. 2-1 indicate that the intensity after the sixth flash is equal to or greater than that after the fifth flash. In order to insure complete illumination of these optically dense samples, the chlorophyll concentration for the samples in this experiment was decreased to approximately 0.8 mg/ml; nevertheless, it is still possible that each flash did not illuminate the sample completely.

2.3.b Decay of the Multiline Signal between 250 and 295 K

To obtain the spectra shown in Fig. 2-1, samples were rapidly frozen in liquid nitrogen immediately following illumination with flashes at 273 K. If the length of time between illumination and freezing is increased, the observed signal amplitude decreases. The decay of the species which gives rise to the multiline signal was monitored by varying the length of time between illumination and freezing; the multiline signal observed at 10 K was quantitated by summing the intensities of the 3 largest peaks downfield from g 2 (Fig. 2-2). At 295 K the decay has a half-time of approximately 40 s. The temperature dependence of this decay was determined by performing the illumination and incubation at temperatures between 253 and 295 K.

Figure 2-2

Decay of the multiline signal amplitude at different temperatures. Broken chloroplasts suspended at 5 mg(Chl)/ml were given one flash at the specified temperature, then placed in liquid nitrogen after a variable delay. The signal amplitudes were taken as the peak-to-peak height of four lines downfield from g=2. The amplitudes at each temperature are normalized to the maximum value. Spectrometer conditions were as in Fig. 2-1, except that no signal averaging was done.


100
50
10
EPR SIGNAL INTENSITY
0
200
400
600
TIME (sec)
253 K
263 K
273 K
279 K
289 K
295 K

As the temperature is decreased in this region, the rate of deactivation becomes dramatically slower. At 253 K the half-time for the decay is about 40 min, and virtually no decay in signal amplitude is observed during 1 h at 243 K.

2.3.c Dependence of the Multiline Signal Amplitude on the Illumination Temperature

2.3.c.i Dark-adapted Preparations (S_1)

While the multiline signal amplitude generated by a single flash given at temperatures between 250 K and room temperature is relatively constant, the signal intensity becomes progressively lower as the illumination temperature is decreased below 250 K (Fig. 2-3). The midpoint of this effect is at about 225 K; virtually no multiline signal is produced by a flash given at or below 190 K.

To determine whether the decline in signal amplitude at lower temperatures of illumination is due to competition with a charge recombination, the temperature dependence of the photogeneration of the multiline signal was examined for multiple flash and continuous illumination. It is observed that the temperature range for which the signal amplitude decreases is lower for more extensively illuminated samples (Fig. 2-4). Illumination with 20 flashes produces maximal signal intensity at temperatures as low as 220 K, and at 190 K, where a single flash produces no multiline signal, approximately half the maximal amount of signal is generated by 20 flashes. Illumination with continuous light for 30 s produces the multiline signal at even lower temperatures; the maximal and half-maximal signal amplitudes are generated by illumination at about 190 K, and 175 K, respectively.

Figure 2-3

Production of the multiline signal in broken chloroplasts, suspended at 4 mg(Chl)/ml, by a single flash at different temperatures. Spectrometer conditions as in Figure 2-1 except that the microwave power was 150 mW and no signal averaging was done.

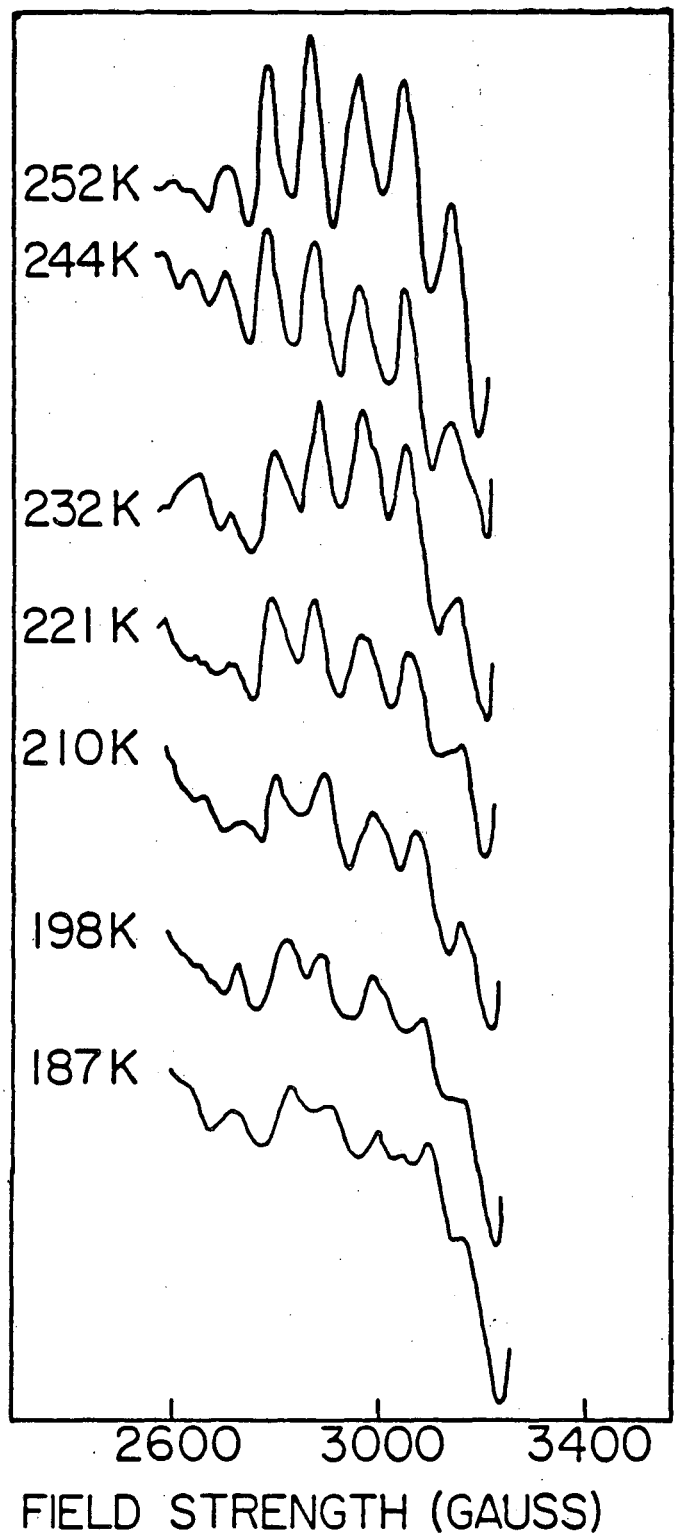
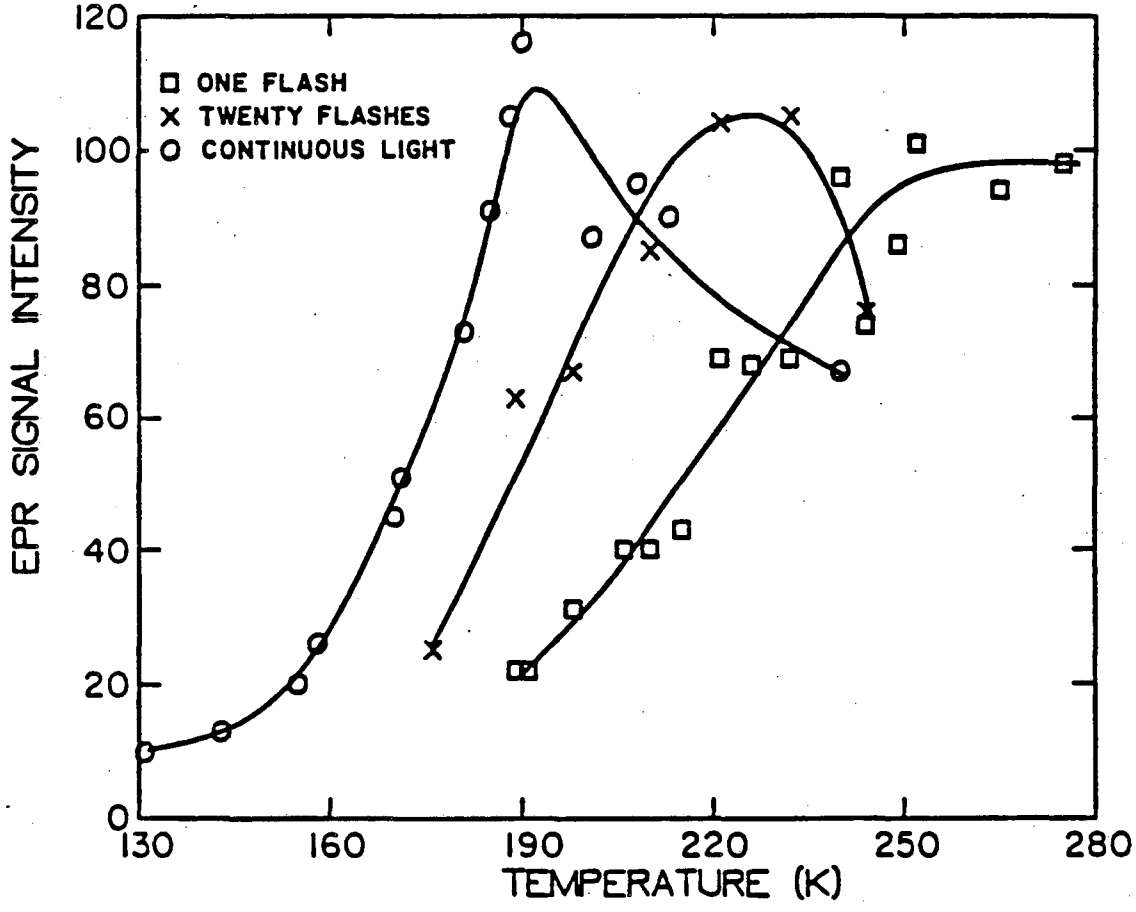

252K
244K
232K
221K
210K
198K
187K
2600
3000
3400
FIELD STRENGTH (GAUSS)

Figure 2-4

Amplitude of the multiline signal produced by a single flash (□), 20 flashes (X), and 30 s continuous light (O) at different temperatures. The signal amplitudes for different illumination schemes have been separately normalized. Spectrometer conditions as in Fig. 2-3, signal amplitude determinations as in Fig. 2-2.


ONE FLASH
TWENTY FLASHES
CONTINUOUS LIGHT
EPR SIGNAL INTENSITY
TEMPERATURE (K)
0
20
40
60
80
100
120
130
160
190
220
250
280

The lower limit for generating the multiline signal with continuous light is approximately 160 K; at 150 K and below no multiline signal is produced. The temperature threshold for generating the multiline signal with continuous illumination is not significantly altered by increasing the illumination time to 2 min. The signal intensities for the three data sets shown in Fig. 2-4 have been scaled to the same maximum value; for concentrations as high as those used for these experiments (between 3 and 5 mg chl/ml), a single flash at 273 K produces about half the signal that is generated by continuous light at 190 K.

2.3.c.ii Preilluminated Preparations (S_2)

The data in Fig. 2-4 show that the signal intensities produced by multiple flashes and by continuous illumination decrease above a certain temperature. The temperature at which this decrease occurs is about 230 K for illumination with 20 flashes, and 210 K for continuous illumination. The lower signal intensity may be the result of further light-induced reactions of the oxygen-evolving system which can occur at the higher temperatures and which deplete the multiline signal species. The reactions on the acceptor side are known to be affected in this temperature region [13]; the primary reduced stable acceptor, Q_A^-, is oxidized only slowly below 238 K, thus preventing the stabilization of more than one oxidizing equivalent by the donor reactions of PS 2 upon illumination below this temperature.

To limit the observed temperature effect to donor reactions, samples were given a preilluminating flash at 273 K, then frozen in liquid nitrogen. This process generates approximately one-half the

maximal amount of the multiline signal species and allows for the reoxidation of the primary acceptor, which occurs in less than 1 ms. Samples were then equilibrated at a chosen temperature between 200 and 250 K for continuous illumination. For samples prepared in this manner the amplitude of the multiline signal remaining after illumination is low for illumination temperatures above 220 K, but increases sharply in the temperature range from 220 to 200 K (Fig. 2-5). Samples illuminated below 210 K exhibit a peak at g=3.1 in the EPR spectrum (not shown), an indication of cytochrome b_{559} photooxidation that is not seen at higher temperatures.

2.4 Discussion

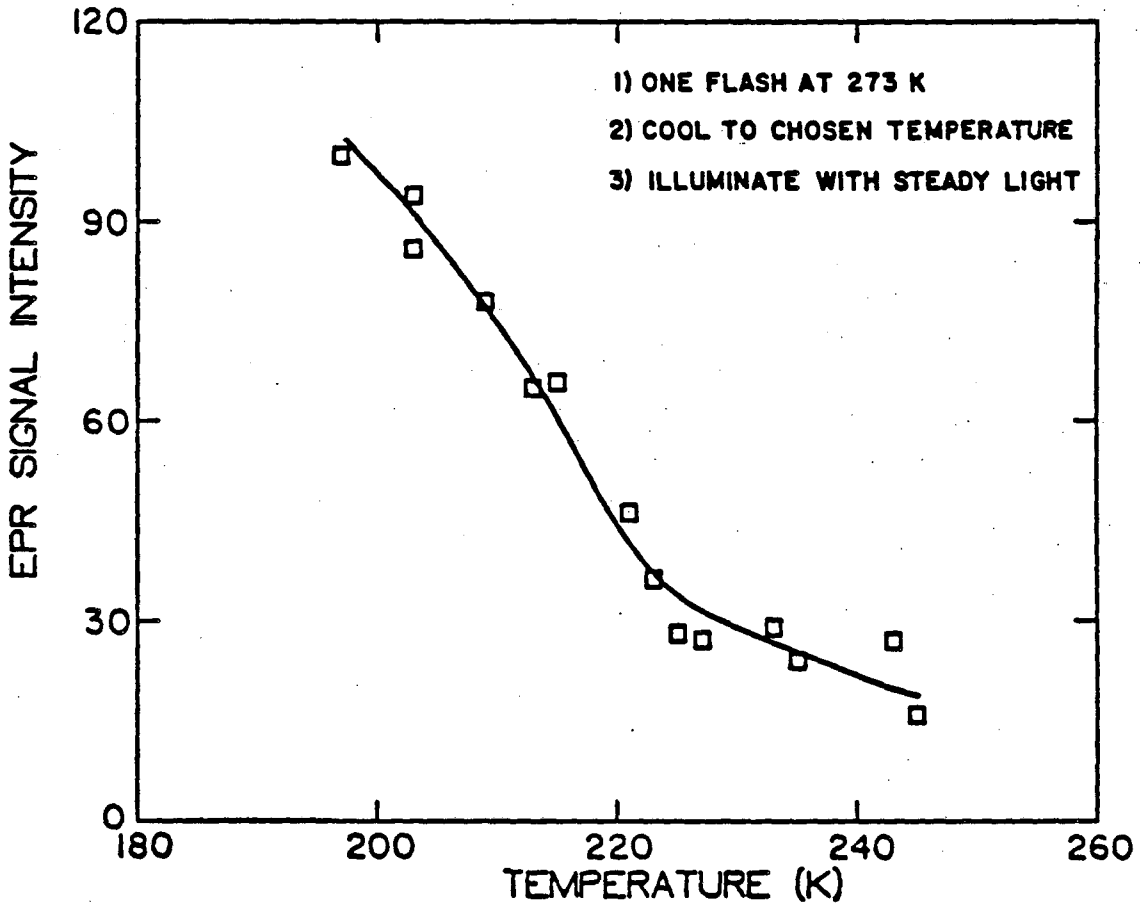
2.4.a Correlations between the Multiline Signal and the S_2 State

The above results demonstrate the ability to reproduce the results of Dismukes et.al. [1,2] in producing the multiline EPR signal, which those authors and others [3] have suggested arises from the S_2 state of the oxygen-evolving system. The signal can be produced by a single flash given near 273 K followed by rapid freezing in liquid nitrogen, or by 30 s continuous illumination at 190 K. Illumination at 190 K gives the largest signal amplitude.

The presence of glycerol in the sample was found to be necessary for observing the signal when samples were illuminated at 273 K and above. If the sample is frozen to 190 K before receiving continuous illumination, the signal can be observed in the absence of glycerol; nevertheless, samples containing glycerol exhibited larger signals. Hansson and Andréasson reported [3] observation of the multiline signal by freezing samples containing no glycerol during

Figure 2-5

Amplitude of the multiline signal produced in broken chloroplasts by a single flash at 273 K followed by continuous illumination at different temperatures. Samples were given an intense flash at 273 K, frozen in liquid nitrogen, then warmed to a specified temperature, at which they received 30 s of continuous illumination. Spectrometer conditions as in Fig. 2-3, signal amplitude determinations as in Fig. 2-2.


1) ONE FLASH AT 273 K
2) COOL TO CHOSEN TEMPERATURE
3) ILLUMINATE WITH STEADY LIGHT
EPR SIGNAL INTENSITY
120
90
60
30
0
180
200
220
240
260
TEMPERATURE (K)

illumination. Thus, although the presence of glycerol is important for observing the signal under some conditions, the ability to observe the signal in the absence of glycerol rules out a direct involvement of glycerol in the multiline signal species. Perhaps the effect of glycerol is to prevent damage due to the formation of ice crystals during freezing, but the exact nature of such damage and the related effects on the photoproduced oxidizing and reducing equivalents are unknown.

The results (Fig. 2-1) for the flash number dependence of the multiline signal in the Triton X-100 photosystem 2 preparation are in qualitative agreement with those reported by Dismukes et.al. [1,2] for chloroplasts. The signal amplitude is maximal on the first flash, and decreases on subsequent flashes, as one expects for the S_2 state. The signal amplitude appears to increase after a fifth flash, as one also expects for the S_2 state, but increases slightly after a sixth flash, contrary to the behavior of S_2. It has been reported [1,2] that the signal decreases again on the sixth flash, although the margin of error in those results is high.

The data for the higher flash numbers are difficult to interpret because it is likely that, due to high optical densities, the flashes do not illuminate the entire sample, effectively leading to a higher miss parameter and rapid dephasing of the separate oxygen-evolving systems in the sample. Thus, although the results in Fig. 2-1 do not clearly demonstrate the expected oscillations in the multiline signal, it is evident that the signal decreases on the second, third and fourth flashes, and increases on flashes at least

three subsequent to the first. This behavior is consistent with that expected for the S_2 state, given the difficulty of achieving complete illumination of the sample with flashes, and rules out the possibility that the signal results from an effect which is specific to the first excitation of a photosystem 2 center and which might not be directly involved in oxygen evolution.

The 40 s half-time of the multiline signal species at 295 K (Fig. 2-2) is evidence that the signal arises from a relatively stable species, rather than from a rapidly transient intermediate. Previous reports of observations of the multiline signal could not rule out the latter possibility, as they involved rapid freezing following flash illumination [1,2] or illumination with continuous light during freezing [3]. The observed 40 s decay time at 295 K is in good agreement with that reported for the S_2 state of the PS 2 oxygen-evolving system in spinach chloroplasts [6-8]; furthermore, the temperature dependence of the decay is similar to that reported for a thermoluminescent species assigned to the S_2 state [8]. The observed behavior of the decay of the multiline signal is thus supportive of the assignment of the signal to the S_2 state of the oxygen-evolving complex.

2.4.b The Effect of Low Temperature on the Donor Reactions of PS 2

The observed difference between the low temperature thresholds for generating the multiline signal by continuous illumination of dark-adapted chloroplasts (ca. 165 K, Fig. 2-4) and for decreasing the multiline signal amplitude by continuous illumination of chloroplasts which had been preilluminated with a single flash (ca.

210 K, Figs. 2-4, 2-5) correlates well with the reported S-state dependence of the threshold temperatures for the decreased kinetics of the fluorescence rise [14,15], cytochrome b_{559} photooxidation [15-18], and the ability to charge thermoluminescence bands associated with the S_2 and S_3 states [8,19]. Thus, the results of the studies on the amplitude of the multiline signal produced by illumination at low temperature are consistent with the proposed involvement of the multiline signal species in oxygen evolution.

Previous investigations of the low temperature photochemistry of PS 2 were based on observations of species or events which are not directly involved in the water-splitting complex (14-19, see Chapter 1). The confirmed assignment of the multiline signal to the S_2 state permits the use of the low temperature illumination behavior of this signal as a probe for a more detailed evaluation of the effects of temperature on the oxygen-evolving system of PS 2. The following model can be used to explain the effects of lowering the temperature of illumination on the generation of the multiline EPR signal:

$$S_i\ D\ P\ Q_A \xrightarrow{h\nu} S_i\ P^+\ Q_A^-$$

$$S_i\ D\ P^+\ Q_A^- \underset{k_b}{\overset{k_{i+1}}{\rightleftharpoons}} S_{i+1}\ P\ Q_A^-$$

$$D \xrightarrow{k_D} D^+$$

In this diagram P and Q are the primary donor and acceptor, respectively; S_1 represents a state of the oxygen-evolving complex, and D represents an alternative donor (possibly cytochrome b_{559}) which is not on the electron-transfer path between the water-splitting complex and P. Since it is the S_2 state of the oxygen-evolving complex which gives the multiline signal, any configuration in which this state is present will exhibit the signal.

The results shown in Fig. 2-4 demonstrate that the threshold temperature for generating the multiline signal with continuous illumination is lower than that for single flash generation of the signal. This result provides evidence for competition between formation of the species which gives the multiline signal and a charge recombination in the temperature range 160 to 240 K. At the high end of this temperature range the forward reaction dominates; k_2 is larger than k_b. At the other end of the range however, k_2 is smaller than k_b, and the back reaction is favored.

The threshold temperature for generating the multiline signal in dark-adapted chloroplasts with continuous light is similar to that reported for the decrease in the rate of the fluorescence rise and for the photooxidation of cytochrome b_{559} [14,15,18]. The simplest explanation for the inability of continuous light to generate the multiline signal below 160 K is that a species, D, competes effectively with the oxygen-evolving complex as a donor to P^+_{680}. According to this scheme, k_D would become larger than k_2 as the temperature is decreased below 160 K. However, no direct evidence is provided for such a scheme in the above results. In fact, although

cytochrome b_{559} is believed to replace the physiological donor at low temperatures, no cytochrome photooxidation was observed by EPR when samples were illuminated near the 160 K threshold temperature.

Further photoreactions decrease the intensity of the multiline signal at temperatures above 200 K (Figs. 2-4, 2-5). It has been reported that both the reoxidation of the reduced primary stable acceptor [13], Q_A^-, by the secondary acceptor, Q_B, and the advancement of S_2 to S_3 (see Ref. 20) are strongly affected by temperature between about 210 and 240 K. Both processes affect the signal amplitude generated upon illumination in this temperature range. It was attempted to observe the effects of temperature on just the donor reactions by preilluminating samples with a single flash at 273 K before illuminating with continuous light at reduced temperature (Fig. 2-5). The threshold temperature for the disappearance of the multiline signal in the preilluminated samples is significantly higher than that observed for the formation of the multiline signal by continuous light in dark-adapted samples. According to the above model, the rates determined by k_3 and k_D become competitive at about 220 K. In this case, it appears that competitive donation from the cytochrome could be a factor in determining the threshold temperature, because cytochrome photooxidation is observed at 210 K in samples given a preilluminating flash at 273 K.

2.5 Conclusion

The agreement between the conditions reported for the formation and decay of the S_2 state of the oxygen-evolving complex and those presented above for the formation and decay of the multiline signal

confirm the previous assignments of the multiline signal to the S_2 state. The decay time of the multiline signal is evidence that the signal results from the final oxidation product of the S_1 state, and not from a prior transient species.

Because the multiline signal has been confirmed to arise from the S_2 state, it has been possible to use the signal as a probe of the effects of low temperature on the reactions of the water-splitting complex. Temperature thresholds of approximately 160 K and 210 K are obtained for the advancement of S_1 to S_2, and of S_2 to S_3, respectively. While the threshold for the advancement of S_2 to S_3 may be due to competitive donation from cyt b_{559}, no evidence exists for such a process near the threshold for advancement of S_1 to S_2. The processes which occur near the threshold temperature for the advancement of S_1 to S_2 are examined more closely in the next chapter.

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CHAPTER 3

THE TRAPPING OF AN INTERMEDIATE BETWEEN THE S_1 AND S_2 STATES

3.1 Introduction

Among the electron carriers between P_{680} and the oxygen-evolving complex, a 16 G wide transient EPR signal centered at g 2.0048, denoted Signal II_{vf}, has been identified [1,2]; it is likely that Signal II_{vf} reflects the oxidized direct donor to P^{+}_{680} [3]. The transient Signal II_{vf} is very similar in lineshape to a signal associated with photosystem 2 which exhibits very slow kinetics and is usually present in dark-adapted samples. The slower kinetic species, denoted Signal II [4], has an undetermined function in photosystem 2 photochemistry. The similar lineshapes of the signals is suggestive that the chemical identities of the different signal II species are similar; the more stable species has been attributed to a plastosemiquinone associated with a divalent cation [5] or to a protonated plastosemiquinone radical cation [6].

There is indirect evidence which is suggestive that additional intermediate electron carriers are involved in transporting electrons from the oxygen-evolving complex to P_{680} [see Ref. 7], but the identities of such species are unknown.

The transition metal manganese appears to be integral to the oxygen-evolving complex, with the number of Mn involved perhaps as high as four [8]. Either a 3Mn(III), Mn(IV) tetramer [9] or a Mn(III) Mn(IV) dimer [10] appear to be consistent with the multiline EPR signal associated with the S_2 state of the oxygen-evolving complex [10-12, see Chapter 2]. The extent of involvement of other metal ions in water oxidation is undetermined. A significant amount of iron (approximately 12 per photosynthetic unit) is typically associated with Triton-extracted, oxygen-evolving PS 2 preparations (D.Goodin, N.Blough, personal communication); but, except for the two heme irons present as cytochrome b_{559} and the single iron associated with the acceptor [13], this iron is unaccounted for in terms of identity or function. Cytochrome b_{559} is associated with the oxidizing side of PS 2, as it is capable of donation to the reaction center at low temperatures, but no precise physiological function has been ascribed to it (see Ref. 14). A major argument against the involvement of the cytochrome in the oxidation reactions leading to the splitting of water is that the reduction potential of the cytochrome (ca. 350 mV) is not high enough to indicate a redox species active in water oxidation.

Studies at low temperature have proven useful in examining the mechanisms of PS 2 reactions and the S states (see Chapter 2 and Refs. 15 and 16). It is shown in Chapter 2 that the threshold temperature for the formation of the S_2 state from S_1 is about 170 K. In this chapter the consequences of illumination at temperatures near the threshold temperature are investigated in an attempt to detect the

formation of intermediate species which are kinetically inert at these low temperatures.

3.2 Materials and Methods

3.2.a Oxygen-evolving Photosystem 2 Preparation

Photosystem 2 preparations were obtained by modifications of two Triton X-100 fractionation procedures [17,18]. Broken chloroplasts were prepared from market spinach. Destemmed leaves were ground at 4°C in a Waring blendor for 10 s in a medium containing 0.2 M sucrose, 5 mM $MgCl_2$, 15 mM NaCl, and 0.05 M HEPES at pH 7.5. The homogenate was strained through three layers of cheesecloth and centrifuged at 5000×g for 8 min. The pellet was suspended in buffer containing 0.15 M NaCl, 5 mM $MgCl_2$, 5 mM EDTA, and 0.05 M HEPES at pH 7.5. The loose pellet from a 5 s spin at 1000×g was discarded and the broken chloroplasts were pelleted by a 8 min 5000×g spin. The pellet was washed once with suspension buffer which contained 15 mM NaCl, 5 mM $MgCl_2$ and 50 mM MES at pH 6.0 and spun again at 5000×g for 8 min. The pellet was suspended in the same buffer, with 5 mM sodium ascorbate and 25:1 (w:w) Triton X-100 per chlorophyll added. The pellet from a 2 min spin at 1000×g was discarded before spinning the supernatant for 10 min at 35000×g. The pellet from this spin was taken up in the MES suspension medium and spun again at 35000×g for 10 min after the pellet from a 2000×g spin was discarded. All samples were suspended at a final concentration of 3-5 mg chl/ml and contained approx. 50% glycerol in the final suspension medium. Oxygen evolution rates of 300 to 500 μmol O_2 (mg chl)$^{-1}$ h^{-1} were obtained when control preparations were assayed in suspension buffer containing

2,5-dichloro-p-benzoquinone.

3.2.b Illumination and EPR Spectra

For EPR measurements samples were placed in quartz tubes (3 mm inner diameter) and kept in the dark for 15 min before freezing in liquid nitrogen. Sample handling and measurement were completed within 48 h. Continuous illumination was for 30 s from a 400 W tungsten source. The sample temperature was maintained with a continuous stream of liquid-nitrogen-cooled nitrogen gas or a dry ice/methanol bath.

EPR spectra were recorded with a Varian E 109 spectrometer equipped with a Liquid Transfer Heli-Tran Refrigerator (Air Products, Model LTR). Spectra were recorded on a digital oscilloscope (Nicolet Explorer IIIa) and stored in a signal averager. Signal-averaged spectra were transferred to a VAX 11/780 computer for manipulation and display. Spectra were typically obtained by averaging 4 or more sweeps of the magnetic field.

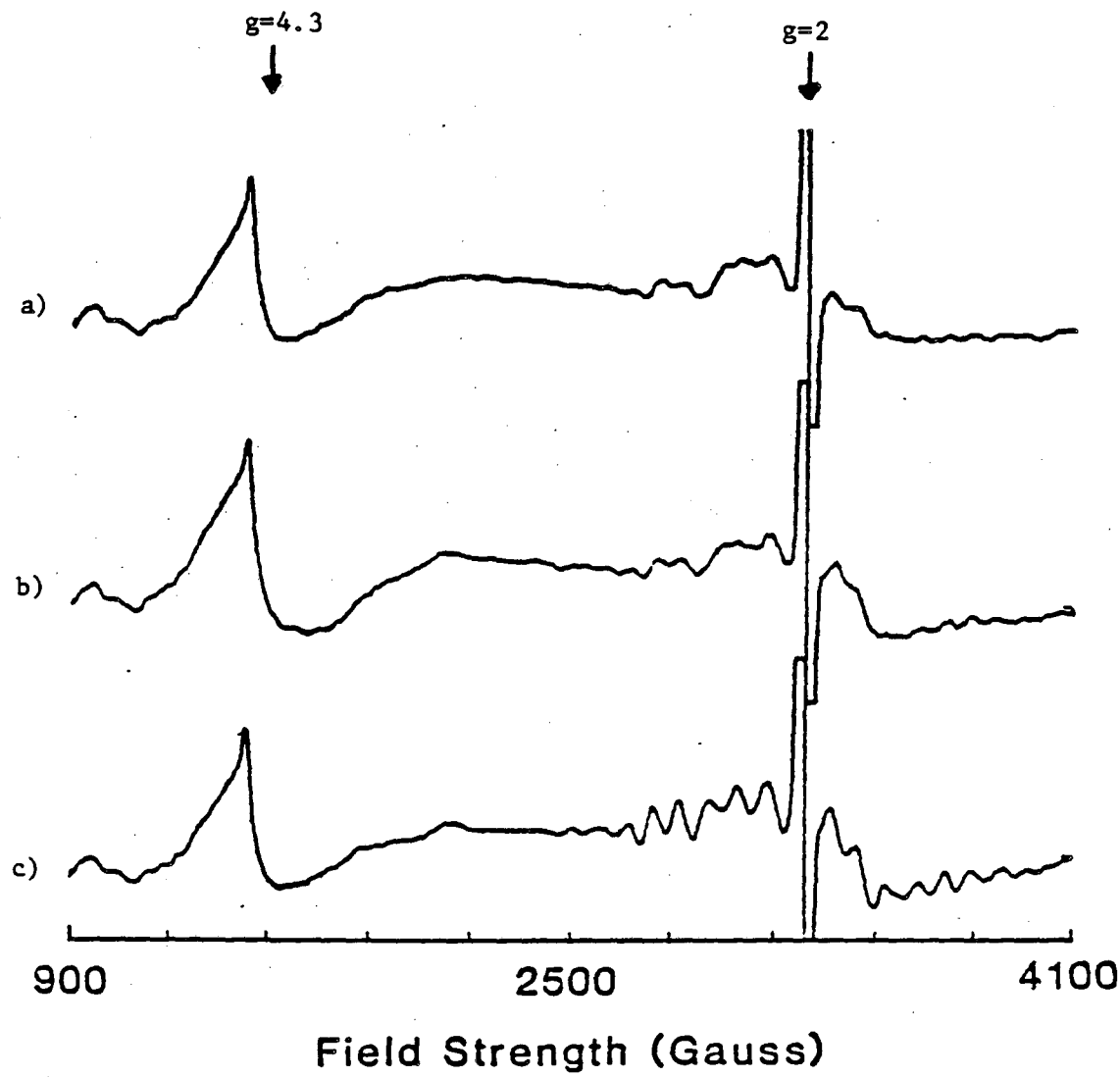
3.3 Results

3.3.a Evidence for the Trapping of an Intermediate State S_1*

Figure 3-1 shows the EPR spectra of a photosystem 2 preparation recorded at 10 K and 50 mW microwave power. The spectrum of a dark-adapted sample is included as a reference (Fig. 3-1a); the most obvious features are the large signal at g 4.3, which is due to ferric iron in a rhombic environment, and the intense signal at g 2, called Signal II, which is due to a species of unknown function which is associated with photosystem 2. The origin of the features in the

Figure 3-1

EPR spectra of a photosystem 2 preparation recorded at 10 K. a) dark-adapted, b) illuminated at 140 K for 30 s, c) warmed to 190 K in the dark for 90 s after illumination at 140 K. Spectrometer conditions: microwave power, 50 mW, microwave frequency, 9.2 GHz; field modulation, 25 G; modulation frequency 100 kHz; sweep rate, 2500 G/min (4 scans); time constant, 64 ms.


g=4.3
g=2
a)
b)
c)
900
2500
4100
Field Strength (Gauss)

vicinity of the g 2 signal is unknown. The intensities of these smaller features and of the large signal at g 4.3 are variable for different dark-adapted photosystem 2 preparations.

Fig. 3-1b shows the EPR spectrum obtained at 10 K after illumination of the photosystem 2 preparation at 140 K. As reported in Chapter 2 for chloroplasts, illumination below the threshold temperature of 160 K produces very little of the multiline signal associated with the S_2 state. Indeed, the entire spectrum appears to be relatively unaffected by illumination at 140 K.

The same sample which produced the spectrum in Fig. 3-1b was warmed to 190 K in the dark; the spectrum taken at 10 K after equilibration at 190 K is shown in Fig. 3-1c. The most striking change in the spectrum is the appearance of the multiline signal between 2600 and 4100 G. This signal, which is identical to the multiline signal which has been confirmed to arise from the S_2 state of the oxygen-evolving complex [10-12 (see Chapter 2)], was produced by following an illumination below the S_2 formation threshold temperature with a dark equilibration at a temperature above the threshold. This result is suggestive that an intermediate state S_1*, between the S_1 and S_2 states, was generated and trapped by the illumination at 140 K.

3.3.b Detection of EPR Signals Which Correspond to the State S_1*

Because such an intermediate is most likely paramagnetic, an EPR signal associated with it might be detectable. Attempts to identify such signals, which may be due to either organic radicals or

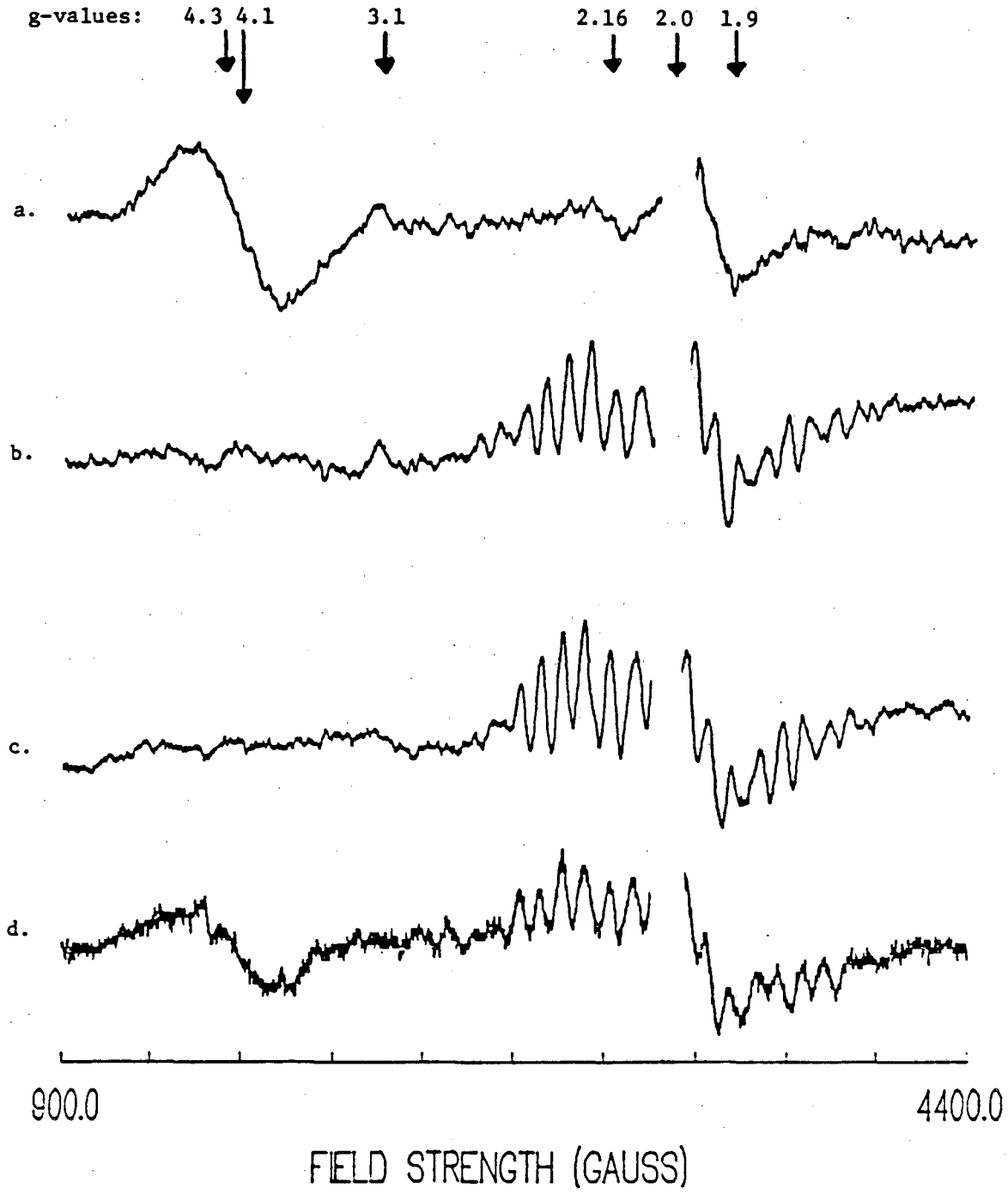
transition metal complexes, are presented below. Two sets of spectrometer conditions have been employed: 1) conditions such as those used for the observation of the spectra shown in Fig. 3-1 are useful for the detection of paramagnetic transition metal complexes, such as the species which gives the multiline signal and cytochrome b_{559}; 2) lower modulation amplitudes, lower microwave power, and higher temperature are more useful for the detection at g 2 of organic radical species, such as Signal II.

3.3.b.i Wide Scans at Low Temperature and High Power

Although no obvious signals were produced in the 10 K EPR spectrum by illumination at 140 K (Fig. 3-1b), the following results show that subtraction of the spectrum of the dark-adapted sample from the spectrum of the illuminated sample yields the observation of an EPR signal which may be due to the trapped intermediate. Fig. 3-2a shows the difference spectrum obtained by subtraction of the dark-adapted spectrum from the spectrum of a PS 2 preparation which was illuminated at 140 K. The prominent features produced by the low temperature illumination are a 320 G wide, structureless, derivative-shaped signal centered at about g 4.1, a peak at g 3.1, another derivative-shaped signal at g 2.16, and a negative peak at g 1.9; as noted for Fig. 3-1b, there was only a small amount of the multiline signal formed. The g 3.1 and g 2.16 features are due to the oxidized high potential form of cyt b_{559} [19]; the negative feature at g 1.9 is possibly due to the reduced PS 2 acceptor [13]. The light-induced g 4.1 feature has not been previously reported; this feature appeared on top of a fivefold larger background signal with a

Figure 3-2

Effects of illumination temperature and warming on the EPR spectrum of a photosystem 2 preparation. Dark-adapted spectra have been subtracted. a) Illuminated at 140K for 30 s, b) warmed to 190 K in the dark for 90 s after illumination at 140 K, c) illuminated at 190 K, d) illuminated at 170 K. Spectrometer conditions as in Fig. 3-1. All spectra were recorded at 10 K.


g-values: 4.3 4.1 3.1 2.16 2.0 1.9
a.
b.
c.
d.
900.0
4400.0
FIELD STRENGTH (GAUSS)

different lineshape (see Fig. 3-1a) which made detection of the light-induced signal difficult in the absence of subtraction.

Fig. 3-2b shows the difference spectrum obtained by subtraction of the spectrum of a dark-adapted sample from the spectrum of a sample which was warmed to 190 K in the dark after being illuminated at 140 K. There are two major changes produced in the spectrum by the warming process; the light-induced g 4.1 feature disappears and, as also shown in Fig. 3-1c, the multiline signal appears. The amplitude of the multiline signal obtained by illumination at 140 K followed by warming to 190 K is approximately 75% of that produced by illumination of a dark-adapted sample at 190 K. The features at g 3.1, 2.16 and 1.9 are unaffected by warming to 190 K, although the latter two are somewhat obscured by the presence of the multiline signal.

The difference spectra in Figs. 3-2d and 3-2c show that progressively less of the g 4.1 signal and more of the multiline signal are photoproduced as the temperature of illumination is increased to 190 K. Little or none of the light-induced g 4.1 signal is trapped by illumination at 190 K, which is above the threshold temperature, and illumination at the mid-threshold temperature of 170 K produces approximately half the maximum amount of both the g 4.1 and multiline signals. The degree of photooxidation of cytochrome b_{559}, as determined by the signals at g 3.1 and 2.16, is also dependent on the illumination temperature between 140 and 190 K, being small at 170 K, and decreasing to zero as the illumination temperature is raised to 190 K.

The ability to produce the g 4.1 signal by illumination below the threshold temperature for advancement of S_1 to S_2, and the concomitant disappearance of this signal and rise of the multiline signal upon warming in the dark above the threshold temperature are suggestive that the light-induced g 4.1 signal is due to an intermediate state S_1* between the S_1 and S_2 states. Further support for this hypothesis is the observed complementarity between the amplitudes of the multiline signal and the g 4.1 signal produced by illumination at temperatures between 140 and 190 K.

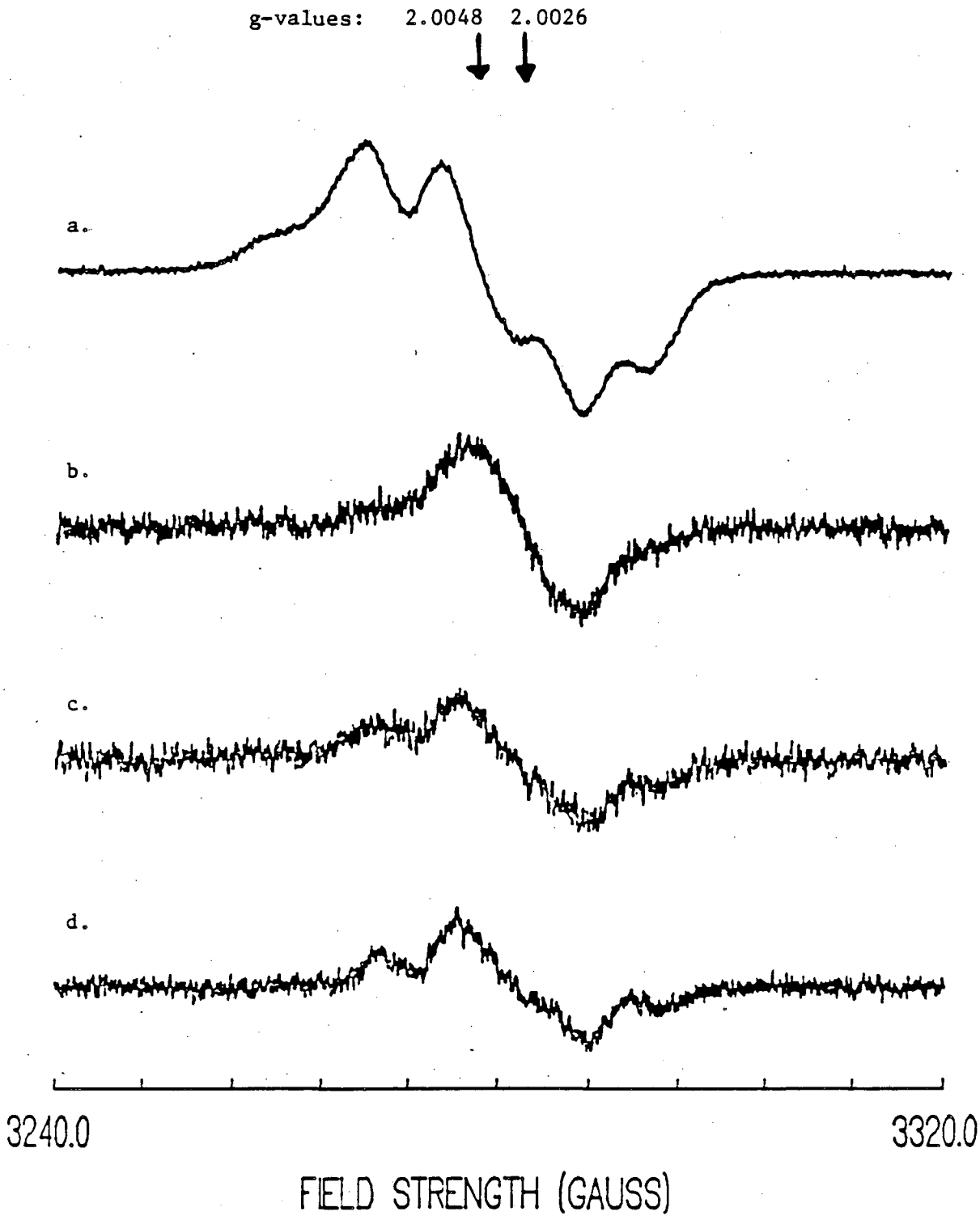
3.3.b.ii The g 2 Region

The g 2 region of the 80 K EPR spectrum of the dark-adapted photosystem 2 preparation is shown in Fig. 3-3a; 80 K was chosen as the observation temperature because at lower temperatures the signal amplitudes are sensitive to small temperature fluctuations, thus making it difficult to obtain reliable difference spectra. The observed broad structured signal in Fig. 3-3a is Signal II [4]. Illumination at 140 K produces a signal at g 2 which is observed after subtraction of the spectrum of the dark-adapted sample (Fig. 3-3b); the light-induced signal is centered at g 2.0026 and is approximately 10 G wide, with no apparent structure. There is little or no increase in the amplitude of Signal II upon illumination at 150 K.

As shown by the subtracted spectra in Fig. 3-3c, warming the sample in the dark to 190 K after illumination at 150 K results in the disappearance of the light-induced signal, and the appearance of a small signal which is similar to Signal II. Illumination of a dark-adapted sample at 190 K produces just the small amount of Signal

Figure 3-3

Effects of low temperature illumination and warming on the g 2 region of the EPR spectrum. a) dark, b) illuminated at 150 K for 30 s minus dark, c) warmed to 190 K in the dark after illumination at 150 K minus dark, d) illuminated at 190 K minus dark. b thru d are expanded twofold relative to a. Spectrometer conditions: microwave frequency, 9.2 GHz, modulation amplitude, 2.5 G; modulation frequency, 100 kHz, microwave power, 100μW; scan rate, 25 G/min; time constant, 64 ms; sample temperature, 80 K.


g-values: 2.0048 2.0026
a.
b.
c.
d.
3240.0
3320.0
FIELD STRENGTH (GAUSS)

II, and none of the 10 G wide species (Fig. 3-3d). The behavior of the 10 G wide species, then, is very similar to that expected for the S_1* state.

3.3.c Effects of Inhibition with 0.8 M NaCl on the Light-induced g 4.1 and g 2 EPR Signals

Exposure of the photosystem 2 preparation used in these studies to high concentrations of NaCl (ca. 0.8 M) inhibits oxygen evolution by approximately 80% [20]. Fig 3-4 shows the results obtained when samples which had been treated with 0.8 M NaCl were illuminated at about 140 K. Virutally no multiline signal is produced by illumination at 190 K (not shown) or by warming to 190 K of a sample which had been illuminated at 140 K (Fig. 3-4c). The difference spectrum in Fig. 3-4b shows that photoproduction of the g 4.1 feature at 140 K is also nearly completely abolished by the high salt treatment. In contrast to these results, the photoproduction of the 10 G wide feature at g 2 is unaffected by the inhibitory treatment (Fig. 3-5b). The light-induced g 4.1 feature thus appears to be more closely associated with oxygen evolution than the 10 G wide g 2 species. The light-induced g 4.1 signal is investigated in more detail below.

3.3.d Further Characterization of the Light-induced g 4.1 Signal

Additional characterizations of the light-induced g 4.1 feature have included quantititation of the g 4.1 signal species by the integration of the signal, and the recording of higher resolution spectra by using 6.3 G field modulation.

Figure 3-4

Effects of treatment with 0.8 M NaCl on the light-induced EPR signals. Dark spectra have been subtracted in all cases. a) control sample illuminated at 140 K, b) 0.8 M NaCl-treated sample illuminated at 140 K, c) 0.8 M NaCl-treated sample illuminated at 140 K, then warmed to 190 K in the dark. Spectrometer conditions as in Fig. 3-1, except: modulation amplitude, 32 G.

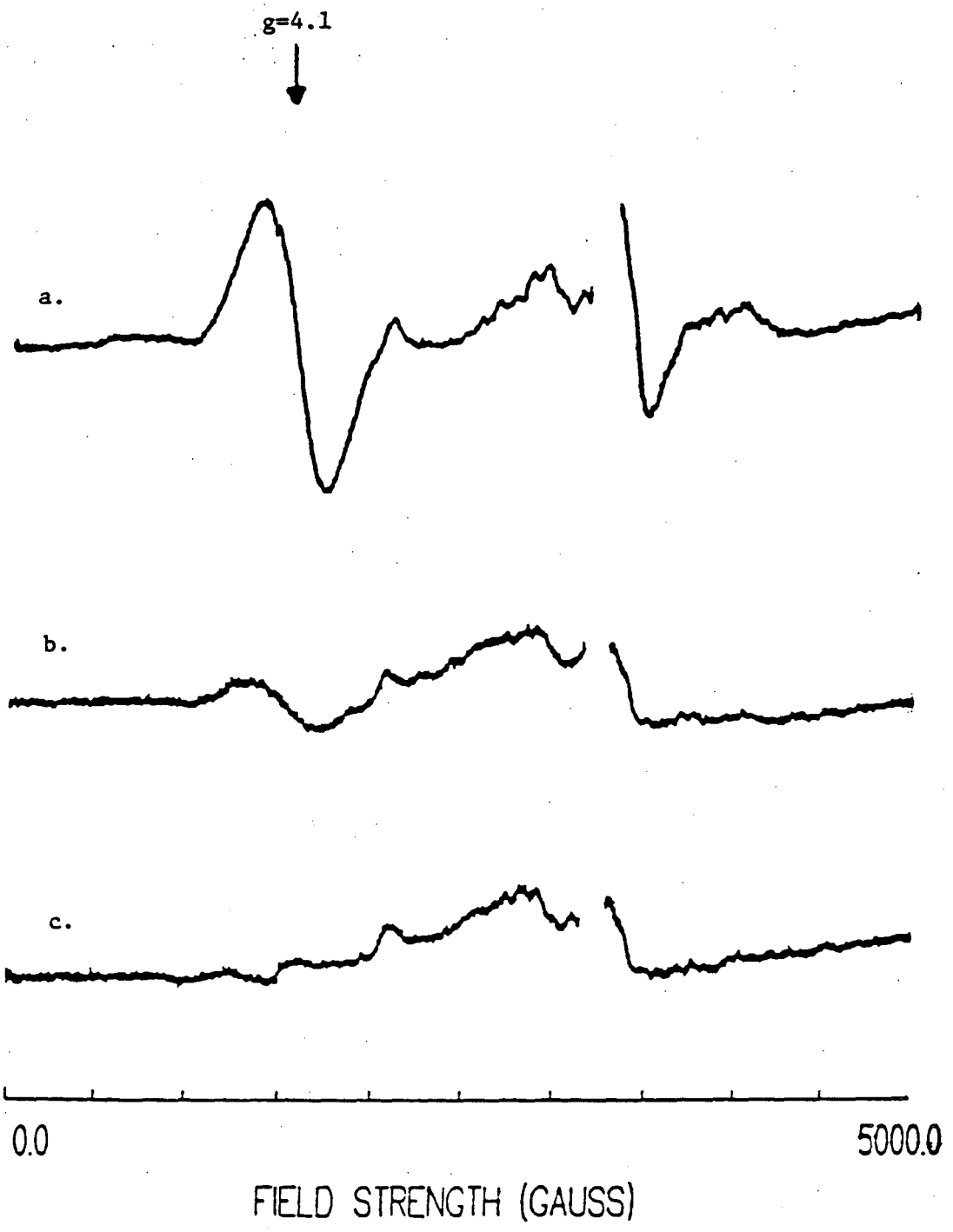
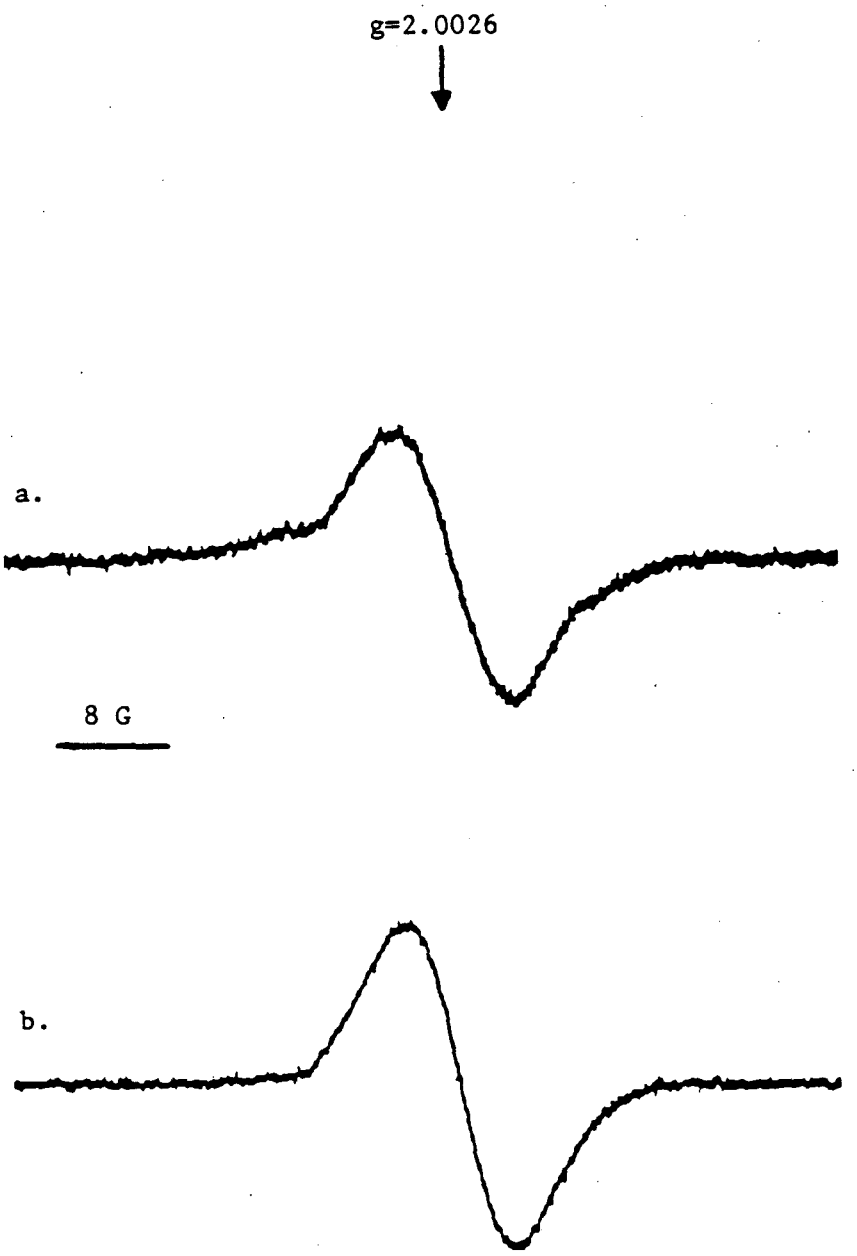

g=4.1
a.
b.
c.
0.0
5000.0
FIELD STRENGTH (GAUSS)

Figure 3-5

Effects of high salt treatment on EPR signals in the g 2 region. Samples were illuminated at 140 K; dark spectra have been subtracted. a) control, b) treated with 0.8 M NaCl. Spectrometer conditions as in Fig. 3-3.


g=2.0026
a.
8 G
b.

3.3.d.i Quantitation of the g 4.1 Signal by Integration

A likely origin of the light-induced g 4.1 feature is a molecular species with spin 5/2 in a rhombic crystal field [21-23], because such species are known to give EPR signals near g 4.27, the effective g-value for a system of perfectly rhombic symmetry. Support for such an assignment and for the proposed involvement of the g 4.1 signal in oxygen evolution might be obtained by a comparison of the integrated intensity of the signal with that of a standard. Ferric EDTA prepared at pH 3.5 was used as the standard. Care was taken to obtain the Fe-EDTA spectrum at a microwave power below the saturation level for the compound. Because the EPR spectra reported here are equivalent to the first derivative of the absorption, double integration is required. The integration was done after subtraction of a baseline, either by double integration of the spectrum or by the integration of the calculated first moment of the spectrum [24]. Both methods provided the result that the number of species giving rise to the g 4.1 signal is approximately 0.3 per photosystem 2 unit, assuming 250 chl per photosynthetic unit [17]. It should be noted that for spin 5/2 species there are other transitions which underly the large signals at g 4.3 and are thus included in the integration; the contribution of these very broad transitions to the integration is small, however, and the difference between these contributions for the light-induced signal species and the Fe^{3+}EDTA standard is expected to be even smaller.

3.3.d.ii High Resolution Spectrum of the Light-induced g 4.1 Signal

Ferric iron is known to give signals near g 4.3 in a wide

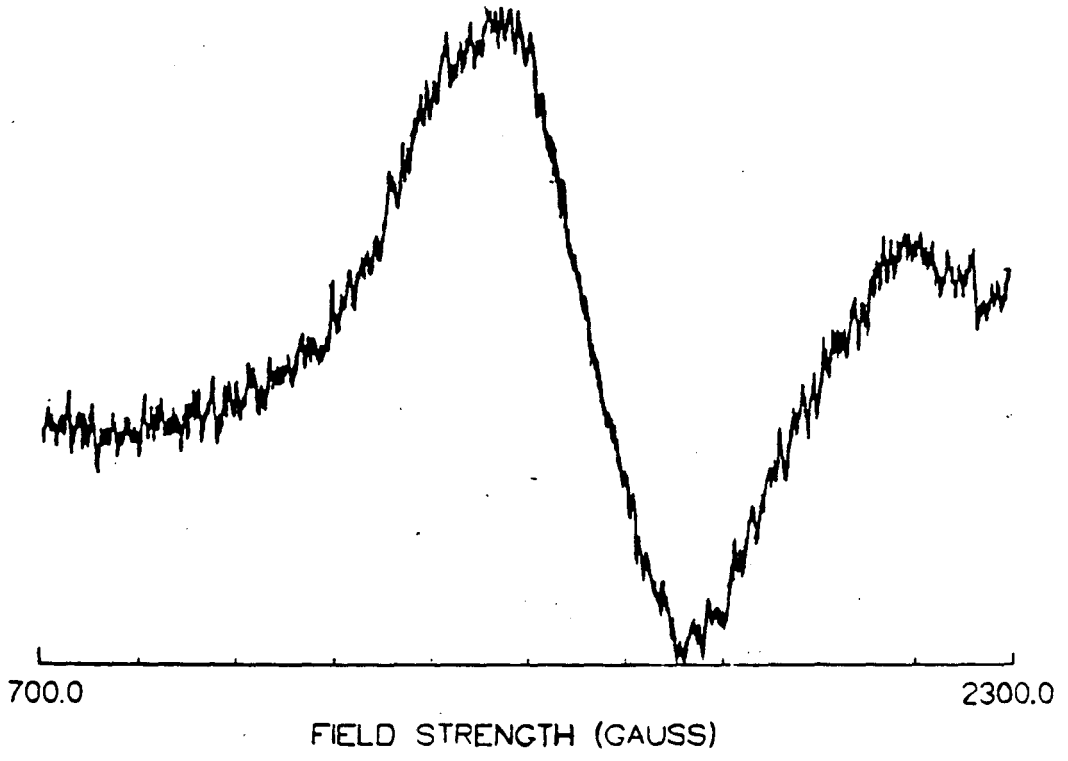
variety of environments, such as the iron-sulfur protein rubredoxin, the enzyme protocatechuate 3,4 dioxygenase [22] and the iron-transport protein transferrin [23z]. However, as the manganous ion has the same electron configuration as the ferric ion, it is conceivable that the signal could be due to manganese or iron. To attempt to distinguish between these alternatives, the spectrum of the light-induced g 4.1 feature was obtained at low modulation amplitudes. Manganese occurs in nature as the isotope ^{55}Mn, which has a nuclear spin of 5/2. This nuclear spin splits the electron spin levels, resulting in the frequent observation of six or more hyperfine lines in the EPR spectra of Mn compounds. The most abundant isotope of iron, ^{58}Fe, has no nuclear spin; thus hyperfine lines are not usually observed in the absence of isotopic enrichment. The low modulation amplitude spectrum of the light-induced g 4.1 signal is shown in Fig. 3-6; neither fine structure nor hyperfine structure is evident. In this expanded scale, however, it is evident that the signal is somewhat asymmetric; this asymmetry may be due to a slight anistoropy of the line.

3.4 Discussion

As reported for chloroplasts in the preceding chapter, illumination of an oxygen-evolving photosystem 2 preparation below 150 K produces none of the multiline EPR signal [10-12] associated with the S_2 state (Figs. 3-1b, 3-2a). However, a sample which had been illuminated at 140 K exhibits the multiline signal upon warming in the dark to a temperature above the threshold, prior to observation at 10 K (Figs. 3-1c, 3-2b); the observation of the multiline signal indicates that the oxygen-evolving complex is advanced to the S_2

Figure 3-6

Spectrum of the light-induced feature at g 4.1 at higher resolution. Photosystem 2 preparation was illuminated at 140 K; the dark spectrum taken under the same spectrometer conditions has been subtracted. Spectrometer conditions as in Fig. 3-1 except: modulation amplitude, 6.3 G; scan rate, 250 G/min (8 scans).


700.0
2300.0
FIELD STRENGTH (GAUSS)

state. Thus, the results shown in Figs. 3-1 and 3-2a,b demonstrate the ability to trap an intermediate state S_1* between the S_1 and S_2 states of the Kok scheme [26, see Chapter 1] by illumination of dark-adapted PS 2 preparations below the threshold temperature for the advancement of S_1 to S_2. The intermediate state, which is stable to recombination with the reduced acceptor at 140 K, is capable of advancing completely to the S_2 state only at temperatures above the threshold of about 170 K. The results are summarized by:

$$S_1 \xrightarrow{h\nu} S_1^*$$

$$S_1^* \longrightarrow S_2 \qquad T > \text{ca. } 170 \text{ K}$$

The designation S_1*, is of course highly schematic (see Chapter 1), and does not indicate the identity of the oxidizing species created and trapped by the low temperature illumination. S_1* could represent an oxidizing equivalent stored in the oxygen-evolving complex itself, but in a chemical form different from the S_2 state; other candidates for this species, discussed in section 3.1, include the oxidized primary donor, the Signal II_{vf} species, $Z^{\bullet +}$, and oxidized cytochrome b_{559}.

3.4.a Signals at g 2 Are Not Involved in the Intermediate State

The oxidized primary donor, P^+_{680}, and the Signal II_{vf} species, $Z^{\bullet +}$, were monitored by observation of the low temperature light-induced changes in the EPR spectrum at g 2.0 (Fig. 3-3). Illumination at 150 K produces a structureless 10 G wide radical centered at g 2.0026 (Fig.3-3b). This signal disappears upon warming above the 190 K (Fig. 3-3c) threshold temperature, and is not trapped

following illumination at 190 K (Fig. 3-3d). The behavior of this signal is thus similar to that of the intermediate state S_1*. Although the linewidth and g-value of this light-induced signal are consistent with an assignment to oxidized chlorophyll, the light-induced signal in Fig. 3-3b is most likely not due to the oxidized primary donor, P^+_{680}, for the reduced acceptor, Q_A^-, which is not oxidized by the secondary acceptor Q_B at these low temperatures of illumination, has been shown to react with the oxidized primary donor with a half-time of 4 ms [27].

A signal similar to the light-induced 10 g wide g 2 signal has been reported for a species which is photogenerated near 10 K in a barley mutant lacking photosystem 1 [28], and in chloroplasts poised at high reduction potentials (E > +450 mV) [29]. The effect of the high potential is uncertain, but it could provide a highly oxidized acceptor for stabilization of the radical pair; although such a stabilization might allow the trapping of P^+_{680}, the assignment of the signal to oxidized P_{680} is not certain, for P^+_{680} could readily oxidize a neighboring reaction center chlorophyll, which would give a similar EPR signal.

The most likely explanation for the light-induced g 2 signal is the oxidation of a chlorophyll species close to the reaction center in a reaction which is not on the path to water oxidation. Supportive of this conclusion are the results in Fig. 3-4, and 3-5, which show that the photogeneration of the g 2 signal is not affected by treatment of samples with 0.8 M NaCl, which inhibits oxygen evolution and the formation of the multiline signal. The formation of this signal could

thus represent a reaction which is competitive with the formation of the S_1* state. That the amplitude of the multiline signal obtained after warming a sample which had been illuminated at 140 K is approximately 75% of that which is produced by illumination at 190 K is evidence that competitive reactions become more effective at lower temperatures (see Chapter 4). At 190 K the multiline signal is produced to its fullest extent because the competitive reaction is ineffective at this temperature, as demonstrated by the absence of the photogeneration of the g 2 signal at 190 K (Fig. 3-3d). The observed decrease in the amplitude of the light-induced 10 G wide g 2 signal upon warming to 190 K (Fig. 3-3c) is not necessarily inconsistent with the proposed assignment of the signal to the oxidation of a reaction center chlorophyll in a competitive side reaction. It is possible that the oxidized chlorophyll species are capable of further reactions at 190 K which result in the loss of paramagnetism and the disappearance of the signal.

No light-induced changes are observed in the intensity of Signal II upon illumination at 150 K (Fig 3-3a,b); however, warming of a sample which had been illuminated at 150 K and illumination at 190 K produce an increase of 10 - 20% in the amplitude of Signal II over that present in the dark (Figs. 3-3b,c). The behavior of Signal II is thus more like that of the multiline signal rather than a species associated with the intermediate state S_1*. The relation between this result and other reports [28,30] that illumination at 13 K induces an increase in the amplitude of Signal II is unclear; some of the preparations used in the reported studies [30] were

different from those used in this work in that they were not competent in oxygen evolution, and the largest light-induced increase in the amplitude of Signal II appeared in samples that were frozen during illumination before receiving additional illumination at 13 K. In any event, it is clear from the spectra in Fig. 3-3 that the Signal II species is not responsible for the intermediate state S_1*.

3.4.b The Light-induced Signal at g 4.1 Is Correlated with S_1*

The spectra presented in Fig. 3-2 show that a number of light-dependent spectral features are affected by the illumination temperature near the 170 K threshold for the formation of the S_2 state. The peaks at g 3.1 and g 2.16 indicate that cyt b_{559} is photooxidized to a progressively greater extent as the temperature is decreased below 170 K. This observed behavior of the cytochrome agrees qualitatively with that reported based on optical measurements of the oxidized cytochrome [31]. Unlike the expected behavior of an intermediate state S_1*, however, no decrease in the amplitude of the oxidized cytochrome peaks occurs upon warming to 190 K (Fig. 3-2b). The cytochrome is thus ruled out as a possibile molecular species associated with the state S_1*. Further evidence for this conclusion is the observation that chemical oxidation of the cytochrome prior to low temperature illumination has no effect on the low temperature behavior of the multiline signal (not shown).

In addition to the cytochrome peaks, illumination at 140 K induced the appearance of signals at g 4.1 and g 1.9 (Fig. 3-2a). The signal at g 1.9 is not responsible for the intermediate state, because it is produced by illumination at 190 K (Fig. 3-2c), and does not

appear to be affected by warming to 190 K after illumination at 140 K. The most likely assignment of this signal is to the reduced photosystem 2 acceptor, Q_A^-, although the difference in g-value, g 1.9 vs. g 1.82, from that reported [13] make this assignment tentative.

The broad derivative shaped feature at g 4.1 which is generated upon illumination at 140 K (Fig. 3-2a) has not been previously reported. The behavior of this signal correlates very well with the expected behavior of the intermediate state S_1*. The signal is produced to a progressively greater extent as the illumination temperature is decreased between 190 K and 140 K (Fig. 3-3), and disappears (Fig. 3-2b) on warming a sample which was illuminated at 140 K to above the 170 K threshold for the formation of the S_2 state, as determined by the appearance of the multiline signal. Furthermore, in contrast to the behavior of the 10 G wide g 2 species (Fig. 3-5), photogeneration of the g 4.1 signal is inhibited by treatment of the photosystem 2 preparation with 0.8 M NaCl (Fig. 3-4b), which inhibits oxygen evolution and the formation of the multiline signal.

As will be discussed in more detail below, the signal at g 4.1 is most likely due to a spin 5/2 species in a rhombic environment. Confirmation of this assignment and of the involvement of the species in photosystem 2 photochemistry is provided by the comparison of the integrated intensity of the light-induced signal in the photosystem 2 preparation with that of Fe^{3+}EDTA, which has S=5/2 and gives a signal at g 4.3. The integration yielded a value of 0.3 species per photosynthetic unit, assuming 250 chl per photosystem 2. Several factors may contribute to the observation that this value is less than

unity. 1) The photosystem 2 preparations used in these experiments were typically suspended at concentrations of 3 - 5 mg/ml of chlorophyll, with resultant very high optical densities; it is thus probable that the illumination of the samples was incomplete, giving a low estimate for the relative amount of light-induced signals in the sample. 2) The amplitude of the multiline signal which is generated by warming a sample which was illuminated at 140 K is approximately 75% of that generated by illumination at 190 K (compare Figs. 3-2b and 3-2c); if the formation of the multiline signal species from the intermediate state is completely effective once the intermediate is generated, the relative amount of intermediate created at 140 K should not exceed approximately 0.75 species per oxygen-evolving center. The quantitation of the signal is thus consistent with an assignment of 1 S=5/2 species per photosystem 2 center.

Further confirmation of the S=5/2 origin of the signal would be provided by the detection of an additional small signal at approximately g 9 which is sometimes observed from such species (see below). Initial attempts have been made to observe a g 9 feature associated with the g 4.1 signal. The small peak at about 600 G in the control sample spectrum for the 0.8 M NaCl treatment (Fig. 3-4a) may be the sought-after g 9 feature, but this result has not been confirmed.

3.4.c The Origin of the Light-induced g 4.1 Signal

The light-induced signal at g 4.1 is similar to that which would be produced by a S=5/2 species in an environment with rhombic symmetry. The Hamiltonian which describes a spin 5/2 species is

described to second order by Eq. 3-1 (see Refs. 21-23,32). In this

$$H = g\beta B \cdot S + D\{S_z^2 - S(S+1)/3\} + E\{S_x^2 + S_y^2\}$$

equation, g represents the g-value of the species (The g-value for spin 5/2 species is actually very close to the free electron value because of the minimal amount of spin orbit coupling in S-state ions.), β is the Bohr magneton, B is the external applied field, S is the electron spin, D and E are parameters which represent electron-electron interactions due to the crystal field. A system with an axial crystal field, such as a porphyrin system in a heme, is described by E=0; in this case the EPR spectrum consists of a derivative-shaped signal at g 6.0 and a negative peak at g 2.0. Rhombic distortions of the field are represented by nonzero values of E; the derivative peak near g 6 becomes split, and the peak at g 2 moves to higher field. When the ratio of E to D is close to 1/3, the system is said to be rhombic.

The energy levels produced for a rhombic crystal field are shown in Fig. 3-7 for three orientations of the external field; the splittings at zero external field in this case are assumed to be greater than the microwave frequency of the spectrometer. The splitting of the middle doublet by the applied field is independent of orientation, and gives a derivative shaped signal at g 4.27. The transitions within the other levels are extremely dependent on orientation, and are thus very broad; small features are expected at the turning points near g 9.7, 0.86, and 0.61. These signals are not readily detected in relatively dilute or randomly oriented samples

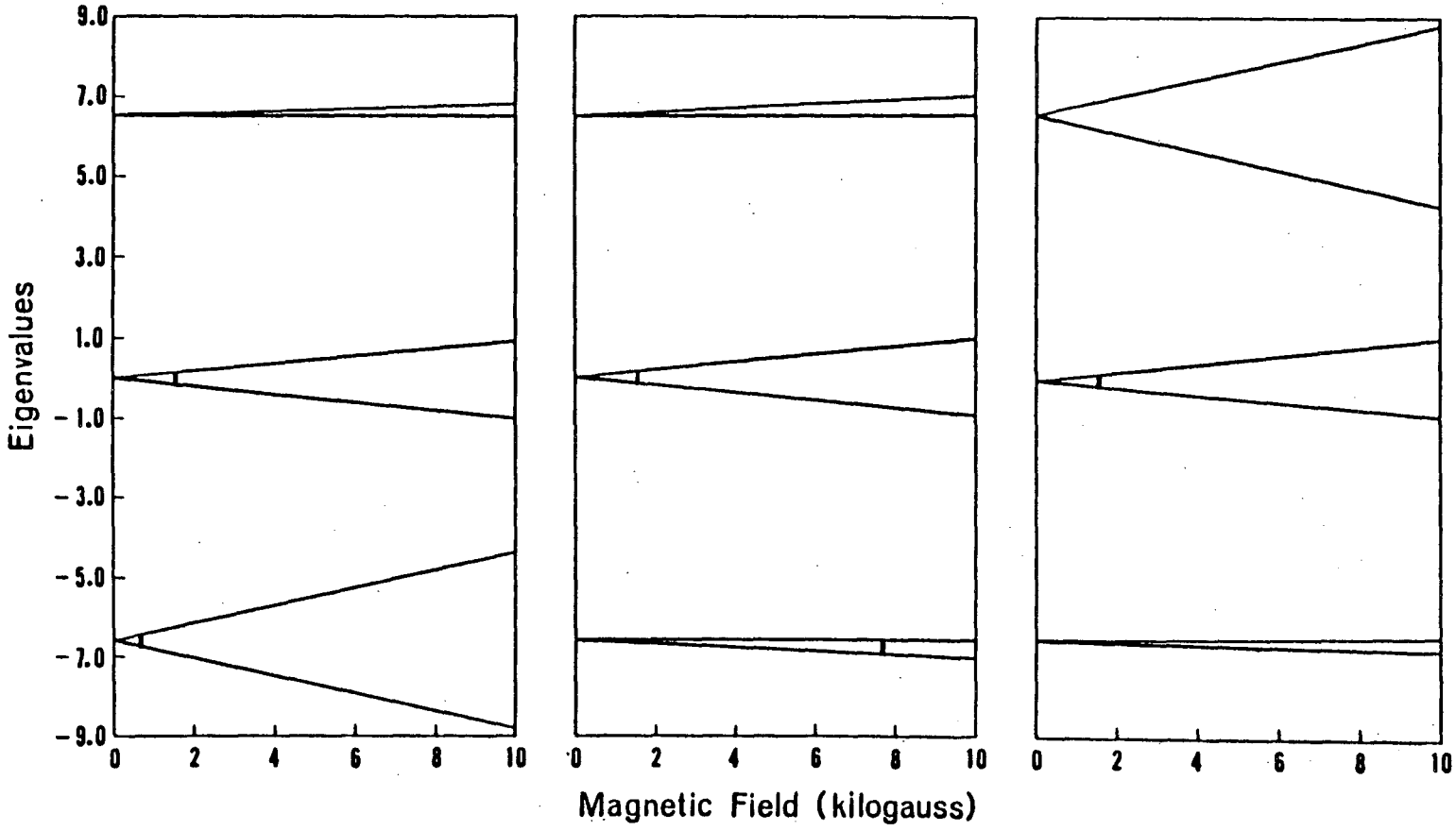


Figure 3-7

Energy levels for the three principle directions for S=5/2 system in a rhombic environment. Vertical bars indicate energy spacings which correspond to the resonance frequency of the spectrometer. From Ref. 21.

such as those used in this investigation. If the splittings between the levels at zero field are comparable to or smaller than the microwave energy, transitions between the different zero field levels will occur, and the spectrum will become very broad and very complex.

3.4.d Is There a Role for Non-heme Iron in Oxygen Evolution?

A ferric complex is the most likely origin for the light-induced g 4.1 signal, for ferric complexes are known to give signals near g 4.3 in a wide variety of systems, both biological and non-biological, and there is sufficient iron of undetermined function associated with photosystem 2 to account for the assignment of the g 4.1 feature to an iron complex. The signal at g 4.3 in dark-adapted photosystem 2 preparations (Fig. 3-1a) is likely also due to ferric iron, but the role in photosynthesis, if any, of this iron is undetermined. Examples of g 4.3 signals from Fe^{3+} in a functional biological environment are the iron sulfur protein rubredoxin, the enzyme protocatechuate 3,4 dioxygenase [23] and transferrin [25]. The environment of the iron in these cases is rhombic; a complex such as a heme, which has a high degree of axial symmetry, will not give a signal near g 4.3.

The chemistry of iron is consistent with the proposed involvement of iron in the S_1* state, for iron has several accessible oxidation states, which are involved in many biological oxidation reactions. The reduction potential of the Fe^{2+}/Fe^{3+} couple varies over a wide range, and includes, as in the case of the o-phenanthroline complex (E_m = +1.14 V), the high oxidizing potentials required for the oxidation of water. Further, the ability of iron to

bind water, hydroxide, and oxidation products of water, including oxygen, could play an important role in the chemistry of oxygen evolution. Thus, the involvement of iron in the intermediate state S_1* could include more than simple electron transfer reactions; it is conceivable that ligand transfers, such as occur in inner-sphere electron transfer reactions [33,34], are accomplished in the advancement of S_1* to S_2.

Iron is not the only transition metal which could give rise to the light-induced g 4.1 signal. Manganous ion has the same electronic structure as Fe^{3+} and is thus theoretically capable of giving a g 4.1 signal, although reports of such signals from Mn^{2+} complexes are rare. The spectrum of the light-induced signal taken at high resolution (Fig. 3-6) exhibits no structure which might be attributed to hyperfine interactions on manganese, but this negative result does not conclusively rule out the involvement of manganese in the complex which gives the signal, because broadening of the hyperfine lines can occur to such an extent that they are not observed. Nevertheless, the involvement of Mn^{2+} in the intermediate state S_1* seems unlikely, because the intermediate is formed as the result of an oxidation reaction and Mn^{2+} is the lowest oxidation state of Mn which might be involved in oxygen evolution.

3.5 Conclusion

An intermediate state S_1*, between the S_1 and S_2 states of the oxygen-evolving complex, can be trapped by illumination at 140 K, which is below the 170 K threshold for the formation of the multiline EPR signal associated with the S_2 state. Once formed, this

intermediate state is capable of advancing to the S_2 state upon warming in the dark to 190 K, which is above the threshold temperature. Neither Signal II nor the EPR signal of cytochrome b_{559} were observed to correlate with S_1*, but other EPR signals were observed which did exhibit behavior similar to that expected for S_1*. One of these was a 10 G wide signal at g 2 which exhibited a temperature dependence similar to that expected for S_1*, but was not affected by treatment with 0.8 M NaCl, which inhibits oxygen evolution and the formation of the multiline signal; this g 2 signal may be due to an oxidized reaction center chlorophyll. A light-induced signal at g 4.1, which is best observed after subtraction of the dark-adapted spectrum, exhibited the best correlation with the expected behavior for S_1*. This signal is proposed to originate from non-heme ferric iron.

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CHAPTER 4

FUNCIONAL INVOLVEMENT OF THE g 4.1 SIGNAL SPECIES IN OXYGEN EVOLUTION

4.1 Introduction

The results presented in the previous chapter demonstrate the involvement of a light-induced EPR signal at g 4.1 in the advancement of the state of the oxygen-evolving complex from S_1 to S_2. The assignment of this signal, which is proposed to be due to a non-heme ferric iron complex, to an intermediate state S_1* is based on the complementary temperature dependence for the generation of this signal and the multiline signal between ca. 150 and 190 K, and on the observation that the appearance of the signal is inhibited by 0.8 M NaCl, which inhibits oxygen evolution and the formation of the multiline signal. Further characterizations of the functional involvement in oxygen evolution of the species which gives the light-induced g 4.1 signal in dark-adapted samples are presented in this chapter.

Cytochrome b_{559} is known to be a competitive donor to the reaction center at low temperatures, with the maximal cytochrome photooxidation occurring at temperatures below about 110 or 120 K [1,2]. In Chapter 3, results are presented which demonstrate that cytochrome photooxidation occurs upon illumination at 150 K. Does the extent of cytochrome photooxidation increase as the temperature of

illumination is decreased, and if so, does such an increase in cytochrome photooxidation lead to a decrease in the extent of formation of the g 4.1 signal upon illumination and of the multiline signal upon subsequent warming? The effects of illumination at still lower temperatures than those employed in the previous chapter are explored to determine the relation between the donation to the photosystem 2 reaction center by the g 4.1 signal species and the cytochrome and to determine more completely the photochemistry of photosystem 2 at low temperature.

Treatment of the photosystem 2 preparation with high salt inhibits oxygen evolution [3], and the formation of both the light-induced g 4.1 signal and the multiline signal (see Chapter 3). While it is known that high salt treatment inhibits oxygen evolution by releasing peptides from the membrane [3], the component of the donor system which is affected by the treatment is undetermined. The effects of two additional inhibitory treatments, which are believed to inhibit the oxidizing photochemistry of photosystem 2 at different sites, are investigated in this chapter. Hydroxylamine affects the photochemistry of photosystem 2 in several ways [4], including the irreversible inactivation of oxygen evolution by the release of Mn. At concentrations in the 100 micromolar range, it has been shown to prevent the reduction of the oxidized primary donor, P^{+}_{680}, by the physiological donor Z [5,6]. The depletion of chloride ion from chloroplast preparations is also known to inhibit oxygen evolution [7,8], but the site of inhibition is not absolutely determined; the inhibition may be at or near the oxygen-evolving complex itself,

because chloride-depleted samples are capable of accumulating at least two oxidizing equivalents [9].

Numerous investigations of the kinetics of photosystem 2 reactions and of the photosystem 2 photochemistry at low temperature have suggested that the donation path of the oxygen-evolving system for the S_0 and S_1 states differs from that for the S_2 and S_3 states (see reviews in Refs. 10 and 11, Chapter 1). The nature of the different donation paths is not yet understood. In Chapter 3 it is demonstrated that an intermediate state S_1* is generated and trapped by illumination below the 170 K threshold for the formation of the S_2 state, and that a light-induced signal at g 4.1 is associated with this intermediate state. Does the species which gives the light-induced g 4.1 signal in dark-adapted samples participate in the advancement of S_2 to S_3? The involvement of the light-induced g 4.1 signal species in the S_2 to S_3 transition is investigated below by the preparation of samples in the S_2 state prior to low temperature illumination.

4.2 Materials and Methods

Photosystem 2 preparations, illumination procedures, and EPR spectrometer conditions are as described in Chapters 2 and 3.

For hydroxylamine treatment, the final suspension buffer contained either 50 μM or 200 μM $NH_2OH.HCl$, as indicated. The rates of oxygen evolution, measured in buffer containing the same concentrations of hydroxylamine, were 75% for 50 μM, and 30% for 200 μM, of control rates, which were typically between 300 and 500

μmol O_2 (mg chl)$^{-1}$ h^{-1}. Rates measured in buffer containing no hydroxylamine were the same as control rates.

Fluoride was substituted for chloride by suspending the photosystem 2 samples in buffer containing 20 mM NaF and 50 mM MES at pH 6.0. These samples were washed twice before final suspension in the same buffer. Rates of oxygen evolution for the fluoride substituted samples, measured in buffer also containing fluoride in place of chloride, were 25% of control rates. Rates measured in the presence of chloride were the same as control.

Samples were prepared in the S_2 state by flash illumination at 273 K or by continuous illumination at 190 K followed by equilibration at 260 K, as described in the text.

Where indicated, samples were chemically oxidized by adding 5 mM $K_3W(CN)_8$ and 5 mM $K_4W(CN)_8$ to the final suspension buffer. $K_3W(CN)_8$ was prepared as in Ref. 12, $K_4W(CN)_8$ as in Ref. 13.

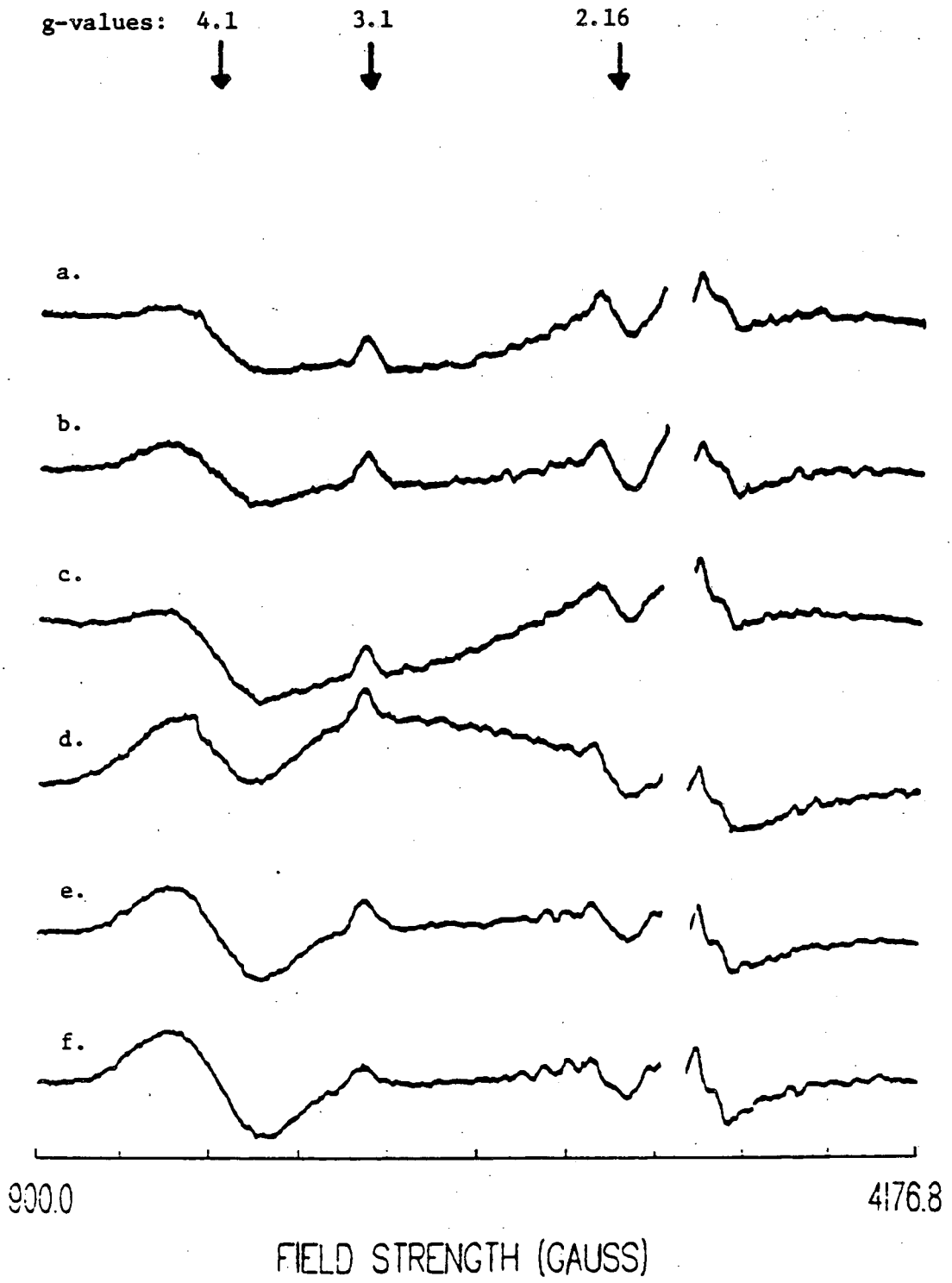
4.3 Results

4.3.a Photogeneration of the Signal at g 4.1 below 150 K.

An examination of the photogeneration of the g 4.1 signal at temperatures between 140 and 190 K is presented in Chapter 3. The investigation of the temperature dependence is extended to about 100 K in the series of spectra, presented in Fig. 4-1, of photosystem 2 samples illuminated between 100 and 150 K. The spectra for the lower temperatures are sometimes complicated by sloping backgrounds, but it is evident that the amplitude of the light-induced g 4.1 signal progressively decreases as the illumination temperature is dropped

Figure 4-1

Effect of the illumination temperature between 100 and 150 K on the EPR spectrum of a photosystem 2 preparation. Spectra of the dark-adapted samples have been subtracted in all cases. Illumination temperatures: a) 100 K, b) 110 K, c) 120 K, d) 130 K, e) 140 K, f) 150 K. Spectrometer conditions: microwave power, 20 mW, microwave frequency, 9.2 GHz; field modulation, 32 G; modulation frequency 100 kHz; sweep rate, 2500 G/min (4 scans); time constant, 64 ms; temperature, 12 K.


g-values: 4.1
3.1
2.16
a.
b.
c.
d.
e.
f.
900.0
4176.8
FIELD STRENGTH (GAUSS)

below about 130 K. The amplitude of the multiline signal produced upon warming to 190 K exhibits the same decrease in amplitude for the lower illumination temperatures as the light-induced g 4.1 signal (Fig. 4-2). Additional illumination at 190 K of samples illuminated at 100 K does not appear to increase the multiline signal amplitude to the level which is observed after illumination at 190 K of dark-adapted samples (not shown).

The extent of cyt b_{559} photooxidation, as determined by the signals at g 3.1 and 2.16, increases slightly in this temperature range, becoming maximal at about 130 to 140 K; this effect is seen most clearly in the spectra taken after warming to 190 K (Fig. 4-2). Chemical oxidation of the cytochrome prior to low temperature illumination does not appear to affect the observed decreased amplitude of the light-induced g 4.1 signal at lower temperatures.

4.3.b Effects of Inhibition of Oxygen Evolution on the Behavior of the Light-induced g 4.1 Signal

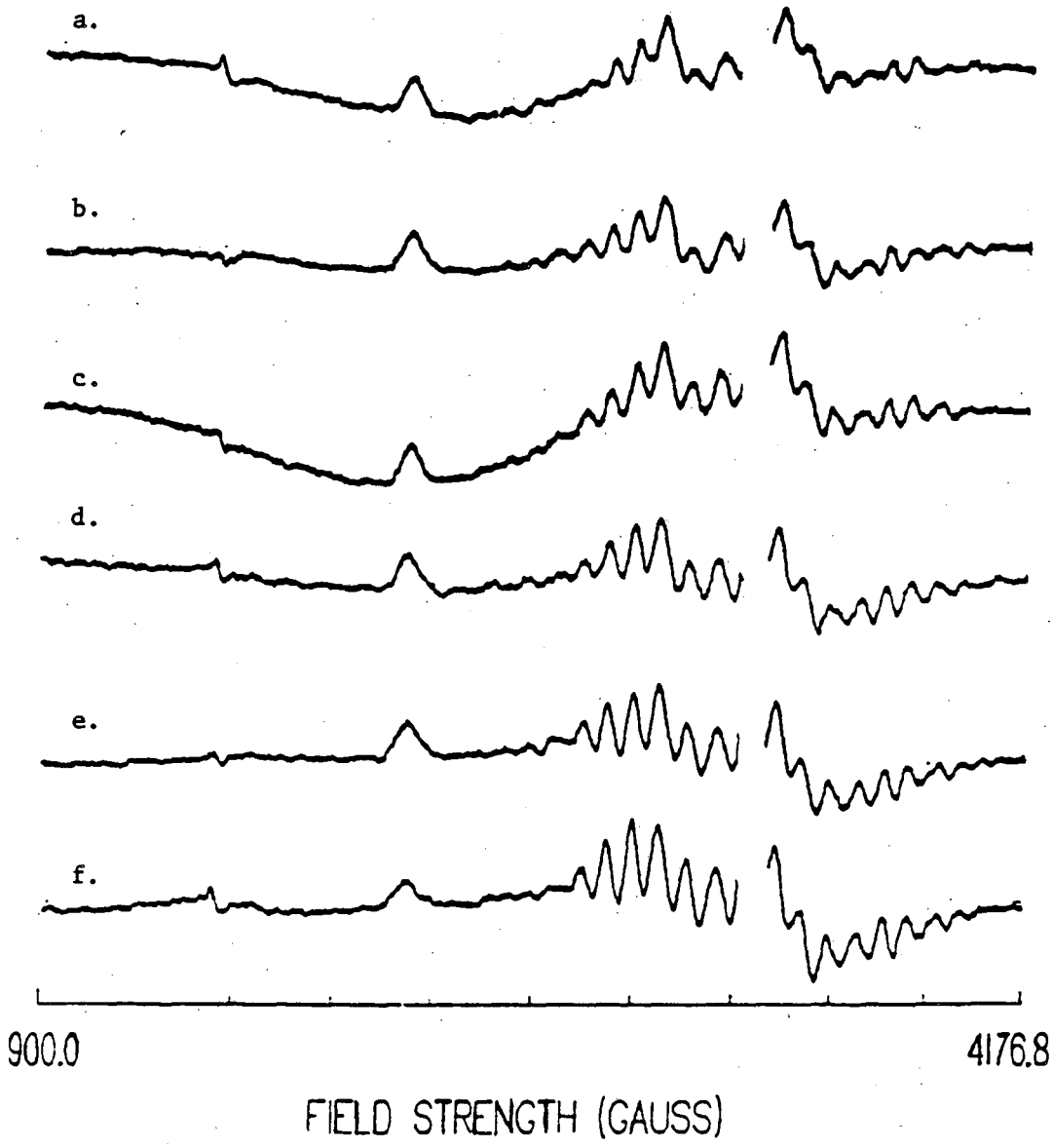
Treatment of the photosystem 2 preparation with high concentrations of NaCl inhibits oxygen evolution, the formation of the multiline signal and the photogeneration of the g 4.1 signal (see Chapt. 3). To assess further the relation between the light-induced g 4.1 signal and oxygen evolution, the effects of two additional inhibitory treatments specific for the oxidizing side of PS 2 have been investigated and are presented below.

4.3.b.i NH_2OH

Addition of 200 μM NH_2OH to photosystem 2 preparations

Figure 4-2

Effect of illumiantion temperature between 100 and 150 K on the EPR spectrum produced by warming to 190 K after illumination. Spectra of dark-adapted samples have been subtracted in all cases. Illumination temperatures: a) 100 K, b) 110 K, c) 120 K, d) 130 K, e) 140 K, f)150 K. Spectrometer conditions as in Fig. 4-1,


a.
b.
c.
d.
e.
f.
900.0
4176.8
FIELD STRENGTH (GAUSS)

reversibly decreased the oxygen evolution rates to less than 30% of control. The difference spectrum in Fig. 4-3c shows that in samples in which oxygen evolution is inhibited by this method the ability to generate and trap the g 4.1 feature by illumination at 140 K is lost; the ability to generate the multiline signal by warming to 190 K after illumination at 140 K is also inhibited (Fig. 4-4c). At a concentration of 50 μM, the extent of inhibition of oxygen evolution is only about 30%; the amplitudes of the light-induced g 4.1 signal and the multiline signal produced by warming are correspondingly larger for the lower level of inhibition (Figs. 4-3b, 4-4b). Analysis of samples treated with 200 μM hydroxylamine showed that less than 10% of the manganese was released by the treatment, and that inhibition of oxygen evolution was reversed by removal or dilution of the hydroxylamine.

4.3.b.ii Fluoride

In photosystem 2 preparations, the removal of chloride by washing with sulfate-containing buffers is not completely inhibitory, but the substitution of fluoride, which cannot reactivate chloroplasts after chloride depletion, achieves the same degree of inhibition as chloride depletion in chloroplasts. The substitution of fluoride for chloride in the final suspension buffer of the photosystem 2 preparation gave inhibitory effects different from those of high salt and hydroxylamine. While substitution of F^- for Cl^- reversibly inhibits oxygen evolution rates by about 75%, the spectrum in Fig. 4-5a shows that the treatment has little effect on the photogeneration of the g 4.1 signal at 150 K. Illumination at 150 K

Figure 4-3

Effect of hydroxylamine inhibition on the light-induced EPR spectrum. All samples were illuminated at 140 K; spectra of dark-adapted samples have been subtracted. a) control, b) 50 μM NH_2OH, c) 200 μM NH_2OH. Spectrometer conditions as in Fig. 4-1 except: microwave power, 50 mW; temperature, 13 K.

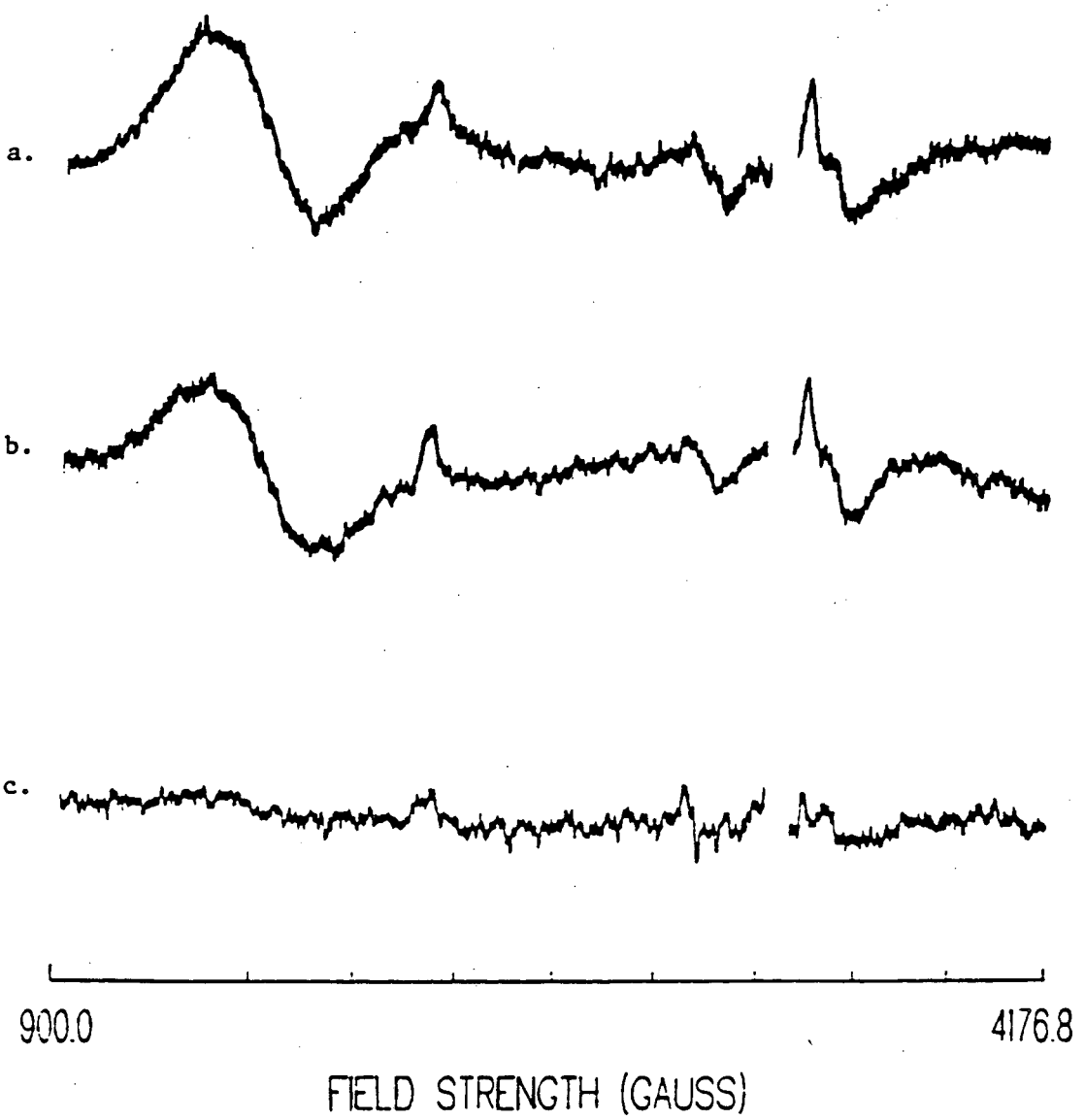

a.
b.
c.
900.0
4176.8
FIELD STRENGTH (GAUSS)

Figure 4-4

Effect of hydroxylamine inhibition on the EPR spectrum taken after warming illuminated samples. All samples were illuminated at 140 K, then warmed in the dark to 190 K; dark-adapted spectra have been subtracted. a) control, b) 50 μM NH_2OH, c) 200 μM NH_2OH. Spectrometer conditions as in Fig. 4-3.

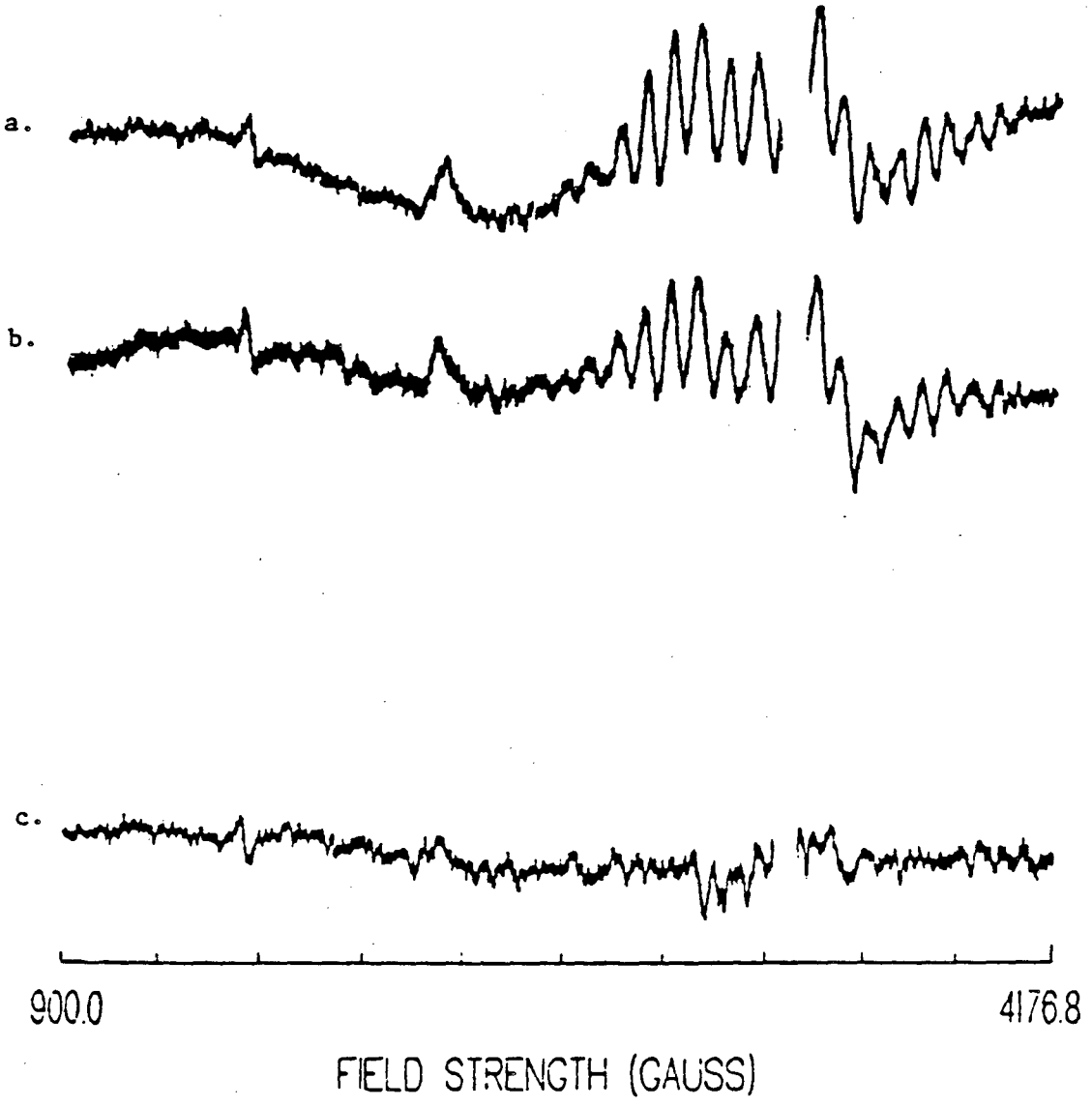
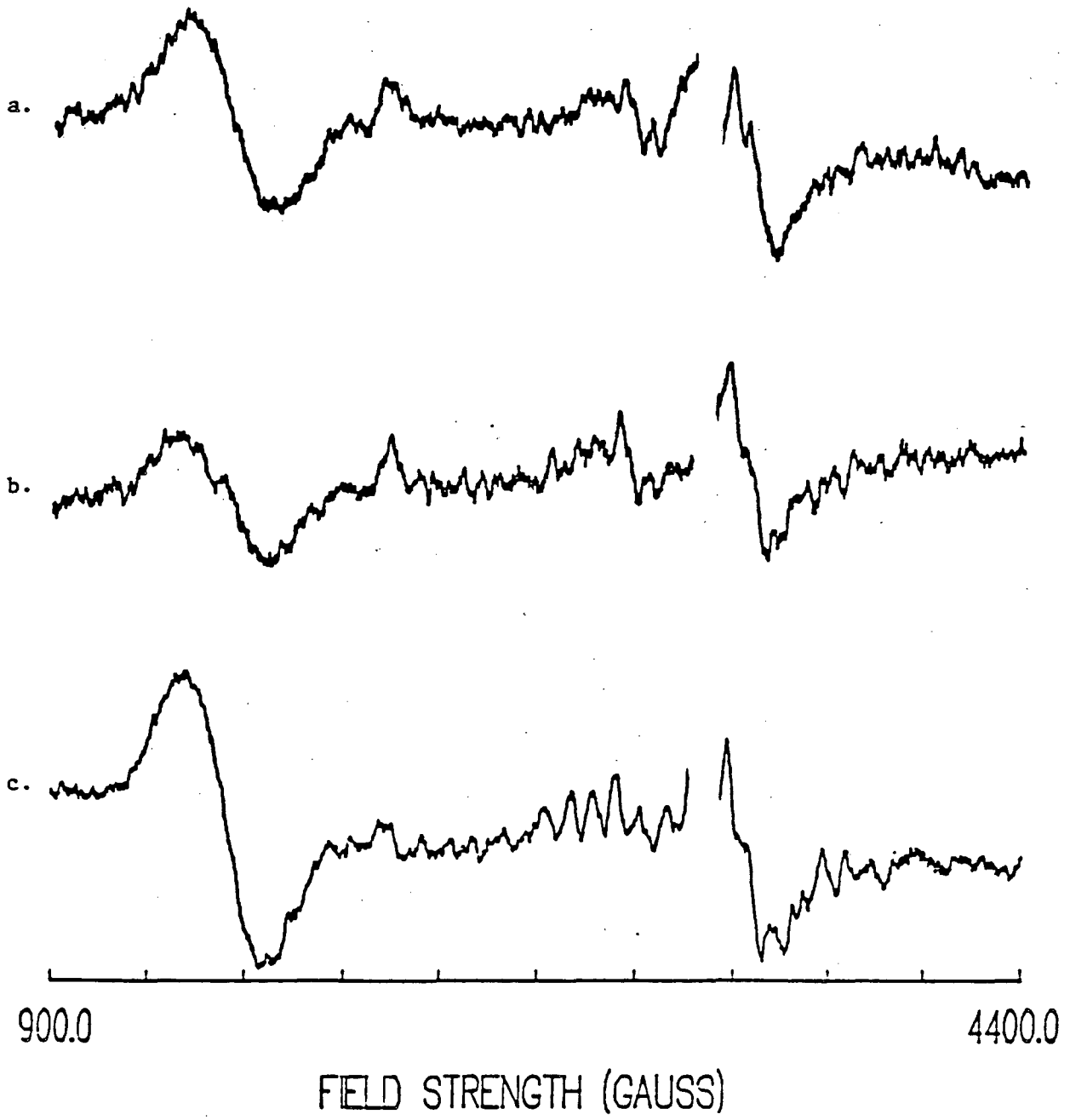

a.
b.
c.
900.0
4176.8
FIELD STRENGTH (GAUSS)

Figure 4-5

Effects of substituting F^- for Cl^- . Spectra of the dark-adapted samples have been subtracted.
a) illumination at 140 K for 30 s, b) warmed to 190 K for 90 s in the dark after illumination at 140 K, c) illuminated at 190 K for 30 s. Spectrometer conditions as in Fig. 4-1 except: modulation amplitude, 25 G; microwave power, 50 mW; temperature, 9 K.


a.
b.
c.
900.0
4400.0
FIELD STRENGTH (GAUSS)

generates and traps the g 4.1 signal in a fluoride-substituted sample to a degree which is about 75% of that observed in non-inhibited samples.

Although fluoride substitution does not appear to affect the photochemistry at 150 K, it does modify the behavior of the system at 190 K. The effect of the fluoride substitution on the behavior of the light-induced g 4.1 signal upon warming the 150 K-illuminated sample to 190 K is shown in Fig. 4-5b; in contrast to the behavior of control samples, approximately two-thirds of the light-induced signal remains after warming to 190 K, and no multiline signal is produced. Further, illumination of dark-adapted fluoride-substituted samples at 190 K produces the g 4.1 signal to the same extent as in control samples illuminatied at 150 K, and less than 25% of the control amplitude of the multiline signal. Close inspection of the light-induced g 4.1 signal in fluoride-substituted samples reveals that the linewidth of the signal is decreased by about 10 to 20 G relative to that in non-inhibited samples.

Fluoride substitution does not appear to significantly alter the pattern of cytochrome b_{559} photooxidation; just as in chloride-sufficient preparations, cytochrome photooxidation is observed at 150 K, but not at 190 K.

4.3.c Attempts to Generate the g 4.1 Signal in Samples Poised in the S_2 State

The ability to trap an intermediate state S_1* in dark-adapted samples, and the assignment of the g 4.1 signal to this state, is

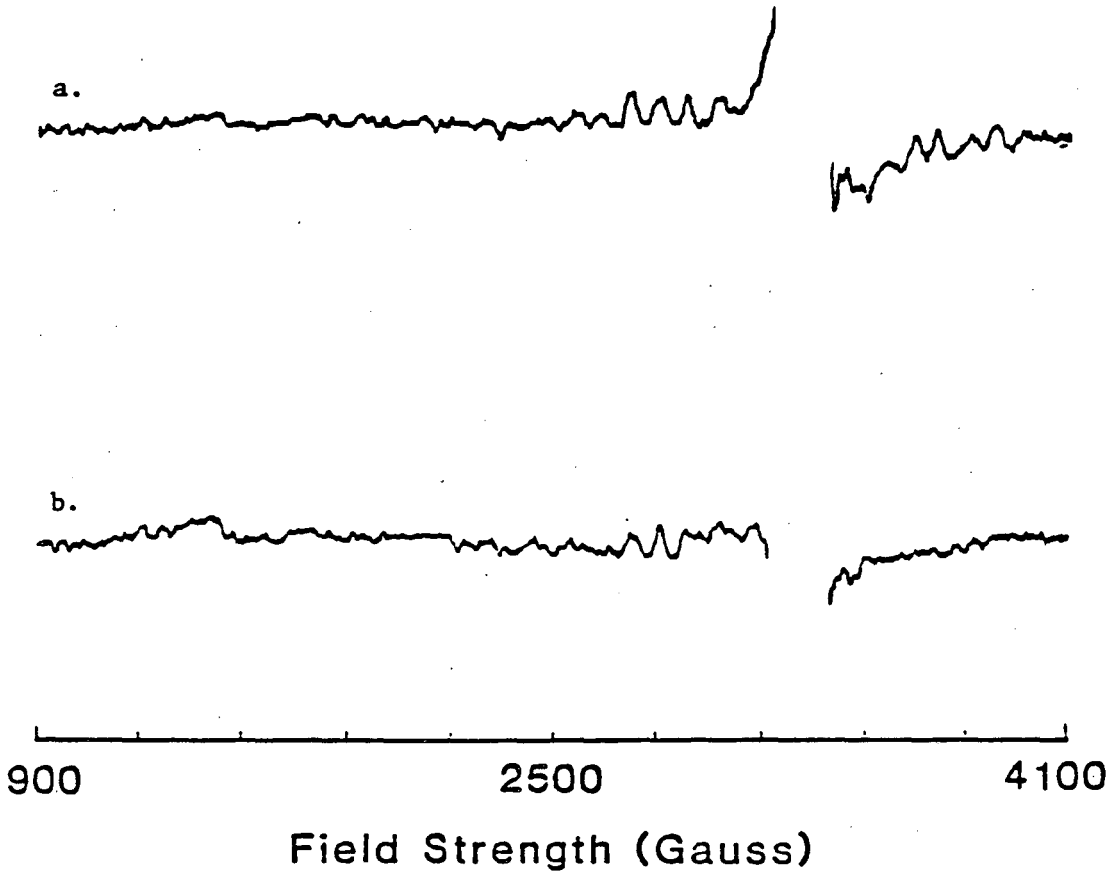
demonstrated in the previous chapter, and the properties of the S_1* state are further explored in the investigations presented above. The data presented below examine whether the species which is responsible for the stored oxidizing equivalents in the S_1* state, and which gives the light-induced g 4.1 signal in dark-adapted samples, is involved in the advancement of the other S-states. The behavior of the photosystem 2 donor reactions in the advancement from S_2 to S_3 was the most readily studied, for although it is difficult to prepare a sample entirely in the S_2 state, it is easier than preparing the system in S_3 or S_0.

Samples were prepared in the S_2 state in one of two ways. The simplest procedure was to illuminate with a single flash at 273 K and freeze rapidly in liquid nitrogen. The disadvantage of this method is that, in order to obtain uniform illumination, samples must be diluted to less than 1.0 mg chl/ml. The second method involved illumination at 190 K, followed by warming in the dark to 260 K. The illumination advances the oxygen-evolving complex to the S_2 state, and the warming allows the reoxidation of the acceptor Q_A, which occurs only very slowly at 190 K [14]. For both preparations, cyt b_{559} was chemically oxidized by the addition of 5mM $K_3W(CN)_8$ and 5mM $K_4W(CN)_8$ to the PS 2 preparation prior to illumination to prevent competitive donation.

After generation of the system in the S_2 state, samples were illuminated a second time at 190 K, which is below the threshold temperature of 220 K for S_2 to S_3 advancement (see Chapter 2). The effect of the second illumination on the EPR spectrum is shown in Fig. 4-6a for the sample prepared in S_2 by the low temperature

Figure 4-6

Illumination and warming of the S_2 state. Samples were prepared initially in S_2 as described in the text. Spectra of samples in S_2 have been subtracted.
a) illuminated at 190 K for 30 s, b) warmed to 260 K in the dark for 90 s after illumination at 190 K.
Spectrometer conditions as in Fig. 4-1 except: modulation amplitude, 32 G; microwave power, 50 mW.


a.
b.
900
2500
4100
Field Strength (Gauss)

illumination procedure. The results were the same for the preparation of S_2 by flash illumination at 273 K, and for low temperature illumination at 150 K. No changes were produced in the g 4.1 region of the spectrum of the oxidized sample by the second illumination. There was a small increase in the multiline signal amplitude, which is probably due to regeneration of a small amount of S_2 which had been lost upon warming to 260 K. The multiline signal amplitude did not decrease, because the second illumination was carried out at 190 K, which is below the S_2 to S_3 threshold temperature. Fig. 4-6b shows the difference EPR spectrum obtained by following the second illumination with an equilibration in the dark at 260 K, which is well above the threshold for the advancement of S_2 to S_3. An advancement to the S_3 state would be accompanied by a decrease in the multiline signal present in the sample (no signal is evident in the figure because of the subtraction); however, because no change in the spectrum is produced by the warming process, it is evident that the system was not advanced to the S_3 state by this procedure.

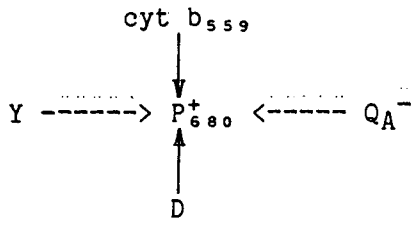
4.4 Discussion

4.4.a Photochemistry Below 150 K

Further evidence that the g 4.1 signal is due to the intermediate state S_1* is provided by the observed correlation between the amplitudes of the g 4.1 signal produced by illumination below 150 K and the multiline signal produced by warming to 190 K after the illumination. This correlation is evident on comparing the spectra in Figs. 4-1 and 4-2; lower illumination temperatures yield progressively smaller amplitudes of both the light-induced g 4.1

signal and of the multiline signal produced by warming to 190 K after illumination.

The origin of the decreased amplitudes is uncertain; the most likely causes are competitive donation from donors which are not on the path to water oxidation, and competition with charge recombination. These possibilities are shown schematically below.



In this diagram, Y represents the donor which gives the g 4.1 signal when oxidized and is on the path to water oxidation, D represents a donor which is not on the path to water oxidation; cyt b_{559}, P^+_{680}, and Q_A^- have been described in Chapter 1. The observation that a second illumination at 190 K of a sample illuminated below 130 K does not increase the amplitude of the multiline signal to the level observed for a single illumination at 190 K is evidence that charge recombination is not the dominant process leading to the decreased amplitudes of the g 4.1 and multiline signals produced by illumination below 130 K; it is thus likely that a competitive donor exists.

Cyt b_{559} is believed to act as a competitive donor to the photosystem 2 reaction center at low temperatures, and significant cytochrome photooxidation, as monitored by the signals at g 3.1 and

2.16, is observed at 150 K. This level of photooxidation increases somewhat as the temperature is decreased to 140 K, but, while the amplitude of the light-induced g 4.1 signal and the warming-produced multiline signal continually decrease as the illumination temperature is dropped below 130 K, no additional increase in cytochrome photooxidation is observed below this temperature. Furthermore, the decreased amplitude of the light-induced g 4.1 signal is not affected by the chemical oxidation of the cytochrome prior to illumination. The role of cyt b_{559} as a comptetitive donor is thus unclear.

A possible explanation for the observed cytochrome behavior is that the cytochrome is capable of acting as a competitive donor when in the high potential, or native, configuration, but loses this ability when it is converted to the low potential form. It has been shown that for a photosystem 2 preparation similar to that used in this study, approximately 80% of the cytochrome is present as the low potential form [15]; the presence of a large amount of low potential cytochrome could therefore explain the lack of an observed correlation between the cytochrome photooxidation and the decreased photoproduction of the g 4.1 and multiline signals.

If there is a competitive donor responsible for the decreased signal amplitudes below 130 K, it is likely that the 10 G wide g 2 species represents the oxidized donor. Although the behavior of this signal was not extensively investigated at temperatures below 130 K, the light-induced amplitude of this signal has been observed to be greater at 130 K than at 150 K (unpublished data).

4.4.b Effects of Inhibitors on the Light-induced EPR Signals

4.4.b.i Hydroxylamine

The inhibitory effects of hydroxylamine are known to be complex (see Refs. 4-6). At micromolar concentrations, hydroxylamine appears to bind tightly to a component of the photosystem 2 donor reactions and is oxidized by light excitation of the photosystem 2 reaction center; water oxidation is delayed until the hydroxylamine is oxidized [16]. At millimolar concentrations, however, hydroxylamine is known to release Mn from the oxygen-evolving complex, with a consequent irreversible loss of oxygen evolution activity [4]. The inhibitory effect of hydroxylamine on the photogeneration of the g 4.1 and multiline signals (Figs. 4-3, and 4-4) is observed at concentrations below those required for Mn release, and, in fact, very little Mn was observed as $Mn(H_2O)_6^{2+}$ after treatment; the effect of hydroxylamine on the low temperature photochemistry is thus not directly related to Mn release.

At concentrations similar to those used in this study, hydroxylamine has been shown [6] to inhibit oxygen evolution reversibly by preventing electron transfer between the primary donor P_{680} and Z, the species which gives the room temperature EPR transient signal II_{Vf} and has been suggested to be the immediate donor to P_{680}. That the light-induced g 4.1 signal could not be generated in the presence of 200 μM NH_2OH (Fig. 4-3) is consistent with that finding and indicates that the species which gives the g 4.1 signal is on the path to water oxidation between the species Z and the oxygen-evolving complex. The effects of hydroxylamine treatment thus provide

additional evidence confirming the involvement of the g 4.1 signal species in oxygen evolution. The total inhibition of the formation of the light-induced g 4.1 feature by NH_2OH concentrations which leave approximately 30% of control oxygen evolution activity is not necessarily inconsistent; oxygen evolution measurements monitor the system in the steady state while low temperature illuminations are akin to single flash conditions.

4.4.b.ii Fluoride

It has been known for some time that depletion of chloride ion from chloroplast preparations inhibits oxygen evolution [7,8]. Usually the depletion is carried out by washing with buffer containing sulfate in place of chloride. This procedure has been found to be ineffective for photosystem 2 preparations, perhaps because the lower pH typically used for handling the photosystem 2 preparation, which is sensitive to higher pH, is not optimal for the removal of chloride. In this investigation, for which photosystem 2 preparations are superior to chloroplasts because of the lack of interfering EPR signals from photosystem 1, chloride is depleted by using fluoride, rather than sulfate, to replace chloride ion. It has been demonstrated that fluoride is ineffective in restoring oxygen evolution in chloride-depleted chloroplasts.

The replacement of chloride with fluoride is effective in inhibiting rates of oxygen evolution in the photosystem 2 preparation. The effects of fluoride substitution on the low temperature photochemistry are much different from those produced by inhibition with high salt concentrations, and with 200 μM NH_2OH. As shown in

Fig. 4-5a, the fluoride substitution does not prevent the light-induced formation of the signal at g 4.1 at 150 K. However, upon warming to 190 K in the dark approximately two-thirds of the light-induced signal at g 4.1 remains, and no multiline signal appears (Fig. 4-5b). This difference from the behavior of control samples was investigated further by illuminating samples at 190 K.

As might be expected from the inability to generate the multiline signal by following illumination at 150 K with equilibration at 190 K (Fig. 4-4b), illumination of fluoride-substituted samples at 190 K (Fig. 4-4c) produces little multiline signal, and as much of the signal at g 4.1 signal as is generated by illumination at 150 K in control samples. These results indicate that the locus of the fluoride substitution effect is between the species which gives the light-induced g 4.1 signal and the manganese of the oxygen-evolving complex; S_1* cannot be advanced to S_2. The cause for the blocking of the advancement of S_1* to S_2 is unclear, but the observation that the linewidth of the light-induced g 4.1 signal is slightly different for fluoride-substituted samples is suggestive that fluoride substitution alters the conformation of the species which gives the signal, possibly by binding to it.

The relation between the effects of fluoride substitution reported here and the effects of the more often used procedure of depleting chloride by substitution of sulfate for chloride are not clear. Muallem et. al. [6] have proposed that chloride depletion by sulfate inhibits O_2 evolution by blocking the advancement of S_3 to S_4. Perhaps the effects of fluoride substitution on the oxygen-evolving

complex are different from those of chloride depletion by sulfate. It was suggested above that fluoride inhibits by binding to the species which gives the signal; such a mode of inhibition by sulfate seems unlikely, but hydroxide, which has been suggested to play a role in the inhibition by chloride depletion, could conceivably bind in a manner similar to that proposed for fluoride.

Alternatively, the apparent differences in behavior could be ascribed to different methods of observation. In this investigation, the state S_2 is monitored directly by the presence of the multiline EPR signal, while in other reports [9] the S states were monitored indirectly by the observation of oxygen yields after flashes and thus might not be able distinguish between normal and altered charge accumulation in the oxygen-evolving complex prior to the release of oxygen. It is conceivable that chloride depletion both by sulfate and by fluoride substitution change the nature of the S states in such a way that electrons can be removed from photosystem 2 but the chemical nature of the stored oxidizing equivalents is different from those in chloride sufficient preparations; these modified S states would be incapable of fully oxidizing water to oxygen. Indeed, evidence has been presented that the S states in chloride-depleted chloroplasts are unusual; the deactivations of the S_2 and S_3 states are considerably slower [9] in chloride-depleted chloroplasts, and it has been shown that the ability to generate the multiline signal associated with the S_2 state is lost in chloride-depleted chloroplasts (unpublished results, V. Yachandra, personal communication). Further investigations of fluoride-substituted and chloride-depleted

preparations are necessary to fully resolve the nature of the halide effect in oxygen evolution.

4.4.c Effects of Preillumination on the Low Temperature Illumination Behavior - the Low Temperature Photochemistry of the S_2 State

Samples prepared in the S_2 state do not exhibit photogeneration of the g 4.1 signal when given further illumination at 190 K (Fig. 4-5a), which is below the S_2 to S_3 advancement threshold temperature. The lack of change in the multiline signal amplitude upon warming to 260 K (Fig. 4-5b) indicates that warming above the S_2 to S_3 threshold after the illumination does not advance the system to the S_3 state. It is reasonable to conclude that no intermediate state S_2* is generated by illumination of the S_2 state below the threshold temperature. That the species which gives the g 4.1 signal could be generated and trapped by illumination of dark-adapted samples at 150 K, which is below the threshold for the S_1 to S_2 and S_2 to S_3 advancements, but not by illumination at 150 K or 190 K of samples prepared in the S_2 state, is evidence that this species is not involved as an intermediate between the S_2 and S_3 states.

A number of studies have concluded, based on low temperature studies and room temperature kinetic measurements of the photochemistry of the S_1 and S_2 states, that the donation path of S_1 to P_{680} differs from that of S_2 to P_{680} (see Ref. 11 and Chapter 1). The results of this study support the proposal that there are different donation paths. The intermediate donor which gives rise to the light-induced g 4.1 signal operates when the system is in the S_1 state but not when it is in the S_2 state. The species which gives the

light-induced g 4.1 signal may thus be equivalent to the donor $Y_{0,1}$ in the model presented in Fig. 1-3 of Chapter 1.

A reasonable explanation for the fact that the g 4.1 signal species functions when the oxygen-evolving complex is in the S_1 state but not in the S_2 state is that advancement from S_1* to S_2 involves transfer of a ligand from this species to the manganese complex; the proposed assignment of an iron complex to this species is consistent with such an exlanation. Eq. 4-1 is a schematic representation of

$$S_1 \quad \ell\text{-}Y \xrightarrow{h\nu} S \quad \ell\text{-}Y^+ \longrightarrow S_2\text{-}\ell \quad Y \qquad \text{(Eq. 4-1)}$$

this model; here, S_1 and S_2 are states of the oxygen-evolving complex according to the Kok scheme [17, see Chapter 1]; Y represents the species which, when oxidized, gives the signal at g 4.1, and ℓ represents a ligand. In this ligand-transfer hypothesis, the iron center in the S_2 state would differ from the iron center in the S_1 state by the loss of a ligand. The absence of the ligand on Y when in the S_2 state could easily change the reduction potential of the center and the rate at which it is oxidized by the primary oxidants. Such a ligand transfer could involve an inner sphere mechanism for the electron transfer from S_1 to Y^+ (see Refs. 18,19). If the ligand is an anion, it would provide a counterion for an S state advancement in which no proton is released from the oxygen-evolving complex. Chloride or hydroxide derived from water are likely candidates for a ligand in this situation, but the actual identity is a matter of speculation.

4.5 Conclusion

The light-induced g 4.1 signal, which is assigned to an intermediate state S_1* in Chapter 3, is shown to exhibit further correlations with the expected behavior of S_1*. The amplitudes of both the light-induced g 4.1 signal and the warming-produced multiline signal are smaller for illumination temperatures below about 130 K. The decreased amplitudes at lower illumination temperatures are most likely due to a competitive donation reaction. The role of the cytochrome as the competitive donor is unclear, because the cytochrome exhibits maximal photooxidation at 130 - 140 K, above the threshold for the stabilization of oxidizing equivalents on the path to water oxidation. Similar to the effects of high salt treatment (see Chapter 3), inhibition of oxygen evolution with 200 μM NH_2OH prevents the formation of the light-induced g 4.1 signal and the multiline signal. Inhibition by substitution of fluoride for chloride blocks the formation of the multiline signal, but does not prevent the formation of the light-induced g 4.1 signal; the inhibitory effect of fluoride substitution may thus result from the blocking of the advancement of S_1* to S_2. While the g 4.1 signal can be generated in dark-adapted samples by illumination below the threshold for the formation of S_2, it is not formed by illumination of samples prepared in the S_2 state. It is concluded that the species which gives the g 4.1 signal is involved in the advancement of S_1 to S_2 but not of S_2 to S_3.

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CHAPTER 5

CONCLUSION

5.1 Presentation of a Model

The results presented in Chapters 2,3, and 4 are summarized in the model shown in Fig. 5-1; Figure 5-1a represents photosystem 2 when the oxygen evolving complex is in the dark-adapted state S_1, and Figure 5-1b represents the system in the S_2 state. The photosystem 2 reactive components are represented as follows: P_{680} is the primary donor; Q_A and Q_B are the primary and secondary quinone acceptors, respectively; Z is the direct donor to the oxidized primary donor, and gives rise to the EPR transient Signal II_{vf}; Y is the species which gives the light-induced g 4.3 when oxidized; S_1 (Fig. 5-1a) and S_2 (Fig. 5-1b) are states of the manganese-containing oxygen evolving complex; cyt is cytochrome b_{559}; and D is a chlorophyll species which is capable of donating to the reaction center at low temperature. The vertical positioning of the different components is intended to correspond to the reduction midpoint potentials. The rate constants for the electron transfer reactions are denoted k_{ab}, where species a donates an electron to species b.

In the initial light-induced reactions, P_{680} donates an electron to the acceptor Q_A; this electron transfer occurs via the intermediate carrier, I, which is not shown in the model. The reduced

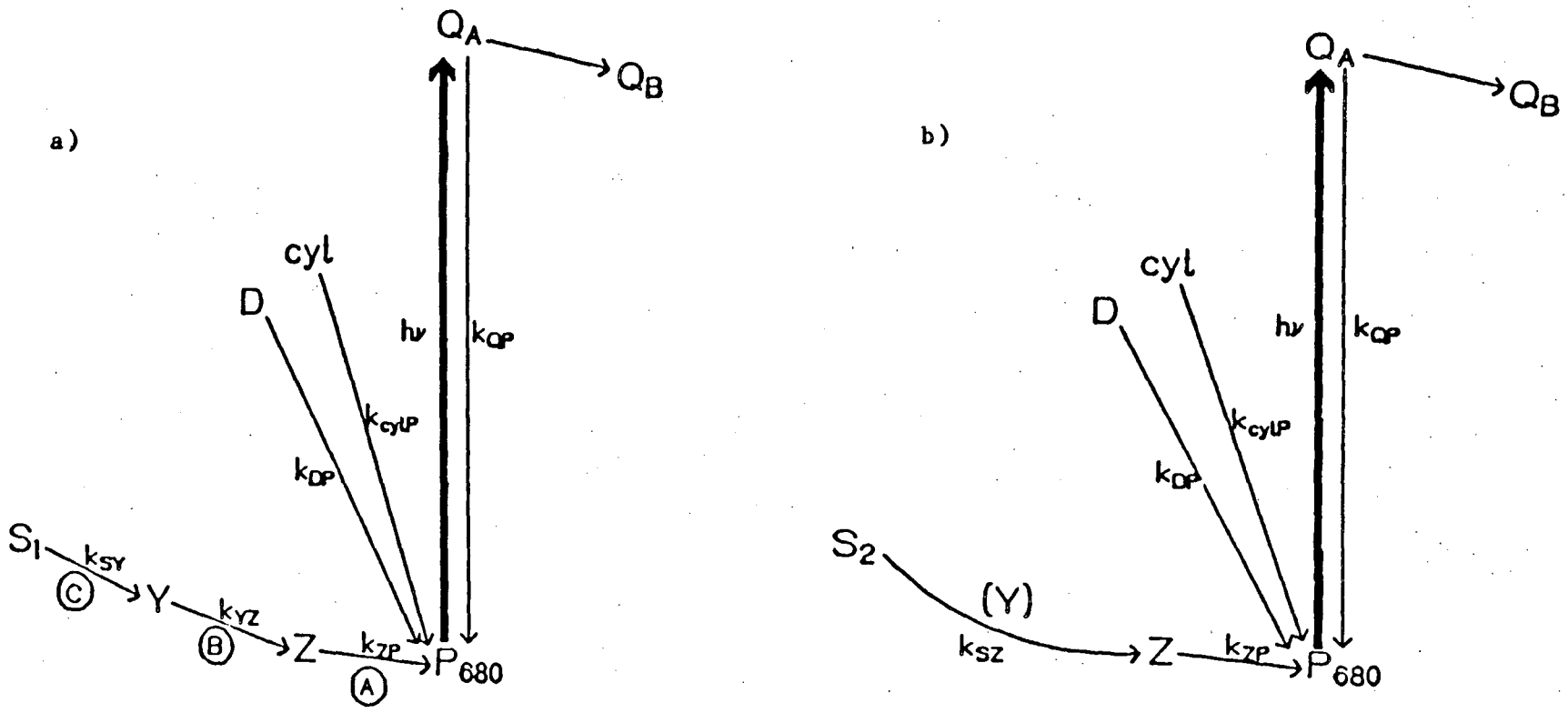

Figure 5

Model for the photochemsitry of photosystem 2. See text for definition of symbols. a) dark-adapted state S_1, b) preilluminated state S_2.

acceptor Q_A^- is reoxidized by the acceptor Q_B in a strongly temperature-dependent reaction, which occurs only very slowly at 200 K [1]. Q_A^- can also reduce the oxidized primary donor P^+_{680}; this process occurs in 200 µs at physiological temperatures [2], and in about 4 ms at liquid nitrogen temperatures [3].

The oxidized primary donor can be reduced by several species; the rates of these reductions are determined by the rate constants k_{QP}, k_{cytP}, k_{DP}, and k_{ZP}. At physiological temperatures, k_{ZP} is the largest of these rate constants, thus P^+_{680} is reduced by Z, the Signal II_{vf} species [4]. It is believed that the equilibrium constant between $Z^{\bullet+}$ and P^+_{680} is close to 1 (see Ref. 5), thus the rate constant k_{YZ}, which determines the rate of reduction of $Z^{\bullet+}$ by Y, must also be greater than the constants k_{QP}, k_{cytP} and k_{DP} in order to withdraw electrons from the oxygen evolving system.

5.1.a The Effects of Reduced Temperature on the First Charge Stabilization in Dark-adapted Preparations

Four temperature regions are apparent in examining the effects of temperature on the donor reactions in dark-adapted preparations: 298 K to 250 K, 250 K to 190 , 190 K to 150 K, and 150 to 100 K. Although the decay of S_1 to S_2 is strongly temperature dependent between 250 and 298 , the forward reaction exhibited no dependence on temperature in this region. Below 250 K, however, effects on the forward donor reactions become significant; these effects are discussed below.

5.1.a.i 250 To 190 K

Results are presented in Chapter 2 which indicate that a charge recombination becomes competitive with the formation of the S_2 state below temperatures of about 250 K; according to the model in Fig. 5-1, the rate constants determined by k_{ZP} or k_{YZ} would become comparable to k_{QP} in this temperature range. At 225 , the forward and reverse rates are approximately equal, because a single flash produces half the maximal amount of the multiline signal, while at 190 K, the rate constant k_{QP} dominates, for no multiline signal is generated by a single flash at 190 K. However, the rate of donation by the oxygen-evolving complex through Y and Z is still faster than that by the cytochrome and the additional donor, D, at 190 K, because continuous illumination at this temperature generates the multiline signal to a full extent.

5.1.a.ii 190 To 150 K

In Chapter 2 it is demonstrated that the amplitude of the mutiline signal produced by continuous illumination decreases as the illumination temperature is dropped from 190 K to 150 K. A decrease in any of the rates k_{ZP}, k_{YZ}, or k_{S_1Y} could yield such a result. The investigations reported in Chapter 3 indicate that it is the rate determined by k_{S_1Y} which is responsible, because it is possible to trap an oxidized intermediated donor, as indicated by the ability to produce the multiline signal by warming to 190 K in the dark after illumination at 150 K. The oxidized intermediate is not the Signal II species Z, because the amplitude of Signal II is not affected by the illumination. The species Y in Fig. 5-1a represents the intermediate

donor; Y^+ gives a broad EPR signal at g 4.1 which had not been previously observed, and may be due to the involvement of non-heme iron in the photochemistry leading to oxygen evolution.

In addition to the decreased rate of donation from the S_1 state, the rate of donation from Y becomes slower between 190 K and 150 K, for donors which are not on the path to water oxidation are observed to be photooxidized in increasing amounts below temperatures of about 170 K. These donors are the species D, which gives a 10 G wide EPR signal at g 2.0026 when oxidized and is most likely a reaction center chlorophyll, and cytochrome b_{559}. The rates of donation from these species, determined by k_{DP} and k_{cytP}, thus become competitive with that of the physiological donors Y and Z, which are determined by the rate constants k_{YZ} and k_{ZP}, near 150 K. The competitive donations from D and the cytochrome may be responsible for the inability to generate the full extent of the multiline signal by warming to 190 K after illumination at 150 K.

5.1.a.iii 150 To 100 K

Below 150 K, the degree of photooxidation of Y becomes progressively lower, indicating that below this temperature the donations from the side-path donors D and cyt begin to dominate the donation from the species on the path to water oxidation. The decreased rate of donation from the physiological path could be due to a decrease in either k_{YZ} or k_{ZP}. The relative contributions of cyt and D to the observed decrease of the light-induced amplitude of the g 4.3 signal below 130 K are not completely clear, but the lack of an effect of chemical oxidation of the cytochrome prior to low

temperature illumination is suggestive that D plays the primary role (see Chapter 4). It should be noted that the extent of the role of the cytochrome as a competitive donor may be greater in chloroplast preparations relative to photosystem 2 preparations due to the presence of a larger amount of high-potential cytochrome in chloroplasts.

5.1.b The Effects of Inhibitors on the Low Temperature Donor Reactions

The effects of inhibitors on the donor reactions were investigated in Chapters 3 and 4. The possible sites of inhibition of the donor reactions in the model are denoted in the diagram by a circled A, B, and C (Fig. 5-1a). Both hydroxylamine and 0.8 M NaCl prevent the oxidation of Y. Hydroxylamine is known to inhibit at site A, as demonstrated by other workers, but 0.8 M NaCl could inhibit at either site A or site B. Substitution of fluoride for choride inhibits by blocking the donation of S_1 to Y, because the g 4.1 signal associated with Y^+, but not the multiline signal associated with the S_2 state, is produced by illumination at 190 K.

5.1.c The System in the S_2 State

The results presented in Chapter 2 indicate that the threshold temperature for the advancement of S_2 to S_3 is higher than that for the advancement of S_1 to S_2. According to the models in Fig. 5-1, the rate of donation of S_2 to Z (or possibly Y) becomes slow at a higher temperature than does the rate of donation of S_1 to Y. In Chapter 4 it is demonstrated that Y is not active as a donor to Z when the oxygen evolving system is in the S_2 state; it is not known whether a

second intermediate donor exists for the S_2 state or if the S_2 state donates directly to Z.

5.2 Outlook for Future Investigations of the Oxygen Evolving Complex of Photosystem 2

The mechanism of positive charge accumulation in photosystem 2 has been of great interest for some time. The involvement of manganese has been documented extensively, although the exact nature of the role of this element in the S-states of the Kok scheme for the photosynthetic oxidation of water is still unknown. The recent observation of the multiline EPR signal associated with the S_2 state was a significant advance, demonstrating conclusively the involement of manganese in the oxygen evolution system. Now that this multiline EPR signal has been confirmed to arise from the S_2 state and the conditions for generation and observation have been determined, additional magnetic resonance techniques and optical methods may be employed to determine the detailed structure of the complex.

The observation of the multiline signal has provided us with the opportunity to monitor directly at least a part of the reactions of the oxygen evolving complex. The temperature dependence of the photogeneration of this signal was useful in determining conditions for generating and trapping an intermediatiate state S_1*. This state gives a previously unreported signal at g 4.1 which, unlike the multiline signal, has no apparent hyperfine structure. While little evidence has been presented in the past concerning the involvement in oxygen evolution of transition metal ions other than manganese, the signal at g 4.1 raises the possibility that non-heme ferric iron plays

a role.

Both the chemical nature of the intermediate state and the functional role in oxygen evolution of the species involved in the intermediate state remain to be determined. Perhaps the best method for identifying the transition metal ions in the complex and exploring their function is x-ray absorption spectroscopy. The position and shape of the K-edge is sensitive to both oxidation state and coordination changes. Observation of the iron and manganese edges under appropriate conditions should allow a definitive determination of the transition metals involved in the intermediate state. A possible complication for the detection of changes in the iron edge is the presence of a ten-fold excess of apparently non-functional iron.

Further EPR studies will also provide more information on the nature of the intermediate state. Confirmation of the S=5/2 origin of the signal by the observation of other spectral features, particularly near g 9, should be possible by using combinations of very concentrated preparations, pumped liquid He temperatures, and oriented samples. The temperature and orientation dependence of the EPR signal could yield information on the energetics and structure of the complex.

In addition to further characterizations of the intermediate species by EPR, it is important to search for optical changes at low temperature and at physiological temperatures which correlate with the observed EPR signals. A number of optical absorbance changes in the near UV have been reported, but the relation of these signals to

species in the path to water oxidation is still unclear. Do any of these signals correspond to the intermediate species? The detection of optical signals which correspond to the EPR signals may also provide the ability to conduct kinetic measurements which are not possible in the case of EPR detection.

An additional approach to the nature and function of the precursor is afforded by the effect of F^-, which seems to stabilize the intermediate state. It is not known whether F^- inhibits by binding to the species which is involved; special EPR methods such as Electron Nuclear Double Resonance (ENDOR) or Electron Spin Echo Spectroscopy (ESE) will be useful in approaching this question, as well as in determining the identity of other ligands.

The functional role in oxygen evolution of the species which is responsible for the intermediate state is also yet to be fully determined. The precursor is involved in the advancement of S_1 to S_2 but not in the advancement of S_2 to S_3. The S_0 to S_1 and S_3 to S_4 advancements have not been investigated, mostly because of the difficulty of preparing samples in a nearly pure S_0 or S_3 state. Methods should be developed to overcome the problems of extreme optical densities inherent in using highly concentrated samples, and thus facilitate the preparation of the S_0 and S_3 states. The mechanism by which the precursor species is involved in the S_1 to S_2 transition but not in the S_2 to S_3 transition is a mystery, but is presumably closely related to its function. It is conceivable that the precursor species is involved in the transfer of ligands as well as electrons. Perhaps comparisons of the ENDOR, ESE and EXAFS of the

multiline signal and manganese complex with those of the intermediate state species will provide the information needed to resolve this question.

The effects of illumination at low temperature have been valuable in furthering our understanding of the reactions on the path to water oxidation. These studies can be pursued even further to investigate more fully the light-induced changes in the g 2 region of the EPR spectrum at low temperatures. The behavior of Signal II and a reaction center chlorophyll donor, in particular, warrant additional study.

There are now five EPR detectable species associated with the oxygen evolving complex of photosystem 2: the primary door, P^{+}_{680}; the oxidized direct donor to P^{+}_{680}, $Z^{\bullet +}$ (Signal II); oxidized cytochrome b_{559}; the S_2 state of the oxygen-evolving complex (multiline signal); and the intermediate state S_1* (g 4.1 signal). The behavior of the signals associated with S_2 and S_1* have been explored in this study. It is now important to detail the behavior of these signals, to determine the relation between some of these signals and transient optical signals which have been reported, and to observe EPR and other spectroscopic signals from the other S-states.

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