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STRUCTURE AND FUNCTION OF THE MANGANESE COMPLEX INVOLVED IN PHOTOSYNTHETIC OXYGEN EVOLUTION DETERMINED BY X-RAY ABSORPTION SPECTROSCOPY AND ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

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R.D. Guiles
(Ph.D. Thesis)

April 1988

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Structure and Function of the Manganese Complex Involved
In Photosynthetic Oxygen Evolution Determined by X-ray Absorption Spectroscopy and Electron Paramagnetic Resonance Spectroscopy

Ronald Davis Guiles
Ph. D. Thesis

April, 1988

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This work was supported by the U.S. Department of Energy under contract number DE-AC03-76SF00098
Structure and Function of the Manganese Complex Involved in Photosynthetic Oxygen Evolution Determined by X-ray Absorption and Electron Paramagnetic Resonance Spectroscopy

by Ronald Davis Guiles

Abstract

Water is the terminal electron donor in the linear light-driven electron transport chain used by higher plants, cyanobacteria and green algae to fix carbon dioxide, the first step in the photosynthesis of carbohydrates. Electron paramagnetic resonance spectroscopy (EPR) and X-ray absorption spectroscopy have demonstrated the involvement of a membrane-bound manganese-containing protein complex at the site of water oxidation within the photosystem II (PSII) reaction center. The photosynthetic oxidation of two molecules of water to molecular oxygen is believed to involve five intermediate states, called S-states (S₀ ... S₄), of the oxygen evolving complex (OEC). The discovery of a multiline EPR signal associated with Mn and assigned to the S₂ state has greatly facilitated structural characterization of the Mn complex within the OEC. This thesis contains a description of methods used to cryogenically stabilize PSII preparations suitable for X-ray absorption spectroscopy in the S₁, S₂ and S₃ states as well as a state induced by hydroxylamine resembling the S₀ state of the OEC.

Studies of the Mn K-edges of PSII preparations indicate that a light-induced oxidation of Mn occurring during the S₁ → S₂ state transition corresponds to a formal valence change from Mn(III) to Mn(IV). The absence of a change in the energy and shape of the Mn K-edge of PSII preparations poised in the S₃ state relative to preparation poised in the S₂ state indicates that Mn is not oxidized during the S₂ → S₃ state transition. The light-induced shift to lower Mn K-edge inflection energy observed for PSII preparations containing low concentrations (40 - 60 μM) of hydroxylamine indicates a mechanism of action for hydroxylamine involving a two-electron reduction of the OEC resulting in the production of N₂, two protons and the S₀ state. Based on this hydroxylamine-mediated light-induced shift to lower Mn K-edge energy, it is suggested that the S₀ → S₁ state transition also involves oxidation of the Mn complex.

An analysis of the extended X-ray absorption fine structure (EXAFS) of the Mn complex within PSII preparations poised in the S₁, S₂, S₃ and hydroxylamine-induced
So states indicates that the four manganese present are organised as two di-\(\mu\)-oxo bridged binuclear manganese complexes. An essential component of the analysis of the EXAFS was a parallel analysis of a set of crystallographically characterized multinuclear \(\mu\)-oxo bridged manganese complexes. Based on conclusions drawn from the analysis of the Mn K-edge and EXAFS of PSII preparations cryogenically stabilized in the S-states described above, a model for the mechanism of photosynthetic water oxidation is presented.
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Acknowledgements

I am grateful for the opportunity to thank the many people who have not only made this work possible, but have also made my stay at Berkeley a rich and rewarding experience. First and foremost, I thank my advisors, Mel Klein and Ken Sauer, whose unique combination of talents made this project feasible. Mel’s wide ranging interests and unbounded curiosity was a constant source of inspiration. I will be forever indebted to him for his unshakable confidence in my abilities. I am indebted to Professor Sauer whose unparalleled knowledge and dedication to the field of photosynthesis has enabled him to provide the insights necessary to the effective investigation of a system as marvelously complex as higher plant photosynthesis.

I thank Professor Bill Armstrong and Professor Richard Malkin for reading this manuscript and for providing many useful suggestions. I also wish to thank Professor Armstrong for many useful discussions concerning physical inorganic chemistry in relation to metalloenzymes.

I also consider myself fortunate to have had the opportunity to work with a group of creative young scientists whose infectious enthusiasm for research was a constant source of inspiration. In particular I thank Dave Britt for numerous discussions concerning paramagnetic resonance spectroscopy and for many practical suggestions regarding the design and construction of equipment required for the X-ray work. I am indebted to Dr. Vittal Yachandra for instruction and guidance in the analysis of the X-ray absorption spectra. I also wish to thank Vittal for making those many long nights in that cold dark room endurable. I am especially grateful to Dr. David Goodin for laying the foundation for much of the work described in this thesis. He also taught me the value of that last cappucino just before Cafe Roma closed. I also thank my friend Jean-Luc Zimmermann for many useful discussions regarding the EPR of PSII preparations and for taking the time to read this manuscript. Without his help and support I think much of the work within this manuscript would not have been completed. I am particularly indebted to Dr. Ann McDermott for numerous insightful discussions regarding every aspect of the experimental work described in this thesis. Her creativity and boundless energy (even at 4 AM on the beam line after 6 - 8 hours of trying to find the whiteline) were essential to the success of this work. There were times, however, when I wish she could have slowed her data transfer rate to around 1200 BAUD.
I also thank the other members of the "EXAFS" group who shared my enthusiasm for this unique brand of experimental science. I thank Sue Dexheimer for assistance with the programming, insights on Fourier transforms and other esoteric areas of mathematics and for help with the data analysis and acquisition. I thank Jean-Luc Zimmermann, Vickie Derose, Jim Cole, Dave Britt, Sun Un and Rick Storrs for assistance with the data collection. I also thank Sun for sharing my pride in the art of machining scientific works of art.

I thank Professor George Christou, Professor Karl Wieghardt and Professor Bill Armstrong for providing the crystallographically characterized manganese model complexes which were essential to the analysis of the Mn complex in PSII.

I thank members of the staff at the Laboratory of Chemical Biodynamics for their help and support. I thank Gary Smith and Phil Eggers for help with numerous technical aspects of the work described in this thesis.

I was also delighted to have the opportunity to discuss many aspects of the experimental work with several staff scientists at Lawrence Berkeley Laboratory. I thank Dr. Al Thompson and Dr. Joe Jaklevic for assistance with the X-ray detection systems. I also thank Don Landis and Norm Madden for providing custom built NIM modules on demand or repairing ailing electronics at a moments notice.

I am grateful to the staff at the Stanford Synchrotron Radiation Laboratory for their help and support. I thank Terry Troxel, Britt Hedmann and Glenn Kerr for assistance above and beyond the call of duty.

I am also indebted to many of my fellow scientists who made my stay at Berkeley an enjoyable experience. In particular I would like to thank Neil Blough, John Casey, Ti-Sheng Young, Tom Pratum, Dave Pearlman, Emil Scaffone, Craig Ogata and Bart de Vos for their help, support and for many interesting diversions.

Finally, I thank my wife Maggie for her love, encouragement, for holding down the Fort during difficult times and for bringing three wonderful blessings into the world, Hilary, Johnny and Isaac.
This work was supported by a grant from the National Science Foundation (PCM 82-16127 and PCM 84-16676) and by the Director, Office of Energy Research, Office of Basic Energy Sciences, Division of Biological Energy Conversion and Conservation of the Department of Energy under contract DE-AC03-76SF00098. Synchrotron radiation facilities were provided by the Stanford Synchrotron Radiation Laboratory which is supported by the U.S. Department of Energy, Office of Basic Energy Sciences, and by the NIH Biotechnology Program, Division of Resources.
Abbreviations

chl, chlorophyll;
cyt b_{559}, cytochrome b_{559};
bipy, 2,2'-bipyridine;
BFBT, best fit based on theory;
DCBQ, 2,6-dichloro-p-benzoquinone;
DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea;
DMSO, dimethylsulfoxide;
EPR, electron paramagnetic resonance;
EXAFS, extended X-ray absorption fine structure;
FABM, fine adjustments based on models;
FT, Fourier transform;
MES, 4-morpholineethanesulfonic acid;
HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid;
MLS, multiline signal;
OEC, oxygen-evolving complex;
phen, 1,10-phenanthroline;
PPBQ, phenyl-p-benzoquinone;
PSI, photosystem I;
PSII, photosystem II;
Q_{A} and Q_{B}, primary quinone acceptors of PSII;
Q_{400}, the high potential acceptor of PSII;
tacn, 1,4,7-triazacyclononane.
Chapter 1

Introduction to Photosynthetic Oxygen Evolution

1.1 Oxygen-Evolving Photosynthesis and Aerobic Respiration

Photosynthesis is the fundamental process which, through the conversion of solar energy to useful forms of chemical potential energy, provides the reduced forms of carbon upon which all forms of life depend. The oxygen in the air we breathe and upon which the myriad of aerobic life forms depend is a by-product of this fundamental energy transduction process carried out by higher plants, algae and cyanobacteria. Oxygen-evolving photosynthetic bacteria are believed to have first evolved approximately 2.3 billion years ago (for a review see Schopf, 1978). It is reasonable to speculate that the ability of these organisms to utilize water as a source of electrons, ultimately for the fixation of CO₂ represented a major adaptive advantage for two reasons: 1) the abundance of water freed these photosynthetic bacteria from the limitations imposed by the lower abundance of H₂ and H₂S which were presumably used as the terminal electron donors by their evolutionary predecessors, and 2) one of the products of photosynthesis, oxygen, was probably toxic to the competitive non-oxygenic photosynthetic organisms. The development of oxygen-evolving photosynthesis dramatically altered the course of evolution. Within a few hundred million years of this development, the atmosphere of the earth was transformed from a reducing environment to an oxidizing one and resulted in the formation of the ozone layer which blocked the intense injurious flux of UV radiation. Oxygen-evolving photosynthesis effected a new cycle of biological evolution. With the development of an oxygen atmosphere a new class of organisms evolved which could utilize the products of photosynthesis 18 times more efficiently (based on ATP produced per mole of glucose consumed) than their fermentative predecessors. It is not surprising that these aerobic respiring organisms have come to predominate.

The process of aerobic respiration is precisely the reverse of photosynthesis (Eqn. 1-1). The products of aerobic respiration, CO₂ and H₂O, are the reactants of the photosynthetic reaction. These two complementary reactions which are the basis of the cycle of life are inextricably linked evolutionarily and chemically by molecular oxygen. The process of photosynthesis has been a source of intrigue to chemists since the classical experiments of Priestly who first demonstrated the relationship between
aerobic respiration and photosynthesis in the 1780. However, only recently have we begun to understand the chemical events associated with this light driven process which is now known to involve a membrane bound protein complex which contains manganese.

\[
\text{Photosynthesis} \\
\text{ATR} \\
\begin{array}{c}
\text{CO}_2 + \text{H}_2\text{O} \\
\text{[CH}_2\text{O}]_n + \text{O}_2 \\
\text{(Carbohydrates)}
\end{array}
\text{hv}
\]

1.2 Higher Plant Photosynthesis

1.2.1 Chloroplast Structure and Organization

Higher plant photosynthesis takes place within a highly structured organelle known as a chloroplast. The chloroplast consists of an outer membrane and highly invaginated inner membrane known as the thylakoid lamella (see Figure 1-1.). The architecture of the chloroplast membrane exhibits a remarkable degree of complexity simultaneously optimizing the cooperative interaction of pigment-protein complexes which mediate physical and chemical processes which together constitute the photosynthetic process. The individual processes involved include light absorption, excitation transfer, charge separation, electron transfer and proton translocation.

The thylakoid membrane is structurally differentiated into two distinct morphological regions, the grana stacks and the stroma lamellae. The protein and lipid composition of the thylakoid membranes also are unusual. More than 50% of the membrane composition is protein. More than 70% of the lipids present are diacyl lipids and more than 10% are sulfolipids. Phosphatidyl choline is a minor component, comprising only about 6% of the lipids present. Interestingly, there appears to be a marked lateral heterogeneity in the lipid composition similar to the heterogeneity in protein complexes described below. The appressed regions of the membrane are depleted in acyl lipids and are enriched in chlorophyll relative to the nonappressed regions (Murphy, 1986).

There are three separate types of supramolecular protein complexes essential to electron transport within the membrane. Each of these complexes is comprised of
Figure 1-1. Schematic diagram of the organization and structure of the chloroplast. Also shown is a cartoon of the structure of the thylakoid membrane containing a model of the distribution of protein complexes. Adapted from Anderson, 1982.
several extrinsic and intrinsic polypeptides, which are coded for by nuclear or organelle genes. Two of these supramolecular pigment-protein complexes are associated with photochemical reaction centers which mediate the oxidation of water and the reduction of \( \text{NADP}^+ \). The current model of the linear electron transport from water to the low potential acceptor \( \text{NADP}^+ \) involves the cooperative interaction of these two reaction centers; Photosystem II (PSII) and Photosystem I (PSI). The third intrinsic membrane complex essential to photosynthetic electron transport is known as the cytochrome \( b_6-f \) complex. A detailed description of the intermediate electron carriers contained within these complexes is deferred until the next section. It suffices to note at this point, that the organization of electron carriers within these complexes results in a vectoral flow of electrons across the membrane. In addition, protons are translocated from the outer thylakoid surface to the intrathylakoid space (the lumen). Additional protein-pigment complexes, the light harvesting chlorophyll proteins (LHCPI and LHCPm), do not display any photo-chemical activity and are believed to function as an additional light harvesting assemblies.

We are only just beginning to understand the nature of the interactions between these supramolecular complexes which presumably mediate assembly of the differentiated thylakoid structure and regulation of the process of photosynthesis under different conditions. For example, it has been shown that the surface-exposed regions of LHCP complexes are involved in cation induced stacking \textit{in vitro} (Mullet & Arntzen, 1980; McDonnel & Staehelin, 1980). It has also been suggested that phosphorylation of LHCP complexes may provide a regulatory mechanism whereby plants adapt to different lighting conditions (Allen et al., 1981). Bennet et al. (1980) have observed that the main polypeptides of LHCP complexes are reversibly phosphorylated at threonine residues. Curiously, the redox state of the plastoquinone pool (the mobile electron carriers which mediate electron transfer between PSII and the cyt \( b_6-f \) complex) appear to regulate both phosphorylation of LHCP complexes and the distribution of light energy between the two photosystems.

In addition to the required transverse asymmetry of electron carriers cross the membrane, the distribution of these supramolecular complexes exhibits a distinct lateral heterogeneity. As shown in the chloroplast cartoon (Figure 1-1.), the grana regions of the thylakoid are enriched in PSII complexes and the associated LHCP complex
while the stroma thylakoids are enriched in PSI. In contrast, the intermediate electron transport complex, the cytochrome $b_{6}-f$ complex, is rather uniformly distributed. In addition, it is known that the ATP synthetase complex, $\text{CF}_1 + \text{CF}_0$, is located only on the outer surface of the stroma and grana thylakoids. Evidence of the lateral heterogeneity of these complexes comes from freeze fracture electron microscopy (Staehelin, 1976, Miller & Staehelin, 1976), phase partition fractionation and SDS polyacrylamide gel electrophoresis (Anderson, 1982).

In view of the lateral heterogeneity of the three supramolecular complexes involved in electron transport, it is clear that the universally accepted picture of the linear electron transport scheme (the Z scheme) involving cooperative interaction between the two photosystems is not totally adequate. The varying stoichiometries of these complexes under different environmental conditions supports this assertion. Alternative views have been put forth, suggesting that the two photosystems operate synchronously, in parallel (Arnon, et al., 1981). In this scheme PSI acts in a cyclic manner analogous to the cyclic photophosphorylation scheme known in purple non-sulfur bacteria. Arnon et al. envisioned the two photosystems acting essentially independently, except for the regulatory links, such as the plastoquinone pool, and the soluble electron carriers, plastocyanin and ferredoxin. In the conventional linear electron transport scheme described below, plastoquinone, plastocyanin and the soluble ferredoxin are intermediate electron carriers in a linear electron transport scheme.

1.2.2 Electron Transport in Higher Plant Photosynthesis

The initial events in the transduction of light energy into useful chemical potential can be summarized as follows; absorption of light by a collection of protein bound pigments known as antennae, excitation transfer to a unique pigment or pair of pigments known as a trap (the primary donor), excited state donation of an electron to a low energy acceptor, and stabilization of this charge separation by rapid electron transfer on both the donor and acceptor sides. What follows is a description of the electron transfer components involved in the generally accepted model of the rapid vectoral electron transport events which stabilize the initial charge separation and mediate the removal of electrons from the oxygen evolving complex.

Shortly after the discovery of two separate light reactions (principally the result of
experiments performed by Emerson, 1958), a model of the cooperative interaction of PSII and PSI involving a linear electron transport chain was developed. The essential features of this model are summarized on a plot of increasing midpoint potential of the intermediate electron carriers (See Figure 1-2.). This model, known as the Z-scheme, was first developed by Hill and Bendall (1960).

As described in the previous section, there are three membrane bound protein complexes which are essential to electron transfer in higher plants; photosystem II, the cytochrome $b_6-f$ complex and photosystem I. A pool of quinones, Q, is believed to mediate electron transfer between PSII and the cyt $b_6-f$ complex. The soluble blue copper protein, plastocyanin (Pc), mediates electron transfer between the cyt $b_6-f$ complex and PSI.

The donor side of PSII consists of a series of bound high potential electron carriers which act as intermediates between the terminal donor, water, and the primary donor $P_{680}$. $P_{680}$ is thought to be a chlorophyll dimer. The identity of the intermediate Z, long thought to be a plastoquinone, has recently been shown to be a tyrosine residue (Barry & Babcock, 1987). Several lines of evidence implicate manganese as the principal redox active component of the OEC. A detailed discussion of the evidence indicating the role of manganese in the storage of oxidizing equivalents is deferred until section 1.4.1.

The acceptor side of PSII consists of a pheophytin (the primary acceptor, often called the first intermediate acceptor, I) and a pair of plastoquinones, $Q_A$ and $Q_B$. $Q_A$ is a tightly bound plastoquinone which acts as a one-electron carrier between I and $Q_B$. On the other hand $Q_B$ is a loosely bound two electron acceptor which dissociates upon being doubly reduced and protonated to the quinol form, only to be replaced by another plastoquinone from the lipophilic pool (Crofts & Wraight, 1983). The electron transfers on both the donor and acceptor side of PSII occur on a very fast timescale. The transfer from $P_{680}$ to I occurs in a few picoseconds. The electron transfer from I$^-$ to $Q_A$ occurs in about 200 ps (Nuijs et al., 1986). The electron transfer from $Q_A$ to $Q_B$ occurs in about 100 $\mu$s (Robinson & Crofts, 1983). On the donor side, the shuttling of electron holes also occurs on a fast timescale. Reduction of $P_{680}^+$ by Z occurs in 50 - 250 ns (Van Best & Mathis, 1978). Finally, S state donation to $Z^+$ occurs on the order of 0.5 - 1 ms (Babcock et al., 1976). These events kinetically stabilize the initial charge
Figure 1-2. A diagram of the linear electron transfer model involved in higher plant photosynthesis, known as the Z-scheme. Electron transfer components are plotted on a scale of increasing midpoint potential. In addition to the normal electron transfer components, the non-physiological donor cyt b_{559} and the non physiological acceptor Q_{400} are indicated. For a more detailed description, see the text.
separation.

PSI produces powerful reductants which ultimately provide reducing equivalents used in the fixation of CO₂. Based on the line width of the EPR signal associated with \( \text{P}_{700}^+ \), the primary donor is thought to be a chlorophyll dimer (Norris et al., 1971). \( \text{A}_0 \), the primary acceptor is thought to be a modified chlorophyll monomer. Recent EPR and optical evidence indicates that \( \text{A}_1 \) may be a phylloquinone (Thurnauer et al., 1987; Brettel et al., 1987). The low potential acceptors \( \text{A}, \text{B} \) and \( \text{X} \) are all bound iron sulfur centers. \( \text{A} \) and \( \text{B} \) exhibit optical and EPR spectra typical of \( 4\text{Fe}4\text{S} \) centers. \( \text{X} \) is unusual in its EPR properties, and a recent EXAFS analysis is suggestive of a pair of \( 2\text{Fe}2\text{S} \) centers as the probable structure (McDermott et al., 1987 a,b).

1.3 Oxygen Evolution

The process of water oxidation carried out by higher plants is quite remarkable for several reasons. It is surprising that oxidative equivalents of sufficient positive potential to oxidize water can be stabilized within a protein matrix. The mean reduction potential necessary for the four-electron oxidation of two molecules of water to molecular oxygen at pH 7 is 815 mV (Eqn. 1-2).

\[
2 \text{H}_2\text{O} \rightarrow \text{O}_2 + 4 \text{H}^+ + 4 \text{e}^- \quad \text{E}_m(\text{pH 7}) = 815 \text{ mV}
\]

Further, since the electron transfer steps from the oxygen evolving complex to the primary donor are downhill energetically, the donors \( \text{Z} \) and \( \text{P}_{680} \) must have midpoint potentials in excess of +815 mV. The midpoint potential of \( \text{P}_{680} \) is estimated to be +1000mV. In fact, under nonphysiological conditions, non-specific photo-oxidation of chlorophyll or carotenoid molecules is observed (dePaula et al., 1985). Thus, it has been suggested that the stabilization of these highly oxidizing equivalents is probably a kinetic phenomenon. The process of photosynthetic oxygen evolution is further complicated by the fact that the oxidation of water is a four-electron process. The mechanistic details of the coupling of the one-electron photochemistry of the primary pigment to this four-electron oxidation remains a matter of some controversy. Based on the pattern of protons released during the process of water oxidation, it has been suggested that a concerted four-electron oxidation of two molecules of water is unlikely. However, it should be pointed out that the release of protons during catalytic steps in a redox enzyme does not necessarily indicate partial oxidation of the substrate. Changes in the
basicity of protein derived ligands to a metal center undergoing an increase in valence can result in the release of protons (Stiefel et al., 1977). Based on an examination of the free energies of various possible one-electron oxidation intermediates, two two-electron oxidations or a concerted four-electron process have been suggested to be energetically more favorable (Krishtalik, 1986). As yet no stable intermediates have been detected. Thus there is no direct evidence precluding a concerted four-electron process occurring in the final step.

1.3.1 The Basis of the Current Phenomenological Model of Oxygen Evolution

A significant advance in our understanding of the mechanism of oxygen evolution occurred with the observation of non-uniform pulses of oxygen released from dark adapted Chlorella suspensions which were subjected to a series of short (< 1μs) flashes of light (Joliot et al., 1969). Kok et al. (1970) found (based on similar measurements of dark-adapted chloroplast preparations from spinach) that the yield of oxygen evolved oscillated with a periodicity of four. Based on this remarkable observation, Kok et al. (1970) proposed a model of independently reacting centers in which a donor complex, known as the oxygen evolving complex (OEC), cycled through five intermediate states in a linear light-driven four-step process. Cycling of these five intermediate states, called S-states, $S_0$ - $S_4$, resulted in the oxidation of water and the release of oxygen. The details of this cyclic process are depicted in a model now known as the S-state clock (shown in Figure 1-3.). Based on the observation that the first maximum flash yield of oxygen occurred on the third flash, the $S_1$ state was presumed to be the dominant state present in dark-adapted thylakoid preparations. The $S_2$ and $S_3$ states were assumed to decay to the $S_1$ state in the dark. The $S_0$ state, although more stable than the $S_2$ and $S_3$ states, has recently been shown to undergo slow conversion to the $S_1$ state in the dark (Styring & Rutherford, 1987). The $S_4$ state is a transient intermediate which decays to the $S_0$ state concurrent with the release of oxygen. The $S$ states, $S_0$ - $S_3$ are relatively long-lived (a half time of decay on the order of a minute for the higher states at room temperature) oxidation states of the OEC. In the original formulation, the chemical identity of the terminal donors associated with these states remained unspecified. This relatively simple model has been the primary basis of a large body of physical and biochemical experiments attempting to elucidate the nature of the chemical events involved in the water oxidation process.
Figure 1-3. The current phenomenological model of oxygen evolution, known as the S-state clock. For details, see text.
1.3.2 EPR of Electron Transfer Components of PSII

Recent advances in our understanding of the electron transfer components and cofactors necessary for photosynthetic oxygen evolution are largely the result of physical experiments performed on PSII preparations obtained by selective Triton X-100 solubilization of PSI from chloroplast suspensions (Babcock et al., 1981; Kuwabara & Murata, 1981). Among the physical techniques used to study electron transfer components in PSII, low temperature EPR has been one of the most useful.

The number of electron donors between P₆₈₀ and the OEC has been reviewed by Bouges-Bocquet (1980). There is direct evidence for only one intermediate, Z, under physiological conditions. However, under non-physiological conditions, light induced-generation of EPR signals associated with cyt b₅₅₉, carotenoid or chlorophyll indicates that these species also donate to P₆₈₀ (Malkin & Vanngård, 1980; dePaula et al., 1985). The oxidized form of Z has been identified as an organic radical exhibiting an EPR signal identical to that of the well known PSII radical, D⁺, which gives rise to the so-called signal II, our EPR signal (Babcock & Sauer, 1975). Unlike P₇₀₀, the primary donor in PSII, P₆₈₀, is difficult to trap in the oxidized form. However, the spin-polarized triplet of P₆₈₀ has been detected and its orientation dependence relative to the membrane plane has been suggested to be due to a parallel orientation (Rutherford et al., 1981). The OEC also exhibits characteristic EPR signals in the S₂ state. The discovery of the multiline EPR signal (described in detail in section 1.3.2) has greatly advanced our understanding of the specific chemical species involved in water oxidation.

Components on the acceptor side have also been associated with EPR signals at low temperature. Rutherford and Zimmermann (1984) assigned broad signals in the g=1.82 and 1.9 regions of the EPR spectrum recorded at 4.2 K with the reduced form of the primary acceptor, Fe²⁺QA. Recently, based on a combination of Mössbauer and EPR evidence, Petrouleas and Diner (1986) have associated the non-physiological, high-potential acceptor, QA, with the oxidized form of the high-spin iron associated with the acceptor complex. Thus, virtually all of the electron carriers of PSII exhibit characteristic EPR signals which are convenient indicators of the redox state of each component.

1.3.3 The Chemical Identity of the S-states
An abundance of physical and biochemical evidence demonstrates the involvement of manganese in the storage of oxidative equivalents during the cycle of oxygen evolution (for a review see Babcock, 1987). EPR and X-ray absorption spectroscopy have proven to be some of the most useful spectroscopic tools in demonstrating the involvement of manganese. The first direct evidence came with the discovery of a light-induced multiline EPR signal assigned to a multinuclear manganese complex based on its characteristic hyperfine structure (Dismukes & Siderer, 1981). The discovery of the multiline EPR signal was significant for three reasons: (1) This was the first evidence that the intermediates in the S-state cycle could be cryogenically trapped. (2) It provided a signal which was characteristic of one of the S-states. (3) It clearly associated manganese with the process. The fact that the signal maximized on the first flash and thereafter on every fourth flash, clearly associated the species giving rise to this signal with the $S_2$ state. The remarkable similarity of the 16 line spectra of dimeric di-$\mu$-oxo bridged manganese complexes characterized only three years earlier strongly supported the assignment of a manganese complex (Cooper et al., 1978). These results were also in line with the analysis of the extended X-ray absorption spectra (EXAFS) of the active pool of manganese in chloroplast preparations (Kirby et al., 1981) which indicated the presence of $\mu$-oxo bridged manganese.

Simulations of the 19 - 22 hyperfine line spectrum observed in chloroplasts indicated that a Mn$_2$(III, IV) dimer (Dismukes & Siderer, 1982), a Mn$_3$(II, III) dimer or a tetranuclear Mn$_4$(III,III,II,IV) cluster are suitable models of the EPR active manganese within the OEC. Methods of cryogenically trapping the $S_2$ state characterized by the multiline EPR signal were developed using a low-temperature illumination procedure (Brudvig et al., 1983). The temperature dependence of the formation and decay of the multiline EPR signal confirmed its assignment to the $S_2$ state. This low temperature illumination procedure allowed the preparation of concentrated PSII samples poised in various S-states and suitable for study by X-ray absorption spectroscopy.

A significant result was obtained through an examination of changes in the X-ray absorption edges of PSII preparations poised in the $S_1$ and $S_2$ states. A dramatic shift to higher energy was observed for the $S_2$ state relative to $S_1$ state preparations. This was the first direct evidence of the stabilization of oxidative equivalents on manganese during an $S$ state transition. More recent studies of PSII preparations poised in the $S_1$
and \( S_2 \) states largely confirm our earlier reports of a \( \mu \)-oxo bridged core structure and indicate that the structure of the manganese complex is largely unchanged in advancing from the \( S_1 \) to the \( S_2 \) state (Yachandra et al., 1987).

### 1.3.4 The Mechanism of Oxygen Evolution

Many models of the mechanism of water oxidation have been proposed (e.g. see Govindjee et al., 1977; Renger, 1977; Lawrence & Sawyer, 1978; Wydrzynski & Sauer, 1980; Renger & Weiss, 1983; Goodin et al., 1984; Hansson et al., 1984; Kambara & Govindjee, 1985; Brudvig & Crabtree, 1986). All of these proposed mechanisms include manganese as the binding site of the substrate water (or hydroxide) at some point during the catalytic cycle. As indicated above, the evidence for the role of manganese as the site of stabilization of oxidizing equivalents during at least one of the \( S \)-state transitions is now compelling. The evidence indicating that manganese is the site of binding of the substrate water is less convincing.

The assertion that the substrate water binds to manganese is based on two lines of evidence. Several low molecular weight "water analogs" (e.g. ammonia, hydroxylamine, hydrazine and hydrogen peroxide) are known to inhibit oxygen evolution or alter the normal pattern of oxygen evolved upon a series of short saturating flashes of light. For example, ammonia in the unionized form is believed to bind to manganese (Sandusky & Yocum, 1983, 1984, 1986). These reports are substantiated by the observation of an altered multiline EPR signal in the presence of ammonia (Beck et al., 1986). The changes in the fine structure of the multiline EPR signal upon inhibition of oxygen evolution by ammonia indicate that ammonia binds to the EPR-active Mn present in PSII preparations poised in the \( S_2 \) state.

Another class of small molecules does not inhibit oxygen evolution but causes a two step lag in the normal pattern of oxygen evolved and protons released. This class of reducing "water analogs" includes hydroxylamine, hydrogen peroxide and hydrazine. A detailed discussion of the effect of hydroxylamine, hydrazine and hydrogen peroxide is deferred to Chapter 6. Evidence of water binding at manganese also comes from studies of the multiline EPR spectrum using isotopically substituted water. Hansson et al. (1986) have claimed that line broadening of the multiline EPR signal upon \( H_2^{17}O \) exchange indicates that water binds to the manganese complex in the \( S_2 \) state. Similarly,
Nugent (1987) claims that line narrowing of the multiline signal observed upon $^2$H$_2$O substitution indicates water binding in the S$_2$ state.

Chloride is an essential cofactor for oxygen evolution and has been suggested to be a ligand to manganese (for a review, see Critchley, 1985). Evidence of chloride binding in different S-states comes from $^{35}$Cl NMR line broadening experiments (Preston & Pace, 1985). This has been taken as evidence of binding of Cl$^-$ to manganese during the S$_2$ and the S$_3$ states. Depletion of chloride appears to block the S-state cycle at the S$_2$ state. This is indicated by the fact that Cl$^-$ is required for the formation of the S$_2$ state multiline EPR signal (Yachandra et al., 1986a). More recent studies indicate that an altered, more stable S$_2$ state may be formed in depleted preparations, but advancement to the S$_3$ is blocked (Ono et al., 1986). A number of mechanisms of oxygen evolution involving direct ligation of chloride to manganese have been proposed. Sandusky and Yocum (1983) have suggested that Cl$^-$ may act to facilitate electron transfer between manganese atoms during turnover. Alternatively, it has been suggested that chloride acts as a charge stabilizing counter ion (Kambara & Govindjee 1985). It is interesting to note that bromide may be substituted for chloride in active O$_2$-evolving preparations (Kelley & Izawa, 1978). A comparison of the superhyperfine structure of the multiline EPR signal observed in PSII preparations containing bromide and chloride indicate that chloride is not a ligand to manganese in the S$_2$ state (Yachandra et al., 1986a). A more detailed discussion of these results is contained in Chapter 3. EXAFS studies of the PSII preparations in the S$_1$ and S$_2$ states also indicate that chloride is not a ligand to manganese (Yachandra et al., 1987, also unpublished results regarding the EXAFS of PSII preparations exchanged with bromide and poised in the S$_2$ state). Thus, most current models of the mechanism of water oxidation do not include chloride as ligand to manganese.

All of the models of the mechanism of water oxidation include at least two, and up to four, manganese atoms. This is based partially on the generally accepted stoichiometry of four Mn per PSII reaction center, and partially on the simulations of the multiline EPR signal.

Two of the models recently proposed contain a redox active ligand involved in the stabilization and storage of oxidative equivalents (Goodin et al., 1984; Kambara & Govindjee, 1985). Although there is no direct spectroscopic evidence for this, the
concept is given some support from the observation of reversible redox equilibria in inorganic complexes between quinones and the higher valence manganese atoms to which they are ligated (Lynch et al., 1984).

The model proposed by Crabtree & Brudvig is particularly appealing for a variety of reasons. This model, shown in Figure 1-4, depicts the structure of the manganese complex in each of the S-states in terms of discrete \( \mu \)-oxo bridged manganese complexes involving all four of the manganese present in PSII. The model is based principally on EPR studies of the \( S_2 \) state and chemical reasoning. The principal basis of the cubane-like structure proposed for the \( S_0 \), \( S_1 \) and \( S_2 \) states is the observation of non-Curie like behavior of the multiline EPR signal. An analysis of this temperature dependence indicated that the multiline signal is an excited state spin 1/2 system and that the ground state is a spin 3/2 system. These properties were best accounted for by assuming a tetranuclear cluster of manganese atoms.

Formation of the peroxy bond has been suggested to be a major thermodynamic barrier in the process of water oxidation. In the final step of the mechanism proposed by Brudvig and Crabtree, a substantial rearrangement of the manganese complex occurs which facilitates the formation of a peroxy bond and release of \( O_2 \) in a thermodynamically favorable process involving an intramolecular nucleophilic displacement. Further, discrete chemical models of the proposed structure for the \( S_3 \) state actually exist (Wieghardt et al., 1983). This proposed mechanism is given substantial support by the fact that analogous chemistry is well documented for tetranuclear iron sulfur clusters (Hagen et al., 1981). Hagen et al. found that the reverse reaction (conversion of an adamantane-like iron sulfur cluster to a cubane-like cluster) could be oxidatively mediated by elemental sulfur.

Recently, however, the basis for the proposal of a cubane-like structure for the manganese complex in the \( S_2 \)-state has been questioned. The non-Curie like behavior of the \( S_2 \)-state multiline signal has not been reproduced by two other laboratories (Hansson et al., 1987; Britt, Zimmermann & Klein personal communication). Further, based on our EXAFS analysis of the \( S_1 \) state, we have suggested that the manganese present in PSII is organised as two separate dimers or a single dimer and two monomers (Yachandra et al., 1986a).
Figure 1-4. A proposed mechanism for photosynthetic oxygen evolution. Reproduced from Brudvig and Crabtree, 1986. See text for details.
1.4 Thesis Scope and Format

The body of work presented in this thesis has been directed at obtaining structural information about the manganese complex contained within the OEC of PSII. Differences in the structure of the manganese complex were examined in PSII preparations cryogenically stabilized in several intermediate states of the catalytic cycle in an attempt to deduce details of the mechanism of action. EPR and X-ray absorption spectroscopy were the two principal tools used to explore structural features of the manganese complex. As described in Chapter 3, EPR was used not only as a tool to establish state composition and integrity of the PSII preparations for use in the X-ray absorption experiments, but also as a structural tool, probing the relationship between manganese and the chloride cofactor. An essential feature of the analysis of the Mn X-ray absorption spectra of the PSII preparation has been a comparative analysis of an extensive list of inorganic manganese complexes, spanning a relevant range of oxidation states and nuclearities (described in Chapters 4 and 5). Chapter 6 contains a systematic evaluation of differences between the structure of the manganese complex cryogenically stabilized in the hydroxylamine-induced $S_0$ state and the $S_1$ state of Kok's catalytic scheme. Chapter 7 presents a method of cryogenically stabilizing PSII preparations in the $S_3$ state suitable for X-ray absorption studies, together with a comparative analysis of the X-ray absorption spectrum of the manganese cluster in this state and the $S_2$ state. Finally, Chapter 8 contains a synthesis of the conclusions drawn from these experiments in the form of a model for the accumulation of oxidizing equivalents in the OEC of PSII. Also presented is a speculative model of the manganese complex together with a discussion of changes in that structure which may occur during the course of catalysis.
Chapter 2
An Introduction to X-Ray Absorption Spectroscopy

2.1 Introduction

The development of X-ray absorption spectroscopy as a practical tool occurred concurrently with the development of synchrotron radiation sources. Although X-ray absorption spectra of concentrated, in most cases crystalline, samples were recorded using conventional X-ray sources (e.g. X-ray tubes and rotating anodes) as far back as 1920 (Kossel, 1920), our current understanding of the phenomena associated with structure in the X-ray absorption spectrum is largely the result of high quality spectra obtainable only with the high fluxes of X-rays provided by electron storage rings. In fact, prior to 1970 it was not known whether the fine structure extending as much as 1000 eV beyond the X-ray absorption edge was due to interference effects due to extended order in a crystalline matrix (Kronig, 1930), much like X-ray diffraction, or a short range photoelectron scattering phenomenon consistent with our current theoretical understanding (Sayers, Lytle & Stern, 1971). Thus, in light of the importance of the development of synchrotron radiation to X-ray absorption spectroscopy as well as many other current and potential applications, it is not surprising that circular electron accelerators, such as synchrotrons (machines that briefly accelerate electron beams to high energy) and storage rings (machines that maintain a high energy electron beam circulating for hours), are now operated fully or partially as dedicated synchrotron sources serving a community of more than 5000 investigators worldwide. What follows is a brief description of the generation and properties of synchrotron radiation. For more comprehensive reviews, see Winick (1980, 1987).

2.1.1 Synchrotron Radiation: Sources and Properties

Acceleration of a charged particle results in emission of electromagnetic radiation. Non-relativistic charged particles emit radiation isotropically. However, when a charged particle is accelerated to relativistic velocities, the emission properties change radically. Both the total flux and the directionality increase substantially. The radiation emitted from a charged particle moving at relativistic velocities is emitted in a narrow cone which sweeps out a path tangential to the direction of motion. The divergence of the emission cone is a function of the energy of the emitted radiation. A smaller divergence
angle results for radiation closer to the critical energy (See Figure 2-1.). Beyond the critical energy, the X-ray flux drops very rapidly. The critical energy, $\varepsilon_c$, of emitted radiation from an electron of energy, $E_e$, accelerated in a path with a radius of curvature $\rho$ is given by

$$\varepsilon_c = \frac{(3\hbar c(E_e/mc^2)^2)}{2\rho}$$

where $m$ is the electron rest mass; $\hbar$ and $c$ are Planck's constant divided by $2\pi$ and the speed of light, respectively.

For a 3.0 GeV electron beam moving in a circular path with a radius of 17.7 m (the characteristics of normal operation on a bending magnet at the Stanford Synchrotron Radiation Laboratory (SSRL)), the critical energy is 7.4 KeV. The brilliance of synchrotron radiation as a function of X-ray energy is plotted in Figure 2-1. The brilliance of X-rays is the total flux at a given energy divided by the angle of divergence and is a measure of the effective concentration of X-rays of a given energy. Such curves are of importance to the experimentalist in determining factors such as the flux of harmonics relative to the fundamental frequency of interest. The presence of a substantial fraction of harmonic X-rays can result in distortion of X-ray absorption spectra. The angle of divergence is also significant in that it limits the power transmitted through the monochromator at a given energy (described in detail in section 2.3). At the critical energy, the angular divergence of the beam is given by $\theta = mc^2/\varepsilon_c$. For a 3.0 GeV electron beam, the divergence angle is $1.7 \times 10^{-4}$ rad.

The intense flux of X-rays generated by the flow of electrons in storage rings, which were initially constructed for colliding beam experiments performed by high energy particle physicists, were at first considered a hazardous nuisance. However, as the utility of this high flux source of X-rays as a spectroscopic tool became apparent, methods of increasing the total flux were developed. Magnets, called wigglers, were developed which cause the beam of electrons to oscillate in a sinusoidal path in the plane of orbit. The effect of these many sinusoidal deflections (e.g. direction changes with small radii of curvature) is a substantial enhancement of X-ray flux over what is possible with a bending magnet. The enhancement is roughly twice the number of full oscillations. For comparison, the brilliance of X-rays emitted from a wiggler magnet is also plotted in Figure 2-1. The high fluxes obtainable from such devices made possible
Figure 2-1. A plot of the brilliance of X-rays produced by bending and wiggler magnets at the Stanford Synchrotron Radiation Laboratory (SSRL). Also shown is a cartoon of the emission from a bending magnet and the electron beam path through a bending magnet and a wiggler magnet. Adapted from Bienenstock, 1980. See text for details.
The graph shows the relationship between photon energy (KeV) and photons per second (mrad⁻¹ mA⁻¹) for different magnetic configurations. Two curves are plotted, one for the \( \xi_c \) Wiggler Magnet and another for the \( \xi_c \) Bending Magnet. The x-axis represents the photon energy in KeV, ranging from 0.01 to 100, while the y-axis represents the number of photons per second, ranging from \( 10^{11} \) to \( 10^{14} \).
many of the measurements of X-ray absorption spectra of samples as dilute as 700 \( \mu \)M in the element of interest.

Other properties of synchrotron radiation which are of interest to experimentalists are its high polarization in the plane of motion and its discrete time structure. For example, the high polarization of the X-ray beam permits the assignment of specific transitions in X-ray absorption experiments with single crystals of known structure (Templeton & Templeton, 1980, 1982; Hahn et al., 1983). The pulsed time structure of synchrotron radiation is a consequence of the injection of electrons into the storage ring in discrete bunches separated by well defined intervals. This time structure allows measurements of time resolved fluorescence spectra over an extremely broad range of wave lengths (0.1 nm to \( \sim 1 \) cm).

2.2 Regions of the X-Ray Absorption Spectrum

The use of X-ray absorption spectroscopy is rapidly expanding as a tool in the investigation of electronic structure and the local coordination of specific atoms in biological molecules (for a review, see Powers, 1982). One significant aspect of this spectroscopic tool is its high elemental specificity. The X-ray absorption edges of the elements are in general well separated in energy and, with the advent of high flux synchrotron radiation sources, the spectroscopic properties of individual elements may be easily examined free from the interference of other elements present.

X-ray absorption spectroscopy has been demonstrated to be particularly useful in probing the structure of the metal sites in metalloenzymes. As shown in Figure 2-2, there are two basic regions of the X-ray absorption spectrum: the edge region and a region extending as much as 1000 eV above the edge, known as the extended X-ray absorption fine structure (EXAFS) region. The information obtainable from these two regions is complementary. The edge region contains a number of bound state transitions, superimposed on the continuum edge jump. The energy of the edge inflection can be used to determine the approximate valence of the metal center (Kirby et al., 1981a and Chapter 4). An interpretation of the EXAFS region yields information regarding the number, the distance and the identity of atoms adjacent to the absorbing atom. The edge region also contains information about the symmetry of the metal center. A low intensity transition, just below the edge jump, has been assigned to a 1s \( \rightarrow \) 3d
transition for the first row transition metals (Yafet et al., 1976). The amplitude of this formally forbidden transition indicates the degree of distortion from a centrosymmetric structure. For example, tetrahedral complexes exhibit more intense $1s \rightarrow 3d$ transitions than octahedrally coordinated ions (Roe et al., 1984). A more comprehensive discussion of the intensity and structure of the $1s \rightarrow 3d$ transition as it relates to the electronic structure of the higher valences of manganese is presented in Chapter 5.

2.2.1 Extended X-Ray Absorption Fine Structure (EXAFS)

The EXAFS region of the X-ray absorption spectrum extends from about 70 eV past the ionization threshold to more than 1000 eV past the edge. Our present understanding of the nature of these broad oscillations in the X-ray absorption cross-section stems from the work of Sayers et al. (1971). Several comprehensive derivations of the equations which accurately describe the photoelectron scattering phenomenon which causes this structure have been published. The reader is referred to one of these works for a more formal discussion of the theory (Ashley & Doniach, 1975; Lee, & Beni, 1977; Lee et al., 1981). What follows is a more general discussion of the equations describing the phenomenon, in sufficient detail to allow an understanding of the limitations of the simulation procedures used to derive structural information from EXAFS spectra.

EXAFS is due to a single-electron scattering phenomenon. The interference between the outgoing photoelectron wave with waves backscattered by the potential fields of neighboring atoms results in a modulation of the X-ray absorption cross-section. Constructive interference results in an enhanced absorption cross-section while destructive interference diminishes it. These oscillations are sinusoidal in form when expressed as a function of the photoelectron wavevector amplitude, $k$. The photoelectron wavevector increases with increasing incident X-ray energy and is related to the wavelength of the photoelectron, as shown in Eqn. 2-2. EXAFS oscillations due to backscattering from atoms at a fixed distance vary sinusoidally with a frequency equal to twice the distance.

$$k = \left[2m(E - E_o)\right]^{-1/2} / \hbar = 2\pi / \lambda$$

where $m$ is the electron rest mass, $E$ is the energy of the X-ray photon, $E_o$ is the ionization energy for a $1s$ core electron and $\lambda$ is the De Broglie wavelength of the photoelectron.

The analytical expression for the EXAFS, $\chi(k)$, due to backscattering from $N_f$
Figure 2-2. Regions of the X-ray absorption spectrum. Bound state transitions in the edge region are schematically represented as transitions from the 1s core level to unoccupied Rydberg atomic levels of the Mn ion. See text for details.
neighboring atoms of the jth type at a distance \( r_j \) is given by Eqn. 2-3.

\[
\chi(k) = \sum_j N_j S_j F_i(\pi,k) \frac{e^{-2\sigma_j^2 k^2}}{kr_j^2} e^{-2r_j/\lambda_j} \sin(2kr_j + \alpha_{ij}(k))
\]

In this expression, \( F_j(\pi,k) \) is the backscattering amplitude from the jth atom; \( \sigma_j \) is a Debye-Waller term which takes into account both thermal and static disorder in the distribution of atoms at a distance \( r_j \); \( \lambda_j \) is the mean free path (not to be confused with the photoelectron wavelength in equation 3-2); \( S_i \) is an amplitude reduction factor due to many-body effects, such as a shake-up or shake-down processes (described in greater detail in Chapter 4) occurring at the absorbing atom (denoted by i) and \( \alpha_{ij}(k) \) is the total phase shift experienced by the photoelectron. The observed EXAFS spectrum is the sum of all backscattering effects from the j different types of atoms at distances \( r_j \). A collection of atoms at a given distance is referred to as scattering shell.

The frequency of an EXAFS wave is determined solely by the distance to a given scattering shell. The phase shift, \( \alpha_{ij}(k) \), results from the effect of the movement of a charged particle (the photoelectron) in the potential fields of both the central absorbing atom and the backscattering atoms. The envelope shape of these sinusoidal waves is characteristic of the absorber-scatterer pair. The amplitude of a given wave is determined principally by the number of equivalent scatterers; however, there are several amplitude reduction factors which affect both its total amplitude and shape. The amplitude depends exponentially on the degree of disorder present in a given shell, and thus dramatic amplitude reductions can occur with increasing disorder; for example, with increasing temperature (Brown & Eisenberger, 1979). The affect of the mean free path is less pronounced; however, this ultimately limits the range of the technique. The amplitude reduction term, \( S_i \), is a fixed scaling factor which is mainly dependent on the nature of the absorbing atom. The presence of two or more EXAFS waves, due to scattering from atoms at different distances, strongly affects the overall envelope shape of the composite spectrum because of difference or "beat" effects.

### 2.2.2 Methods of Analysis

The normalized X-ray absorption cross-section, \( \mu(E) \), is obtained by extrapolating a smoothed polynomial fit (approximating the free atom absorption) to the EXAFS a
hundred eV or more above the absorption edge. The amplitude of this extrapolated free atom decay is set equal to unity at the energy corresponding to the maximum peak in the edge region. The X-ray absorption cross-section is then converted to the space of the photoelectron wavevector according to Eqn. 2-3. The normalized EXAFS, \( \chi(k) \), is obtained from the converted X-ray absorption cross-section, \( \mu(k) \), by taking the ratio to the free atom decay, \( \mu_0 \), and subtracting a low frequency background, \( \mu_* \) (Eqn. 2-4).

\[ \chi(k) = \frac{[\mu(k) - \mu_*(k)]}{\mu_0(k)} \]

where \( \mu(k) \) is the measured X-ray absorption cross-section expressed as a function of the photoelectron wavevector. The free atom absorption was obtained from tabulated Victoreen functions describing the decay (McMaster et al., 1969). A cubic spline was used to remove the low frequency background components, \( \mu_* \).

Generally, structural information is obtained from EXAFS spectra by fitting the experimental curves to the known functional form (Eqn. 2-3). Two general approaches have been taken in fitting the EXAFS of an unknown system to Eqn. 2-3: the Hodgson-Doniach method (Cramer et al., 1976) and the Teo-Lee (1979) method. Using the Hodgson-Doniach approach, the backscattering amplitude, \( F_j(\pi,k) \) and phase, \( \alpha_j(k) \), functions are obtained empirically from compounds of known structure. Alternatively, according to the method of Teo and Lee backscattering amplitude and phase functions which have been calculated for the majority of the elements are used. We have used the latter approach; however, improvements upon the structural information determined for the unknown (the manganese complex within the OEC of PSII) were made based on identical analyses of a number of spectroscopically similar inorganic complexes of known structure. This approach using fine adjustments based on models (FABM) was developed by Teo et al. (1983). Estimates of the number of scatterers to within ±20% and distances to neighboring atoms to within ±0.02 Å can be obtained in this manner.

2.3 Instrumentation

A schematic representation of the instrument setup used in the X-ray absorption experiment is shown in Figure 2-3. The components used in the X-ray absorption ex-
periment are similar to the components found in any conventional UV-visible or IR absorption spectrometer. The essential components consist of a source of X-rays; a monochromator; a X-ray detector which monitors the incident flux, \( I_0 \); a sample compartment (in this case, also a cryostat); and a second X-ray detector which monitors the flux of X-rays transmitted through the sample, \( I_1 \). The sensitivity of the technique is extended several orders of magnitude by fluorescence detection of the absorption spectrum (Jaklevic et al., 1977). This enhanced sensitivity is essential to the measurement of X-ray absorption spectra of metalloenzymes.

What follows is a brief description of each component of the X-ray absorption apparatus. Particular attention is paid to specific features, or recent changes, of each component which have significantly improved the signal-to-noise ratio of recently acquired edge and EXAFS spectra of PSII preparations. The characteristics of the synchrotron X-ray sources have been described in detail in section 2.1.1. The use of high flux wiggler beam lines IV-2 and VI-2 at the SSRL has been essential to the acquisition of high quality spectra of samples as dilute in the metal of interest as 700 \( \mu \text{M} \).

The monochromator consists of a pair of monolithic silicon crystals, each cut along a specific crystal plane. A narrow range of X-ray energies may be selected from the broad band synchrotron emission through the principle of Bragg diffraction. The specific X-ray wavelength selected is determined by the angle, \( \theta \), of the incident X-ray beam relative to the crystal plane (see Eqn. 2-5 and Inset of Figure 2-3).

\[
2-5) \quad n \lambda = 2dsin\theta
\]

where \( d \) is the distance between atomic planes in the crystal.

For a flat crystal monochromator, the energy resolution is given by Eqn. 2-6 (Lee et al., 1981). Thus the X-ray energy is continuously tunable over an extremely broad range.

\[
2-6) \quad \delta E = E\cot\theta \delta \theta
\]

The energy resolution of this monochromator is limited by the Darwin reflection width, \( \delta \theta_w \), which is typically \( \sim 5 \times 10^{-6} \text{ rad} \). The intrinsic divergence of the synchrotron beam resulting from a 3.0 GeV electron beam is \( 1.7 \times 10^{-4} \text{ rad} \). This mismatch in angles results in a loss of a factor of about three in transmitted power. The energy
Figure 2-3. A schematic diagram of the instrumental setup used in X-ray absorption experiments. Individual components are not drawn to scale. The expanded view of the Soller slit assembly is adapted from Stem and Heald (1979). $I_0$, $I_1$, and $I_2$ are ionization chambers used to monitor X-ray flux in the synchrotron beam. For further details, see text.
resolution at the iron edge, using Si<220> monochromator crystals, is 1.5 eV. Crystals are designated by the Miller indices of the crystal plane along which the faces are cut.

Two problems commonly arise through the use of this monochromator. Frequently, within the energy range of an EXAFS scan, sharp drops in the transmission of X-rays of a given energy occur due to alignment of crystal planes other than the plane along which the crystal was cut. These so called “crystal glitches” are occasionally sharp enough that they can simply be excised from the broad EXAFS oscillations; however, it is preferable to find a set of crystals which do not contain diffraction glitches in the energy range of interest. For the Mn EXAFS energy region (6500 eV - 7100 eV), Si<400> crystal monochromators have been found to be free of crystal glitches. It should be noted however that the Darwin width of these crystals is a factor of about four smaller than that of the more conventional Si<111> crystals. This results in a reduction in the transmitted flux of a factor of about four. This reduction in transmitted flux made the use of energy resolving solid-state detection systems competitive with faster pulse-counting X-ray detection systems such as plastic scintillators for the measurement of Mn EXAFS in PSII membrane preparations (described in detail in section 2.3.1).

Another problem inherent to this monochromator system is caused by the fact that harmonics of the fundamental frequency of interest also satisfy the Bragg relation. An examination of the plot of X-ray flux shown in Figure 2.1 indicates that for a Wiggler magnet the content of the first harmonic can be nearly equal to the flux of the fundamental frequency in the X-ray region associated with Mn absorption (e.g. 6.5 to 7.0 KeV). The harmonic content is undesirable, for it has been found that it can significantly distort X-ray absorption spectra (Stern & Kim, 1981). Two methods have been found to substantially reduce harmonic content: the use of X-ray mirrors with low energy cutoffs and “detuning” of the crystal monochromator. The use of a mirror will virtually eliminate harmonic content if the high energy cutoff of the mirror occurs at an energy between the fundamental and the harmonic. Grazing angle incidence mirrors are X-ray optical devices available on several of the wiggler beam lines at SSRL. The total X-ray flux at a given energy may be enhanced by a factor of 5 to 6 through the use of a mirror. The high energy cutoff of the mirror on beam line IV-2 at SSRL is ~10.5 KeV and thus will result in substantial attenuation of the harmonics of the Mn EXAFS region. Alternatively, a method known as “detuning” can substantially reduce harmonic content.
The monochromator crystals are generally adjusted to be parallel since this results in the maximum transmission of X-rays. However, as the crystals are rotated away from a parallel configuration, the decrease in flux is much more rapid for the harmonics than it is for the fundamental. Thus it is possible to adjust the monochromator crystals such that a large percentage of the fundamental is transmitted, while most of the harmonics are rejected by detuning.

2.3.1 Detection Methods

There are two basic methods in which an X-ray absorption experiment is performed. Both methods are illustrated in Figure 2-3. The simplest approach, suitable for the measurement of X-ray absorption spectra of concentrated samples, is to monitor the X-ray flux before, I₀, and after the sample, I₁. For dilute samples, fluorescence detection of the absorption spectrum is the method of choice (Jaklevic et al., 1977). In both cases, the ratio of a measure of the transmitted X-ray flux or a measure the fluorescence intensity to a monitor of the incident intensity is taken in order to correct for variations in the flux as a function of energy. In the ideal case, even sharp drops in the intensity of the incident flux (such as the monochromator crystal glitches described in the previous section) would "ratio out". However, in practice, non-linear responses of the detectors result in distortions of the X-ray absorption spectrum with sudden changes in the incident flux.

Because of their durability and high count rate capabilities, ionization chambers are generally used to monitor the incident flux in such experiments. An ionization chamber consists of a gas contained between two parallel plates, separated by about 1 cm, across which a voltage of 300 V is applied. The percentage of the X-ray beam absorbed and the magnitude of the photo-ionization current produced depend on the gas in the chamber.

Several different X-ray detectors have been devised for the measurement of fluorescent X-rays. The ideal fluorescent X-ray detector would have high count rate capabilities, high energy resolution and a large acceptance area (to maximize the observed solid angle of emission from the sample). Ion chambers have high count rate capabilities but no energy resolution. However, ionization chambers for use in the fluorescent detection of X-ray absorption spectra have become popular because of the large solid angle which
can be viewed by appropriately constructed detectors and because of the linearity of the ratio of the response these detectors to the response of ion chambers used in monitoring the incident flux.

Scintillation detectors also have high count rate capabilities and can be constructed into arrays which can view nearly the entire emission of the sample (Cramer & Scott, 1981). Because these devices (ion chambers and scintillation arrays) have very poor energy resolution, methods have been developed to discriminate fluorescent X-rays from elastically or inelastically scattered X-rays. Filtering, using a thin layer of the element of the next lower atomic number (Cr for Mn fluorescence), has been shown to be an effective method of discriminating against scattered X-rays (Stern & Heald, 1979). This filtering scheme can result in a significant secondary background due to Cr filter fluorescence. Since this secondary emission is isotropic from a point closer to the detection system, the total counts of Cr fluorescence seen by the detector may be minimized by viewing the sample fluorescence through a set of plates set to the angle of divergence of the fluorescence emission (See inset of Figure 2.3). This assembly of plates is known as a Soller slit array.

As is indicated by the discussion above, the major source of background noise, and artifacts associated with "crystal glitches" in the fluorescent detection of X-ray absorption spectra is the flux of elastic or inelastically scattered X-rays. Thus any detection system which effectively rejects scattered X-rays would substantially improve the signal-to-noise of fluorescence-detected EXAFS. Since the energy of fluorescent emission for the first row transition metals is approximately 500 eV below the absorption edge, a detector with an energy resolution of at least 250 eV FWHM would be ideal for such discrimination. The energy resolution of solid state detectors can be as high as 180 eV FWHM. Thus, solid state detection systems would seem to be ideally suited for background rejection in the fluorescent detection of EXAFS. For a general discussion of the properties of solid state detectors see Goulding (1978). In the past, the small acceptances of solid state detectors and the count rate limitations imposed principally by the pulse shaping electronics have limited the widespread use of solid state detection systems. In theory, both of these limitations could be overcome by construction of arrays of such detectors and, while such an undertaking might seem prohibitively expensive (due principally to the cost of the associated pulse processing electronics required for
each channel), such arrays have recently been constructed. As mentioned in Section 2.1.1, synchrotron radiation is highly polarized in the plane of motion of the electron beam. As a result, the flux of elastically scattered X-ray is anisotropic; in fact, the flux of elastically scattered X-rays at 90° to the X-ray beam in the plane of polarization is zero. One advantage of detection systems with small acceptances is that they discriminate against elastically scattered X-ray backgrounds.

Recent advances in the design of pulse processing electronics have improved the count rate capabilities of solid state detectors by as much as 40% (Goulding, et al., 1983b). These triangular-pulse shaping amplifiers have increased the count rate capabilities of solid state detection systems by reducing pulse pile up due to overlap of the Gaussian tails which occurred in conventional Gaussian pulse shaping amplifiers. The improvements in pulse processing electronics and the reductions in X-ray flux caused by the required use of Si(400) led us to consider the use of semiconductor detectors in recently acquired EXAFS spectra of PSII membranes.

We found that the use of these energy resolving detectors substantially improved the signal-to-noise of acquired spectra and also eliminated severe artifacts presumably associated with crystal glitches and harmonic distortions. Optimum pulse shaping times were found to be in the range of 2 μsec. The choice of the length of the pulse processing time is important for it determines both the energy resolution and the total linear count rate of the amplifier (Goulding, et al., 1983a). Longer pulse shaping times (>10 μsec) are required for maximum energy resolution; however, these long pulse shaping times limit the linear pulse counting range (counts in vs counts out) to less than 10,000 cps. A pulse shaping time of 2 μsec has allowed an acquisition rate of up to 30,000 cps without substantial deviations from linearity in the response of the detector. The energy resolution at this pulse shaping time is ~ 300 eV FWHM.

We have also found that the use of a scintillation detector in monitoring the incident flux substantially reduced artifacts observed in the ratio of the response of the detectors to sudden changes in the incident flux. This scintillator monitored the incident flux by viewing a mylar film placed directly in the X-ray beam. This method of monitoring the incident flux was first suggested to us by Prof. B. Chance.

Energy calibration in a X-ray absorption experiment is maintained by monitoring the
position of known absorption edges. For example, the absorption edges of Cu or Fe foil are used in calibrating the energy of the monochromator initially. Occasionally, loss of calibration may occur due to mechanical slippage of the stepper motors which drive the rotation of the crystal monochromators. We have found it necessary to maintain energy calibration by simultaneously measuring the intense 1s → 3d transition of a standard potassium permanganate sample (Goodin, et al., 1983).

2.3.2 Sample Handling

As with any spectroscopic measurement, it is essential to verify the integrity of the sample under investigation. This is of particular concern when performing X-ray absorption experiments because of the ionizing nature of the radiation. Since X-rays are known to produce hydrated electrons in many solvents, photoreduction of metal centers is a concern. Considerable attention has been devoted to understanding the specific conditions (e.g. buffer composition, counter ions present, the specific sample under investigation and the temperature of observation) affecting radical yield and X-ray induced reduction (Brudvig, et al., 1980; Chance, et al., 1980). While all of the factors affecting photoreduction are not completely understood, it is generally agreed that keeping the sample cold decreases diffusion of free radical species and thus slows the rate of photoreduction.

All X-ray absorption measurements performed on PSII membrane preparations and inorganic model complexes were performed below -80°C in a home built double Kapton wall cryostat. The cryostat temperature was maintained by a cooled stream of dry N$_2$ gas. Samples used in X-ray absorption measurements were mounted in lucite sample holders which were designed to fit into a liquid He Dewar used in conjunction with a Varian E109 EPR spectrometer. EPR spectra were recorded before and after X-ray beam exposure to ensure sample integrity.
Chapter 3

Studies of the Substructure of the Low Temperature Multiline EPR Signal

3.1 Introduction: The Chloride Effect in Photosynthetic Oxygen Evolution. Is Halide Coordinated to the EPR-Active Manganese in the O₂-Evolving Complex?

It has been known since the early work of Warburg and Luttgens (1944) that chloride is an essential cofactor in photosynthetic oxygen evolution. Recent studies have confirmed that Cl⁻ is closely related to photosystem II (Izawa et al., 1969; Kelley & Izawa, 1978), and it has been suggested that the Mn-containing oxygen evolving complex (Mn-OEC) is the site of Cl⁻ action (Kelley & Izawa, 1979; Muallem & Izawa, 1980; Govindjee et al., 1983; Izawa et al., 1983). Models have been presented suggesting that the chloride ion could be ligated to Mn (Kelley & Izawa, 1978; Sandusky & Yocum, 1983) or may be required to stabilize a positive charge on the Mn-containing oxygen evolving enzyme (Critchley et al., 1982; Baianu et al., 1984). Recent kinetic studies have concluded that Cl⁻ depletion inhibits the advancement of the Mn-OEC beyond the S₂ state (Itoh et al., 1984; Theg et al., 1984), while other studies have implicated a role for Cl⁻ in the S₂ to S₀ transition (Sinclair, 1984). It has also been postulated that Cl⁻ interacts with cytochrome b₅₅₉ (McEvoy & Lynn, 1971; Muallem et al., 1981). Although the requirement of Cl⁻ on the donor side of PS II and its involvement with the Mn-OEC has been established, both its mechanism and site of action are still matters of controversy.

The light-induced multiline EPR signal containing 19 or more lines extending over more than 1000 G in width observed at low temperature in spinach chloroplasts has been assigned to a manganese species on the basis of its hyperfine splittings (Dismukes & Siderer, 1981; Dismukes et al., 1982; Hansson & Andréasson, 1982). The flash dependence and the temperature dependence of its formation and decay indicate that it is correlated with the presence of the S₂ state in the O₂-evolving complex (Dismukes & Siderer, 1981; Brudvig et al., 1983). Using EDTA-washed, Cl⁻-depleted chloroplasts, we have investigated in detail the Cl⁻ requirements for generating the multiline EPR signal. We present evidence to suggest that Cl⁻ is required for the generation of the S₂ state as defined by EPR criteria and also that the fine structure of the multiline signal is invariant (at a modulation amplitude of 4 G) when Cl⁻ is replaced with Br⁻, indicating
that the site of the halide ion is not as a ligand to Mn at the $S_2$ state. However, it cannot be ruled out that the transferred hyperfine coupling with the halogen nuclei is too small to be evident at this resolution.

3.2 Materials and Methods

3.2.1 Halide Exchange Procedures

Broken chloroplasts were prepared from destemmed market spinach by a procedure described elsewhere (Casey & Sauer, 1984). Chloride-depleted chloroplasts were prepared by washing the chloroplasts 3-4 times in a chloride-free buffer containing 50 mM HEPES at pH 8.0, 10 mM $Na_2SO_4$, 5 mM $MgSO_4$ and 0.5 mM EDTA. Fewer washes are required when higher pH(8.3-8.5) buffers are used (Izawa et al., 1983, Theg & Homann, 1982). Two more such washes were carried out in an EDTA-free buffer at pH 7.5. The suspensions were incubated for 5 min each time in the dark before centrifugation. $O_2$-evolution activity of the chloroplasts was monitored in a Cl$^-$ free buffer after each wash; after 3-4 washes the chloroplasts were totally inactivated.

The Cl$^-$ samples were prepared by suspending the Cl$^-$ depleted chloroplasts in buffers containing 50 mM HEPES at pH 7.5, 5 mM $MgSO_4$, 10 mM $Na_2SO_4$ and various concentrations of NaCl ranging from 0.1 to 15 mM. The Br$^-$ samples were prepared by suspending Cl$^-$-depleted chloroplasts in 50 mM HEPES buffer (pH 7.5)/5 mM $MgSO_4$/15 mM NaBr. The F$^-$ sample was prepared in a similar manner except that $MgSO_4$ was not added because of the limited solubility of MgF$_2$. The $O_2$-evolution activity and the amplitude of the multiline EPR signal are reported as a fraction of the control sample, which contained 15 mM NaCl.

The preparation of Cl$^-$-free or Br$^-$- or F$^-$-substituted subchloroplast membranes was accomplished by a modification of the method of Kuwabara and Murata (1982). The method consisted of initially preparing Cl$^-$ depleted chloroplasts followed by suspension in Cl$^-$ free (50 mM HEPES (pH 7.5)/10 mM $Na_2SO_4$/5 mM $MgSO_4$) or Cl$^-$ (50 mM HEPES (pH 7.5)/15 mM NaCl/5 mM MgCl$_2$ or Br$^-$ (50 mM HEPES (pH 7.5)/15 mM NaBr/5 mM $MgSO_4$) or F$^-$ buffers (50 mM HEPES (pH 7.5)/10 mM $Na_2SO_4$/15 mM NaF). PS II particles were then prepared by Triton X-100 treatment of the chloroplasts in the respective 50 mM MES buffers at pH 6.0 (Kuwabara & Murata, 1982) (Exchanged and Triton Washed: the ETW method). This was by far the most efficient method for
preparing functionally Cl⁻ depleted or Br⁻ or F⁻ containing PS II particles, as will be shown in the Results section.

We also prepared Cl⁻ depleted PS II particles by washing PS II particles prepared by the method of Kuwahara and Murata in a Cl⁻-free buffer containing 50 mM MES (pH 6.3)/5 mM MgSO₄/10 mM Na₂SO₄. Dialysis of the PS II particles overnight in a Cl⁻ free buffer with 3 buffer changes yielded similar results. The PS II samples were made by suspending the Cl⁻ depleted PS II particles in 50 mM MES at pH 6.0, 5 mM MgSO₄, 10 mM Na₂SO₄ and various concentrations of NaCl ranging from 0.1 to 15 mM. Bromide or fluoride samples were prepared by suspension in buffers containing 15 mM NaBr or 15 mM NaF. The F⁻ sample did not contain any MgSO₄. We also prepared the F⁻ and Br⁻ samples by directly exchanging the Cl⁻ PS II particles in F⁻ and Br⁻ buffers, (Triton Washed and Exchanged: the TWE method) instead of initially preparing the Cl⁻ depleted PS II particles. The protocol for the preparation of ETW and TWE particles is summarized in Figure 3-1.

Rates of O₂-evolution were measured using a Clark-type oxygen electrode. Illumination was with a 200 W quartz lamp. Chloroplast samples were suspended at 20-30 µg Chl/ml in 50 mM HEPES (pH 7.5)/5 mM MgSO₄/ 3 mM K₃Fe(CN)₆/3 mM K₄Fe(CN)₆/500 µM DMBQ and contained the same concentration of the halide ion as the samples. In the case of PS II particles 50 mM MES at pH 6.0 was used. O₂-evolution rates were typically 250-300 µmol O₂ per mg Chl per h for PS II particles and 150-200 µmol O₂ per mg Chl per h for the chloroplasts.

3.2.2 EPR Measurements

For EPR measurements, samples were placed in quartz tubes and dark adapted for 1 h at 4°C. After equilibration at 190 K in a dry ice/methanol bath, the samples were illuminated with a tungsten lamp for 1 min and then were immediately frozen in liquid N₂.

EPR spectra were recorded with a Varian E109 spectrometer equipped with a model E102 microwave bridge and an Air Products Helitran cryostat. EPR spectra were recorded at 8-10 K using 50 mW microwave power at 9.19 GHz, 100 KHz field modulation of 32 G amplitude, scan time 4 min and time constant 0.25 s. EPR spectra at high resolution (4 G modulation amplitude) were collected using PS II particles.
with 6-8 mg Chl/ml. To achieve a S/N at which the substructure became evident, we collected multiple scans (approx. 60, 4 min scans at a time constant of 0.128 s) using a signal averager built in our laboratory, and the averaged data was then transferred to a VAX 11/780 computer for analysis.

3.3 Results

3.3.1 Oxygen Evolution and the Amplitude of the Multiline EPR Signal

Figure 3-2a shows the amplitude of the multiline EPR signal generated by 1 min of continuous illumination at 190 K plotted as a function of the Cl⁻ concentration. The curve drawn in Fig 3-2a is a hyperbolic plot derived from a linear least squares fit to a Lineweaver-Burk plot of the data. The profile of the curve is similar to that observed in earlier studies (Izawa et al., 1969; Kelley & Izawa; 1978) of O₂-evolving activity. The EPR signal amplitude reaches half maximum at about 0.5 mM Cl⁻ and a maximum at 5 - 10 mM Cl⁻ ion concentration. Figure 3-2b is a plot of the oxygen evolution activity vs. the multiline EPR signal amplitude at each Cl⁻ concentration studied. The line drawn is a linear least squares fit to the data. Both response inhibitions were reversible and strongly correlated.

The results with Br⁻ or F⁻ substituted chloroplasts show that Br⁻ is an effective replacement for Cl⁻ in terms of O₂-evolution activity (95%), but F⁻ does not restore O₂-evolution activity to more than 30% of the control sample. The amplitude of the multiline EPR signal correlates with O₂-evolution activity in both cases, 83% for Br⁻ and 30% for F⁻ (Table 3-1).

Similar studies were carried out using TWE PS II particles. Repeated washing or dialysing against Cl⁻ free buffers decreased the O₂-evolution activity by about 40%, with a corresponding decrease in multiline EPR signal amplitude, but we were unable to prepare PS II particles with total functional impairment by this method. The results are shown in Figures 3-3a and 3-3b. Both the O₂-evolution activity and the amplitude of the multiline EPR signal amplitude reach a maximum at 5-10 mM Cl⁻ concentration.

The Br⁻ and F⁻ substituted particles prepared by the TWE method follow patterns observed with chloroplasts. Br⁻ is an effective replacement for Cl⁻, with O₂-evolution activity of 100% and a multiline amplitude of 84%. The F⁻ substituted particles exhibit an O₂-evolution activity of about 23% and a multiline amplitude of about 46%. It is
Figure 3-1. A Schematic presentation of the TWE and ETW PSII particle preparation procedures.
chloroplasts

\[ \text{Triton-extracted} \rightarrow \text{Cl}^- \text{ particles} \]

\[ \rightarrow \text{washed in Cl}^- \text{ free buffer} \rightarrow \text{Cl}^- \text{-depleted particles} \]

\[ \rightarrow \text{washed in F}^- \text{ buffer} \rightarrow \text{F}^- \text{ particles} \]

\[ \rightarrow \text{washed in Br}^- \text{ buffer} \rightarrow \text{Br}^- \text{ particles} \]

\[ \rightarrow \text{washed in Cl}^- \text{ buffer} \rightarrow \text{Cl}^- \text{ particles} \]

\[ \text{control} \]

\[ \text{Triton extracted} \rightarrow \text{Cl}^- \text{ particles} \]

\[ \rightarrow \text{washed in Cl}^- \text{ free buffer} \rightarrow \text{Cl}^- \text{-depleted particles} \]

\[ \rightarrow \text{Triton extracted in F}^- \text{ buffer} \rightarrow \text{F}^- \text{ particles} \]

\[ \rightarrow \text{Triton extracted in Br}^- \text{ buffer} \rightarrow \text{Br}^- \text{ particles} \]

\[ \rightarrow \text{Triton extracted in Cl}^- \text{ buffer} \rightarrow \text{Cl}^- \text{ particles} \]

\[ \text{control} \]

\[ \text{ETW particles} \]
Figure 3-2. a) The effect of Cl⁻ concentration on the amplitude of the low temperature multiline EPR signal of spinach chloroplasts. Illumination and EPR protocols are described in the text. The amplitudes of the multiline signal were taken as the average peak-to-peak height of 4 lines downfield and 4 lines upfield from g=2.0 and normalized with respect to the Chl content of each sample. Sample buffers contained 50 mM HEPES at pH 7.5, 5 mM MgSO₄, 10 mM Na₂SO₄ and a variable concentration of Cl⁻ ion. The curve drawn is a hyperbolic fit derived from a linear least squares fit to a Lineweaver-Burk plot of the data.

b) The relation between multiline EPR signal amplitude and O₂ evolution activity of spinach chloroplasts at various Cl⁻ ion concentrations. Control Cl⁻ containing chloroplast preparations exhibited oxygen evolution rates between 150 - 200 μmol O₂ per mg chl per h. Illumination, EPR and oxygen activity assay protocols are described in the text. The line drawn is a linear least squares fit to the data. Cl⁻ ion concentrations corresponding to each point plotted are: 1, 0.0 mM; 2, 0.1 mM; 3, 0.2 mM; 4, 0.5 mM; 5, 1.0 mM; 6, 2.0 mM; 7, 5.0 mM; 8, 10.0 mM; 9, 15 mM. EPR spectrometer settings were as follows: microwave frequency: 9.21 GHz; microwave power: 50 mW, field modulation amplitude: 32 G at 100 KHz. Samples were maintained at 8K in an Air Products LTD liquid He cryostat during all EPR measurements.
interesting that the F- substituted particles exhibit a smaller multiline amplitude and O2-evolution activity than the Cl- depleted particles.

The behavior of PS II particles prepared by the ETW method was very different. By this method we were able to prepare Cl- depleted PS II particles with considerably less O2-evolution activity and multiline EPR signal (e.g. 10 - 20%) than the particles prepared using the TWE method (approx. 60%). On readdition of Cl- to Cl- depleted ETW particles only 40-50% of control activity was restored (control was the Cl- ETW particles, see Figure 3-1 for details). It appears that the O2-evolution activity and multiline EPR signal amplitude of the PS II particles can be reduced to only about 60% by washing in a Cl- free medium but, once completely Cl- depleted PS II particles are prepared (by utilizing completely Cl- depleted chloroplasts), only about 50% of the O2-evolution activity and multiline EPR signal can be restored on readdition of Cl-.

The particles prepared using the ETW method resulted in Cl- and Br- particles which were indistinguishable from the particles prepared using the TWE procedure in terms of activity and multiline EPR signal amplitude. The F- samples prepared by the two protocols exhibited distinctly different properties. The O2-evolution activity was less than 5% and we were unable to generate any multiline EPR signal using particles prepared by the ETW method, compared to about 30% activity and multiline EPR signal that we could generate with the TWE method. This raises the possibility that the O2-evolution activity and multiline amplitude in F- samples prepared by washing or exchanging is actually due to the presence of residual amounts of Cl- preparations, which is then completely removed by our newer procedure of preparing F- particles, giving rise to negligible activity and multiline signal. All of these results are summarized in Table 3-1.

3.3.2 Superhyperfine Structure of the Multiline EPR Signal

Other studies (Izawa et al., 1969; Kelley & Izawa, 1978; Kelley & Izawa, 1979; Muallem & Izawa, 1980; Izawa et al., 1983; Govindjee et al., 1983) have implicated Cl- as a ligand to Mn or in the electrostatic stabilization of the complex. These studies and our results presented above suggest that the Cl- requirement for O2-evolution activity is due to the involvement of Cl- in the Mn-OEC. To test for the possibility that the halide is in the first coordination sphere of the metal atom, where it could or might give rise to
Figure 3-3. a) The effect of Cl$^-$ concentration on the amplitude of low temperature multiline EPR signal of TWE PS II particles. Conditions are similar to those described in Figure 3-1a.

b) The relation between multiline EPR signal amplitude and O$_2$ evolution activity of TWE PSII particles at various Cl$^-$ ion concentrations. The line drawn is a linear least squares fit to the data. Cl$^-$ ion concentrations corresponding to each point plotted are: 1, 0.0 mM; 2, 0.2 mM; 3, 1.0 mM; 4, 5.0 mM; 5, 10.0 mM; 6, 15.0 mM. Illumination, EPR and oxygen activity assay protocols are described in the text. EPR spectrometer settings are the same as those described in the Figure 3-2 b caption.
Spinach PSII Particles

% Multiline EPR Signal

mM [NaCl]

Spinach PSII Particles

% Multiline EPR Signal

% Oxygen Evolution Activity
Table 3-1
The Correlation of Oxygen Activity and EPR Multiline Signal Amplitude in Chloroplasts, and TWE and ETW PS II Particles.

<table>
<thead>
<tr>
<th></th>
<th>Chloride depleted</th>
<th>Bromide</th>
<th>Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity  MLS</td>
<td>Activity MLS</td>
<td>Activity MLS</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>5</td>
<td>15</td>
<td>95</td>
</tr>
<tr>
<td>TWE particles</td>
<td>62</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>(Triton Washed and Exchanged)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETW particles</td>
<td>8</td>
<td>18</td>
<td>92</td>
</tr>
<tr>
<td>(Exchanged and Triton Washed)</td>
<td></td>
<td></td>
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</tbody>
</table>

In each case the sample with 15 mM NaCl concentration is the control sample and is explained in Scheme 1. The control O₂ evolution rates were between 250 and 300 μmol O₂ per mg chl per h.

MLS - EPR multiline signal amplitude
Figure 3-4. a) Multiline EPR spectra observed for spinach ETW PS II particles in Cl⁻ and Br⁻ buffers. The EPR spectra were recorded at 7 K using 50 mW microwave power at 9.19 GHz; 100 KHz field modulation at 4 G amplitude, scan time 4 min; time constant, 0.128 s. The spectra shown are the sum of 60 individual scans.

b) The second derivative d²x/dH² of the multiline EPR spectra for spinach ETW PS II particles in Cl⁻ and Br⁻ buffers. The second derivatives were obtained numerically from a 10 point (10 G) zero-order sliding fit to the data.
Figure 3-5. The multiline EPR spectra of spinach TWE PS II particles in Cl⁻/H₂O or in Cl⁻/²H₂O buffers on the low field side of g=2. The PS II particles were washed in ²H₂O buffer 3 times and cycled through the S states by illumination and then again washed in D₂O buffer before dark adaptation and preparation of the S₂ state by continuous illumination at 190 K. The instrument settings were identical to those described for Figure 3-4a.
superhyperfine splittings from the halogen nuclei, we have recorded the multiline EPR signal at high resolution. Figure 3-4a shows the EPR spectra of PS II particles in Cl⁻ and Br⁻ buffers at 4 G modulation amplitude. Distinct fine structure is seen in both spectra and is even more evident in the second derivative presentation shown in Figure 3-4b. Each major line is split into a multiplet of 4-6 lines with splittings of the order of 10-15 G and there are no significant differences between the spectra of the Cl⁻ and Br⁻ samples.

In order to evaluate whether exchangeable protons are coupled to the manganese complex yielding the multiline EPR signal, we examined the effect of ²H₂O exchange. Figure 3-5 shows a portion of the multiline spectrum on the low field side of g=2, on an expanded scale. The peaks are clearly resolved into a multiplet and appear to be more complex than was previously recognized (Brudvig et al., 1983). The multiplet spectrum of a sample in ²H₂O buffer is also shown in Figure 3-5, but no clear differences are seen in the spectra of the Cl⁻/H₂O or Cl⁻/²H₂O samples.

3.4 Discussion

3.4.1 Correlation of Cl⁻ Concentration with Multiline EPR Signal Amplitude

The correlation of the multiline amplitude with the concentration of the Cl⁻ in chloroplasts is a direct indication that Cl⁻ is required for the generation of the S₂ state as defined by EPR criteria, and that its involvement in the Mn-OEC occurs prior to the S₂ state. However, it is possible that Cl⁻ is also required for the generation of the higher S states, as suggested by other studies (Itoh et al., 1984; Theg et al., 1984; Sinclair, 1984). Earlier studies with Cl⁻ depleted chloroplasts indicate that two oxidizing equivalents can accumulate on the oxidizing side of PS II (Muallem et al., 1981). Luminescence studies by Itoh et al. (1984) and Theg et al. (1984) have concluded that Cl⁻ depletion inhibits the advancement beyond S₂ state but does not inhibit the earlier transitions. This discrepancy between the luminescence and EPR studies may be due either to (1) the presence of intermediates between the Mn-OEC and Z on the donor side of PS II or (2) removal of Cl⁻ may alter the structure of the Mn-OEC sufficiently to change its EPR properties, without preventing S₂ or even S₃ formation from a functional point of view. More recent studies of the multiline EPR signal obtained by subjecting chloride-depleted PSII preparations to a series of short saturating flashes of light, followed by
dark repletion of chloride, indicate that Cl\textsuperscript{−} depletion blocks advancement beyond the \(S_2\) state (Ono et al., 1986). Although the multiline EPR signal is not formed in the absence of chloride, these authors also noted that the rate of decay of the \(S_2\) state in chloride-depleted PSII membranes was 20 times slower than that observed in Cl\textsuperscript{−} sufficient preparations. Based on their observation they concluded that an altered, more stable, EPR-silent \(S_2\) state was formed in the absence of Cl\textsuperscript{−}. Whether the oxidizing equivalent stored on the donor side of PSII is stabilized within the manganese complex is not clear from these studies. Work is in progress in our laboratory to resolve this question by monitoring the oxidation state of the Mn-OEC in Cl\textsuperscript{−}-depleted particles by studying the Mn K-edge X-ray spectra, which is an independent measure of the number of oxidizing equivalents stored in the Mn-OEC (Goodin et al., 1984). It should be noted that Ono et al. also found that depletion of chloride did not inhibit the formation of the \(g=4.1\) signal. The \(g=4.1\) signal has been associated with the \(S_2\) state (Zimmermann & Rutherford, 1986), and our recent X-ray absorption edge studies indicate that the \(g=4.1\) species is due to the manganese complex at the same oxidation level as the \(S_2\) state characterized by the multiline EPR signal (Cole et al., 1987).

The results with TWE PS II particles (Table 3-1) also indicate that Cl\textsuperscript{−} or Br\textsuperscript{−} is required for \(O_2\)-evolution activity and that the site of action is prior to the \(S_2\) state. However, the \(O_2\)-evolution activity decreases to only 60% upon washing in Cl\textsuperscript{−}-free buffer, and subsequent washes in Cl\textsuperscript{−}-free buffer are not effective in reducing the activity or the multiline amplitude. One explanation for this behavior is that to maintain the functional integrity of the PS II particles it is necessary to work at a pH of 6 and that at such a low pH it is not possible to deplete the particles of Cl\textsuperscript{−}. It has been reported (Izawa et al., 1983; Theg & Homann, 1982) that high pH facilitates the removal of Cl\textsuperscript{−} in chloroplasts. Similar results have been observed in PS II particles (Sandusky & Yocum, 1983) but are complicated by the fact that at high pH peptides are also released from the PS II particles (Kuwabara & Murata, 1982). Another possibility is that there are two pools of the Mn complex, only one of which is susceptible to Cl\textsuperscript{−} depletion in PS II particles. A proposal for two pools of the Mn-OEC has recently been presented by Beck et al. (1985). This proposal is supported by our observation that Cl\textsuperscript{−}-depleted PS II particles prepared using the ETW procedure showed only 10-20% \(O_2\)-evolution activity and multiline amplitude and only about 40-50% activity could be
restored on readdition of Cl$. However, further experiments are necessary before the observed data can be fully explained. The lack of good quantitative estimates of the halide requirements has been a major obstacle to a better understanding of the role of Cl$ ion in photosynthetic oxygen evolution. Experiments are underway to quantitate the residual Cl$ content of the chloroplasts and PS II particles using X-ray fluorescence techniques.

Addition of F$ to Cl$-depleted chloroplasts decreases the $O_2$-evolution activity and the multiline amplitude to about 30% in exchanged PS II particles and to less than 5% in PS II particles made using F$-substituted chloroplasts. This reduction in activity and in multiline amplitude from that of Cl$-depleted PS II particles indicates a clear inhibitory role for F$ in $O_2$-evolution, possibly at the point of advancement to the $S_2$ state. This observation is in accord with our earlier studies of the EPR signal at $g=4.1$ which pointed to a site of action of F$ between the $S_1$ and $S_2$ states (Casey & Sauer, 1984).

3.4.2 Superhyperfine Structure of the Multiline EPR Signal

A close examination of each of the hyperfine lines (Figure 3-4) indicate a multiplicity of 4-6 lines separated by 10-15 Gauss. Based on models that have been proposed for the Mn site (Dismukes & Siderer, 1981; Dismukes et al., 1982; Hansson & Andréasson, 1982) in PS II it seems probable that most, if not all, of this finer structure can be attributed to a non-degenerate superposition of the hyperfine lines due to Mn alone; this becomes more evident when simulations (not shown) using an antiferromagnetically coupled Mn(III)Mn(IV) binuclear model have been carried to second order (similar to that carried out by Dismukes et al., 1982). The fine structure can also be accounted for by including hyperfine or g-tensor anisotropy or by including nuclear quadrupole interactions. In addition, it can be argued that the EPR substructure is due to ligand superhyperfine interactions. The most likely ligand atoms capable of such magnetic interactions in the context of what is known about the Mn-OEC are H, N and Cl.

In this report we address the question of whether the EPR substructure is due to an interaction of the halide ion with the Mn complex. Our approach to this problem is to look for differences in the high resolution EPR spectrum of Cl$ and Br$ containing ETW particles. Br$ is an effective replacement for Cl$ functionally and in its ability
to generate the multiline signal, so it is reasonable to assume that structurally it plays a role similar to that of Cl\textsuperscript{−}. All stable isotopes of Cl and Br have nuclear spins of 3/2, and the Cl\textsuperscript{−} and Br\textsuperscript{−} nuclei would be expected to split each of the major lines in the EPR spectrum into quartets. However, there are some significant differences in the magnetic properties of these two elements. The magnetic moments of the Br\textsuperscript{−} isotopes (\(\mu\) of \(^{79}\text{Br}=2.09\mu_N\) and of \(^{81}\text{Br}=2.26\mu_N\)) are about 2.5 times larger than the magnetic moments of the isotopes of Cl (\(\mu\) of \(^{35}\text{Cl}=0.82\mu_N\) and of \(^{37}\text{Cl}=0.68\mu_N\)). The difference in the magnitude of the magnetic moments and the natural abundance of the nuclei involved (\(^{35}\text{Cl}/^{37}\text{Cl}=3/1, ^{79}\text{Br}/^{81}\text{Br} = 1/1\)) would lead to very different ligand superhyperfine splitting patterns, when Cl\textsuperscript{−} is replaced by Br\textsuperscript{−} as a ligand.

Some examples of Cl\textsuperscript{−} and Br\textsuperscript{−} ligand superhyperfine coupling parameters (A(Cl) and A(Br)) in transition metal complexes are presented in Table 3-2. In all of these reports the Br\textsuperscript{−} splittings are larger than the Cl\textsuperscript{−} splittings. Unfortunately, we could not find any reports in the literature for A(Br) in Mn complexes; however, there is a report of A(Cl) for Mn\textsuperscript{2+} in NaCl, which is about 2 G (Santucci & Stefanini, 1967). So it is very likely that, if Cl was replaced by Br as a ligand to Mn, a large change in the superhyperfine structure would be evident.

In addition to changes in the superhyperfine structure, changes in the metal hyperfine and g-anisotropy would be expected for a metal complex in which a chloride ligand was replaced by a bromide ligand. Such changes are well documented for a number of mononuclear spin \(\frac{1}{2}\) systems. For example, oxomolybdenum(V) complexes exhibit substantial shifts in both the average g-value and the nuclear hyperfine splitting upon replacement of one chloride by one bromide (Goodman & Raynor, 1970). More subtle changes in the ligation of other metals are also observed to cause substantial changes in both the magnitude of the metal hyperfine splitting and anisotropy of the g-tensor. For example, substitution of nitrogen for oxygen in the coordination of oxovanadium(IV) complexes is observed to cause significant changes in both of these parameters (White & Chasteen, 1979). Similar effects are observed in copper complexes (Vännård, 1972).

It might be argued that the multiline EPR signal is a much more complex spin system than the mononuclear systems cited above. However, all simulations of the substructure of the multiline signal assume that the hyperfine coupling constants and g-anisotropy in this coupled spin system are weighted averages of the individual ion
Table 3-2
Superhyperfine Splittings \( A(\text{Cl}) \) and \( A(\text{Br}) \) in Transition Metal Complexes

<table>
<thead>
<tr>
<th></th>
<th>( A(\text{Cl}) ) Gauss</th>
<th>( A(\text{Br}) ) Gauss</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(^{3+}) in AgCl</td>
<td>2.4</td>
<td>10.6</td>
<td>1,2</td>
</tr>
<tr>
<td>Fe(^{3+}) in AgBr</td>
<td></td>
<td></td>
<td>1,2</td>
</tr>
<tr>
<td>Co(NCCH(_3))(_4)Cl(_2)</td>
<td>13.2</td>
<td></td>
<td>3,4</td>
</tr>
<tr>
<td>Co(NCCH(_3))(_4)Br(_2)</td>
<td></td>
<td>73.7</td>
<td>3,4</td>
</tr>
<tr>
<td>Cu(^{2+}) in CdCl(_2)</td>
<td>9.5</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Cu(^{2+}) in NH(_4)Br</td>
<td></td>
<td>24.9</td>
<td>5</td>
</tr>
<tr>
<td>([\text{Cp}_2\text{MoCl}_2])^+</td>
<td>2.3</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>([\text{Cp}_2\text{MoBr}_2])^+</td>
<td></td>
<td>15.8</td>
<td>6</td>
</tr>
</tbody>
</table>

Cp-cyclopentadiene

References
1. Hayes et al. (1964)
2. Hennig (1964)
3. Maher (1967)
4. Maher (1968)
5. Thornley et al. (1961)
6. Cooper & Green (1967)
terms in the uncoupled representation (Dismukes et al., 1982; Hansson & Andréasson, 1982). Thus changes in the coordination of any single metal center of a coupled spin system would be expected to cause changes in the magnitude of $g$ and $A$ values of the coupled system. However, it is possible that these changes would be too small to be observed.

At 4 G modulation no significant differences are seen in the substructure of the multiline EPR signal for Cl$^-$ or Br$^-$ exchanged samples (Figure 3-4). The criterion we used to arrive at this conclusion is as follows. We compared the difference spectrum of two independent Cl$^-$ samples with the difference spectrum from a Cl$^-$ and a Br$^-$ exchanged sample. The autocorrelation of the residuals obtained from the difference between two chloride spectra (each the sum of 60 individual scans) was a spectrum of points satisfying the 95% confidence interval criterion for white noise (Jenkins & Watts, 1968). This analysis also indicated that no artifacts were introduced in taking the difference between two independently collected spectra. The autocorrelation of the residuals obtained from the difference between the chloride and bromide spectra also yielded a distribution of points satisfying the 95% confidence interval criteria for white noise. Thus we have rigorously demonstrated that there are no statistically significant differences that are detectable under our conditions between the chloride and bromide spectra. This result demonstrates that the substructure of the multiline signal is not due to the exchanged halide ion, and we conclude that exchangeable halide ion is not coordinated to the EPR-active Mn. We qualify our conclusions, by noting that there are two implicit but reasonable assumptions in our rationale, that (1) Cl$^-$ is exchangeable and (2) if the halide is bound to Mn, some unpaired electron density is delocalized on to the ligands.

Recently, we have analysed the EXAFS spectrum of Mn in PS II particles poised in both the S$_1$ and S$_2$ states: the evidence does not support first shell coordination of Cl to Mn either in the S$_1$ or S$_2$ state (Goodin et al., 1984; Yachandra et al., 1986).

Alternatively, the superhyperfine lines might be explained by isotropic superhyperfine coupling to other ligand nuclei. Nitrogen ligands are wide-spread in metalloproteins, and the substructure in the multiline EPR signal might be explained by isotropic coupling to nitrogen nuclei. The magnitude of the observed splittings which are 10-15 G are in the range observed for ligand superhyperfine coupling to $^{14}$N (Goodman &
Raynor, 1970). Couplings to protons could also give rise to splittings of this magnitude. For example, superhyperfine coupling to \(^1\)H of 9.35 G for Mn in AlCl\(_3\)·6H\(_2\)O (Koryagin & Grechushnikov, 1966) and in Ca\(_2\)Mg\(_5\)(NO\(_3\))\(_{12}\)·24H\(_2\)O (Van Ormondt & Thalhammer, 1965) have been attributed to Mn-OH\(_2\) moieties.

If the substructure is due to coupling to exchangeable protons, then deuterium substitution should significantly alter the appearance of the fine structure. In general, deuteron exchange for protons results in a collapse of doublets into unresolved triplets because deuterons have I=1 and the hyperfine splittings by deuterons are seven times smaller than those due to protons. An example of this approach involves the rapid Mo(V) signal of xanthine oxidase, which exhibits proton doublets with 14 G splittings. These splittings collapse to single lines upon exchange in \(^2\)H\(_2\)O, and it was concluded from these studies that the protons or deuterons were not directly coordinated to the Mo atom (Bray & Swann, 1972; Bray & Vännegård, 1969). Similar effects are seen for Mo(V) complexes with coordinated -NH groups, which display proton superhyperfine couplings of 7.4 G that are reduced to 2.0 G on deuteration (Pariyadath et al., 1976).

Figure 3-S shows a portion of the multiline spectrum for a sample which was prepared using \(^2\)H\(_2\)O buffers. It is clear from a comparison of this spectrum with the multiline spectrum of the sample prepared in H\(_2\)O buffers that the substructure is not due to coupling to exchangeable protons; however, it is possible that the splittings are due to non-exchangeable protons or that they are not directly observable. More recently, Nugent (1987) has reported a narrowing of superhyperfine lines in the multiline EPR spectrum of PSII particles prepared from spinach and pea. This increased resolution of superhyperfine lines was more pronounced at higher temperatures of illumination (e.g. 298 K). This has been taken as evidence of direct ligation of exchangeable water to the manganese complex yielding the multiline EPR signal. We are presently addressing the question of ligation and structure of the Mn complex by ENDOR and Electron Spin Echo spectroscopy studies. Preliminary results indicate that the proton couplings to the multiline EPR signal are not due to protons bound directly to a ligand of manganese, but are more probably bulk water protons or exchangeable protons associated with nearby protein residues (Britt & Klein, personal communication).

3.5 Conclusions
1) Cl\textsuperscript{-} is required for the production of the multiline EPR signal, which indicates that it is required for the generation of a \( S_2 \) state as defined by EPR criteria and that its involvement in the Mn containing \( O_2 \)-evolving complex is prior to the \( S_2 \) state.

2) The similarity of the high resolution EPR spectra with Cl\textsuperscript{-} and Br\textsuperscript{-} suggests that the halide ions are not exchangeable ligands of the paramagnetic center of \( S_2 \). However, it is possible that the differences are too small to be evident under the conditions of our experiment.

3) The superhyperfine structure of the multiline EPR signal generated by continuous illumination at 195 K is not due to exchangeable protons.
Chapter 4

Extended X-Ray Absorption Fine Structure Analysis of Multinuclear Oxo-Bridged Manganese Complexes

4.1 Introduction

A variety of physical techniques has established a functional role for manganese in the process of photosynthetic oxygen evolution (see section 1.3.3). EPR and X-ray absorption spectroscopy have been among the most useful tools in establishing the role of manganese in this process. Through an analysis of the extended X-ray absorption fine structure (EXAFS) of the X-ray absorption spectrum (see section 2.2.1) it has been possible to obtain detailed information concerning the structure of the manganese site. The analysis of an unknown site like the manganese complex in PSII is greatly facilitated by parallel analyses of structurally relevant inorganic complexes. The analysis of a set of inorganic complexes can significantly improve the accuracy of the determination of structural features of the unknown environment (Teo et al., 1983) and in some cases allows one to decide whether certain structures proposed for the unknown active site are in fact reasonable.

Until recently, improvements in the analysis of the EXAFS of the manganese site in PSII have been impeded by a lack of structurally characterized multinuclear manganese complexes. However, recently a large number of structurally and physically well characterized inorganic complexes have been prepared. For a list of recent publications see Table 6-1. However, it should be noted that some of the earliest multinuclear complexes synthesized and characterized are still among the best models for the structure of the active site in PSII. For example the phenanthroline (Stebler et al., 1986) and bipyridine (Plaksin et al., 1972) binuclear di-μ-oxo bridged manganese complexes (shown in Figure 4-1) possess many of the essential structural features of the manganese site, as was found in the first EXAFS study (Kirby et al., 1981b). These mixed valence binuclear complexes have also been essential to an understanding of the magnetic properties of the manganese complex in PSII preparations poised in the $S_2$ state (Cooper et al., 1978; Dismukes & Siderer, 1981).

As has been the case with other unknown systems, most notably the active site of nitrogenase (Cramer, 1978; Conradson, 1983) and the metal centers in cytochrome
Figure 4-1. X-ray crystal structures of the (a) bipyridine (complex 1) and (b) phenanthroline di-μ-oxo bridged binuclear manganese complexes. Average distances to each component of each scattering shell are indicated (e.g. average Mn - O bridging distances, Mn - N, Mn - Mn and Mn - C distances).
a) $\text{Mn}_2(\text{II, IV})(\mu-\text{O})_2(2,2'\text{-bipyridine})_4(\text{ClO}_4)_3$
b) \( \text{Mn}_2(\text{III,IV})(\mu-O)_2(\text{1,10-phenanthroline})_4(\text{PF}_6)_3 \)
oxidase (Powers et al., 1981, Scott et al., 1986), improvements in the quality of the X-ray absorption spectra have led bioinorganic chemists to synthesize novel structurally relevant “model complexes”. The analysis of the EXAFS of these model complexes has then led to improvements in our understanding of the unknown metal sites. Generally, the refinement of the analysis of an unknown at some point becomes dependent on parallel analyses of structurally relevant crystallographically characterized inorganic model complexes. Because of the importance of the selection of a good model several criteria have been developed (Cramer et al., 1978; Bunker et al., 1982; Teo et al., 1983). We have taken the approach developed by Teo et al. (1983). They have suggested that a good model must exhibit similar structural parameters, but in addition, they suggested that correlations between parameters observed in the multiparameter fitting procedures used to obtain structural information must also be similar.

What follows is an extensive analysis of a set of ten multinuclear manganese complexes, all of which possess structural or physical properties which have been useful to an understanding of the role of manganese in photosynthetic oxygen evolution. A systematic analysis of correlations between parameters in the multiparameter fitting of the EXAFS is also presented in order to determine which inorganic complexes are most relevant as site models. Refinement of the structural information obtained for the Mn site in PSII based on inorganic model complexes selected as described above, the most probable structure for the site is described.

4.2 Materials and Methods

The inorganic complexes examined in this study are: 1. Mn$_2$(III,IV)(μ-O)$_2$-(2,2'-bipyridine)$_4$(ClO$_4$)$_3$ (Plaksin et al., 1972); 2. Mn$_2$(IV,IV)(μ-O)$_2$(picolinate)$_4$ (G. Christou, personal communication); 3. Mn$_2$(III,III)(μ-O)(acetate)$_2$(1,4,7-triazacyclononane)$_2$(ClO$_4$)$_2$ (Wieghardt et al., 1985); 4. Mn$_2$(III,III)(μ-O)(acetate)$_2$(hydrotris(pyrazolylborate))$_2$ (Sheats et al., 1987); 5. Mn$_3$(II,III,III)(μ$_3$-O)(benzoate)$_6$-(pyridine)$_2$(H$_2$O) (Vincent et al., 1987b); 6. [tetra-N-butylammonium]Mn$_3$(III,III,III)-(μ$_3$-O)(benzoate)$_6$(imidazole·H)$_3$ (ClO$_4$)$_2$ (G. Christou, personal communication); 7. Mn$_4$(III,III,III,III)(μ-O)$_2$(acetate)$_7$(2,2'-bipyridine)$_2$ (Vincent et al., 1987a); 8. Mn$_4$(II,III,III,III)(μ-O)$_2$(acetate)$_7$(2,2'-bipyridine)$_2$ (Vincent et al., 1987a); 9. Mn$_4$(III,IV,IV,IV)(μ-O)$_2$(1,4,7-triazacyclononane)$_4$ (ClO$_4$)$_4$ (Wieghardt & Bossek, 1983); 10. Mn$_4$(III,III,III,IV)(μ-O)$_3$(acetate)$_3$(imidazole·H)(imidazole·H$_2$)$_2$ (Bashkin et al., 1987).
Inorganic complexes will be referred to by the bold face number in the list of complexes above. Complex 1 was prepared by the procedure described by Cooper & Calvin (1977). Complexes 2, 5, 6, 7, 8 and 10 were synthesized and crystallographically characterized in the laboratory of Prof. George Christou, Indiana University, Bloomington. Complexes 3 and 9 were synthesized and crystallographically characterized in the laboratory of Prof. Karl Wieghardt, Ruhr-Universität, Bochum, West Germany. Complex 4 was synthesized and crystallographically characterized in the laboratory of Prof. William Armstrong, University of California, Berkeley.

O₂-evolving PSII subchloroplast membrane preparations were prepared from commercially grown spinach as described in section 3.2.1. PSII preparations suitable for X-ray absorption spectroscopy were poised in the S₁ state by long term (> 1 h) dark adaptation at 277 K. PSII preparations poised in the S₂-state were prepared by continuous illumination at 195 K of dark adapted samples. PSII preparation poised in the S₃ state were prepared as described in section 7.2.2.

X-ray absorption spectra were recorded at the Stanford Synchrotron Radiation Laboratory on beam lines IV-2 or VI-2 using a Si(400) double crystal monochromator. Spectra were collected using a fluorescence detection scheme previously described (Jaklevic et al., 1977). EXAFS spectra of PSII preparations were collected using a lithium-drifted silicon solid state X-ray detector. EXAFS spectra of inorganic model complexes were collected using a plastic scintillation array similar to that described by Powers et al. (1981). All EXAFS analyses presented in this report were performed under identical conditions. All analyses were performed on k³-weighted data over the k-range extending from 3.0 to 10.5 Å⁻¹.

4.3 Results and Discussion

4.3.2 Structural Motifs in Multinuclear μ-Oxo Bridged Manganese Complexes

This section contains a brief description of the variety of multinuclear μ-oxo bridged manganese complexes examined in this study. Figure 4-2 contains a schematic representation of the structure of each type of manganese complex examined. The drawings are accurate in that the structures were constructed from the coordinates of each atom obtained from the X-ray crystallographic characterization of each complex. Average distances to each scattering shell are indicated in Figure 4-2.
Complexes 1 and 2 are both di-μ-oxo bridged binuclear manganese complexes. Although the EXAFS analysis of complex 1 has been reported previously (Kirby et al., 1981b), the spectrum of this complex has been remeasured and analysed under identical conditions to those in our study of the PSII preparations poised in the S1, S2 and S3 states for the sake of comparison. As mentioned in the introduction, complex 1 is still one of the best models for the Mn site in PSII, both in terms of structural parameters and magnetic properties. Complex 2 differs from complex 1 in that the terminal ligation consists of a mixture of heterocyclic ring structures and carboxylate ligands both derived from the bidentate picolinate ligand.

Complex 3 and complex 4 are both mono μ-oxo, di-μ aceto-bridged binuclear Mn(III) complexes. The first coordination sphere of these two complexes is very similar. However, complex 4 differs from complex 3 in that the terminal ligation consists of a chelate containing three pyrazole rings. The terminal ligation of complex 3 consists of a tridentate cyclic saturated triamine. Despite the similarity in the core structure of these two complexes the Mn-Mn distances differ significantly. The Mn-Mn distance in complex 4 is 0.08 Å shorter than that in complex 3.

Complexes 5 and 6 are isostructural triangular trimanganese complexes containing a central three-coordinate (e.g. μ3-O) pyramidally distorted oxo bridge. These complexes differ only in the formal valence of the three manganese atoms. Although the Mn(III) ions in complex 6 are not all equivalent, the Mn-Mn distances within this structure differ by less than 0.02 Å. Complex 5 also possesses inequivalent Mn sites; however, in this structure sites clearly identifiable with each formal valence are present. In this structure Mn-Mn distances differ by nearly 0.2 Å. The effect of this large difference in static disorder in the Mn-Mn distances in these two structures is evaluated in section 4.3.2.2.

Complex 7 is a tetrancular manganese complex which contains a core structure which consists of a binuclear di-μ-oxo bridged unit; however, each of the μ-oxo bridges is actually a μ3-oxo bridge involving another Mn(III) site. Both μ3-oxo bridges are pyramidally distorted like those in the trinuclear structures described above. The structure of complex 7 has been described as an “open butterfly-like” tetrancular structure. Two different Mn-Mn distances which differ by nearly 0.5 Å are present within this structure. The terminal ligation of this structure like that in complex 2 contains a mixture of hete-
Figure 4-2. X-ray crystal structures of a representative example of each type of multinuclear complex examined in this study. The structures shown are of (a) a binuclear di-μ-oxo bridged manganese(IV,IV) complex (complex 2) (b) a binuclear mono-μ-oxo di-μ-aceto bridged manganese(III,III) complex (complex 4) (c) a trinuclear μ3-oxo bridged manganese(II,III,III) complex (complex 5) (d) the open butterfly-like tetranuclear manganese(III,III,III,III) complex (complex 7) (e) the adamantane-like tetranuclear manganese(IV,IV,IV,IV) complex (complex 9) and (f) a distorted cubane-like tetranuclear manganese(III,III,III,IV) complex (complex 10). Mean distances to each scattering shell are indicated in each structure.
a) \( \text{Mn}_2(\text{IV,IV})(\mu-\text{O})_2(\text{picolinate})_4 \)
b) \( \text{Mn}_2(\text{III}, \text{III})(\mu-O)(\text{acetate})_2(\text{hydrotris(pyrazolylborate)})_2 \)
Mn₃(III,III,III)(μ₂-O)(benzoate)₆(pyridine)₂(H₂O)
e) \( \text{Mn}_4(\text{IV}, \text{IV}, \text{IV}, \text{IV})(\mu-\text{O})_6(1,4,7\text{-triazacyclononane})_4(\text{ClO}_4)_4 \)
f) $\text{Mn}_4(\text{III,III,III,IV})(\mu-\text{O})_3(\text{acetate})_3(\text{imidazole}\cdot\text{H})(\text{imidazole}\cdot\text{H}_2)_2$
rocyclic rings (bipyridine) and carboxylates. Although good crystals of complex 8 have been obtained, it has not been possible to characterize this complex crystallographically. Other physical measurements indicate that complex 8 is isostructural with complex 7 but differs in the formal valence of the four manganese present.

Complex 9 is an adamantane-like tetranuclear Mn(IV) complex. Adamantane-like tetranuclear structures have been proposed for the structure of the Mn site in PSII poised in the S₃ state (Brudvig & Crabtree, 1986). Complex 9 is a highly symmetrical structure. Each manganese has three μ-oxo bridges to each of the other three Mn atoms. In addition, each manganese is terminally ligated by the tridentate cyclic triamine 1,4,7-triazacyclononane. Thus from an EXAFS standpoint each Mn possesses equivalent scattering shells containing three oxygens, three nitrogens and three Mn neighbors.

Complex 10 is a mixed valence cubane-like tetranuclear complex. Distorted cubane-like structures have been proposed for the structure of the Mn complex in PSII preparations poised in the S₂ state, based on the EPR properties of this S-state (Brudvig & Crabtree, 1986). It is interesting to note that complex 10 exhibits a multi-line EPR spectrum which contains a wealth of hyperfine structure in the region centered around g=2. At low resolution this spectrum looks similar to the multiline EPR spectrum exhibited by PSII preparation poised in the S₂ state. However, the EPR spectrum exhibited by complex 10 has many more hyperfine lines than does the multiline EPR signal; it also has turning points at lower field (e.g. g ~ 5 and g ~ 10). Tentatively the EPR signal associated with complex 10 has been associated with a S=9/2 ground state (G. Christou, personal communication). Complex 10 is unique among the complexes in this study in that it has six covalently bound chloride ligands in the first coordination sphere, including one μ₃-chlorido bridge. Like complex 7, this complex contains two different Mn-Mn distances.

4.3.2 Advantages and Limitations of the EXAFS Analysis of Multinuclear Manganese Complexes

The major limitation to the analysis of multinuclear complexes which is intrinsic to the EXAFS technique is the fact that EXAFS can yield only radial information which is a superposition of the EXAFS of all centers present. When significantly different metal sites are present within a given structure, large static disorders can result in a substantial
loss of structural information obtainable using EXAFS. Two factors render the analysis of multinuclear $\mu$-oxo bridged multinuclear manganese complexes tractable: 1) The low static disorder and high intrinsic backscattering power of Mn neighbors dominates the EXAFS due to second shell scatterers (e.g. neighboring atoms in the range of 2.6 - 4.0 Å from the absorbing atom), and thus permits a reasonably accurate determination of both the number of and distance to Mn neighbors in multinuclear $\mu$-oxo bridged complexes; 2) The low static disorder and small thermal disorder of the relatively rigidly bound $\mu$-oxo bridges allow a reasonably accurate determination of the number of $\mu$-oxo bridges present in a multinuclear complex.

Because it is generally accepted that there are four manganese atoms present in PSII, we have restricted our analysis to complexes with a maximum nuclearity of four. Thus the static disorder in Mn-Mn distances is generally small. In addition, because the $\mu$-oxo bridges between Mn atoms in these complexes are relatively strong covalent bonds, the thermal disorder is also small. X-ray crystal structures of the complexes examined in this study indicate that light elements (carbon and oxygen) must also contribute to the EXAFS of the second shell. However, the large static disorder present in these light element shells results in substantial self anihilation effects which radically reduce the contribution made by these atoms to the observed EXAFS. One non-parametric method which demonstrates the dominant contribution made by backscattering from Mn neighbors in multinuclear manganese complexes is k-weighting behavior.

4.3.2.1 k-Weighting Behavior

Principally as a consequence of the shape of the envelope of the EXAFS wave, resulting from backscatter from a heavy atom like manganese, the amplitude of peaks in the Fourier transform of the EXAFS due to heavy atoms increases more rapidly with increasing powers of the photo-electron wave vector (k) than do peaks resulting from backscattering from light elements like carbon, oxygen or nitrogen. Figure 4-3 contains several examples of k-weighting behavior. In all cases the Fourier transform spectra are plotted normalized to the first peak. Note that the increase in the amplitude of the manganese peaks exhibited with increasing k-weighting in the Fourier transforms of the EXAFS of the inorganic complexes is very similar to that observed for the peak corresponding to the second shell of the EXAFS of the Mn complex in PSII preparations poised in the $S_1$ state (see Figure 4-3 d). This amplitude enhancement is also evident in
the second shell EXAFS of PSII preparations poised in the $S_2$ state (see Figure 7-7a).

Another important consequence of the dominant contribution of the Mn backscattering in the second shell is that Mn-Mn distances determined by multiparameter fits of Fourier isolates of the second shell tend to be very accurate (e.g. are in good agreement with results obtained by X-ray crystallography). A comparison of the Mn-Mn distances obtained through the simulation of the EXAFS with the distances obtained from X-ray crystallography indicates that agreement to within ±0.03 Å is generally obtainable (see Table 4-1). Ten of twelve crystallographically characterized Mn-Mn distances are predicted to within this accuracy. Note, however, that there are two exceptions. The Mn-Mn distance predicted from the simulation of the EXAFS of complex 5 is 0.04 Å shorter than the distance determined by X-ray crystallography, and the Mn-Mn distance predicted from simulation of the EXAFS of complex 4 is 0.06 Å shorter than that determined by crystallography. For complex 5, this error may be due to the fact that the EXAFS of this complex is inadequately modelled with two Mn waves in that carbon EXAFS must also contribute to this shell.

Two components (e.g. a Mn-Mn and a Mn-C or two Mn-Mn EXAFS waves) were used in the simulation of the EXAFS due to backscattering from the second shell for three reasons: 1) Based on Mn neighbor distances calculated from the coordinates obtained from the X-ray crystal structures of these compounds, light elements like carbon or oxygen must contribute to the EXAFS of the second shell; 2) Fits to the second shell EXAFS generally improve substantially upon addition of a second component; 3) Fits to the second shell EXAFS of the Mn complex in PSII generally also improve substantially upon addition of a second component. Thus, comparisons of the quality of the fits obtained using two components for the inorganic complexes with the fits obtained for the Mn complex in PSII provide one criterion for deciding which inorganic complexes most closely resemble the structure of the unknown Mn site.

It is important to note that for a number of the complexes studied (e.g. 5, 7, 8 and 10) which contain Mn-Mn distances differing by more than 0.2 Å from one another, a Mn-Mn and a Mn-C wave simulation was inadequate to describe the complex beat structure caused by the interference of the two Mn-Mn waves. For example, the Fourier isolates and fits to the second shell of complex 2, 5 and 7 are plotted in Figure 4-4. Simulation results obtained from the two-component fits plotted are contained in
Figure 4-3. k-weighting behavior of the Fourier transforms of the EXAFS of multinuclear $\mu$-oxo-bridged manganese complexes and the Mn complex in a PSII preparation poised in the $S_1$ state. The Fourier transforms of the EXAFS spectra plotted are the $k^1$-weighted (_______), $k^2$-weighted (_____ ...), $k^3$-weighted (____ ...), of (a) a binuclear complex (complex 2) (b) a trinuclear complex (complex 6) (c) a tetranuclear complex (complex 7) and (d) the Mn complex in a PSII preparation poised in the $S_1$ state.
Relative Amplitudes

Apparent Distance $R' \, (\text{Å})$

- $k^1$-weighted
- $k^2$-weighted
- $k^3$-weighted
Relative Amplitudes

Apparent Distance $R'$ (Å)
Table 4-1
Simulation Results for the Second Shell of Ten Inorganic Complexes$^a$

$k^3$-weighted EXAFS Data

<table>
<thead>
<tr>
<th>Complex</th>
<th>N</th>
<th>R(Å)</th>
<th>$\sigma^2$(Å$^2$)</th>
<th>$\Delta E_0$(eV)</th>
<th>N</th>
<th>R(Å)</th>
<th>$\sigma^2$(Å$^2$)</th>
<th>$\Delta E_0$(eV)</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.8(1.0)$^d$</td>
<td>2.72(2.72)</td>
<td>0.005(0.000)</td>
<td>-1.</td>
<td>1.4(8.0)</td>
<td>3.07(3.02)</td>
<td>0.010(0.009)</td>
<td>0.0</td>
<td>24.4</td>
</tr>
<tr>
<td>2.</td>
<td>1.5(1.0)</td>
<td>2.72(2.75)</td>
<td>0.010(0.000)</td>
<td>-10.</td>
<td>1.1(6.0)</td>
<td>2.88(2.89)</td>
<td>0.010(0.0015)</td>
<td>-2.</td>
<td>8.6</td>
</tr>
<tr>
<td>3.</td>
<td>0.9(1.0)</td>
<td>3.11(3.08)</td>
<td>0.005(0.000)</td>
<td>-10.</td>
<td>1.3(6.0)</td>
<td>2.96(3.00)</td>
<td>0.010(0.004)</td>
<td>0.0</td>
<td>4.97</td>
</tr>
<tr>
<td>4.</td>
<td>0.8(1.0)</td>
<td>3.10(3.16)</td>
<td>0.009(0.000)</td>
<td>-8.</td>
<td>0.7(9.0)</td>
<td>3.22(3.15)</td>
<td>0.010(0.020)</td>
<td>-1.</td>
<td>2.31</td>
</tr>
<tr>
<td>5.</td>
<td>1.6(2.0)</td>
<td>3.25(3.27)</td>
<td>0.006(0.000)</td>
<td>-10.</td>
<td>2.0(2.0)</td>
<td>2.87(3.04)</td>
<td>0.010(0.0025)</td>
<td>-1.</td>
<td>36.9</td>
</tr>
<tr>
<td>6.</td>
<td>1.5$^e$(3.0)</td>
<td>3.25(3.22)</td>
<td>0.005(&lt;0.001)</td>
<td>0.0</td>
<td>3.2(6.0)</td>
<td>2.90(2.94)</td>
<td>0.010(0.0026)</td>
<td>-1.</td>
<td>70.3</td>
</tr>
<tr>
<td>7.</td>
<td>0.5(0.7)</td>
<td>3.26(3.22)</td>
<td>0.005(0.000)</td>
<td>-10.</td>
<td>0.8(1.3)</td>
<td>3.44(3.41)</td>
<td>0.005(&lt;0.001)</td>
<td>-10.</td>
<td>6.02</td>
</tr>
<tr>
<td>8.</td>
<td>0.6(0.5)</td>
<td>2.87(2.85)</td>
<td>0.005(0.000)</td>
<td>-4.</td>
<td>0.5(2.0)</td>
<td>3.34(3.34)</td>
<td>0.005(0.0018)</td>
<td>-10.</td>
<td>2.3</td>
</tr>
<tr>
<td>9.</td>
<td>0.4(-)</td>
<td>2.90(-)</td>
<td>0.005(-)</td>
<td>-4.</td>
<td>0.5(-)</td>
<td>3.42(-)</td>
<td>0.005(-)</td>
<td>0.0</td>
<td>1.9</td>
</tr>
<tr>
<td>10.</td>
<td>1.3(1.5)</td>
<td>2.81(2.81)</td>
<td>0.005(&lt;0.001)</td>
<td>-10.</td>
<td>0.4(1.5)</td>
<td>3.26(3.28)</td>
<td>0.005(0.0015)</td>
<td>-10.</td>
<td>10.3</td>
</tr>
</tbody>
</table>

$^a$ The inorganic complexes examined here are referred to by the bold face number in the list of complexes in materials and methods (see section 4.2).
b. Frequently, unconstrained values of the Debye-Waller factor yielded unrealistic results (e.g. 0.0000 or > 0.025 Å²). In such cases the Debye-Waller factor was constrained to some reasonable value (e.g. 0.005 Å for a Mn - Mn shell and 0.010 for the a carbon shell).

c. F is the least squares difference between the Fourier isolated EXAFS and the EXAFS calculated using theoretically derived phase and amplitude functions.

d. Numbers in parentheses are parameters calculated from the crystal structure of each inorganic complex. Only the static contribution to the Debye-Waller factor has been calculated here. Since the thermal contribution is often as large or larger than the static term, the simulated values are invariably larger than the calculated static values.

e. The low number of manganese neighbors predicted by simulation of the EXAFS of this model is principally a consequence of the fact that two waves (i.e. a Mn-Mn and a Mn-C wave) are inadequate to describe the second shell EXAFS of this inorganic complex. A well defined shell of oxygens at 3.44 Å also exists in this complex. Inclusion of a third oxygen wave improves the fit quality substantially (e.g. F=46.) and yields a much better estimate of the number of manganese neighbors (e.g. 2.4).
Table 4-2
Simulation Results for the Second Shell of
PSII Preparations Poised in the $S_1$, $S_2$ and $S_3$ States

$k^3$-weighted EXAFS Data

<table>
<thead>
<tr>
<th>State</th>
<th>N</th>
<th>$R$(Å)</th>
<th>$\sigma^2$(Å$^2$)</th>
<th>$\Delta E_0$(eV)</th>
<th>N</th>
<th>$R$(Å)</th>
<th>$\sigma^2$(Å$^2$)</th>
<th>$\Delta E_0$(eV)</th>
<th>$F^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_1$</td>
<td>1.4</td>
<td>2.72</td>
<td>0.009</td>
<td>-10.</td>
<td>1.2</td>
<td>3.18</td>
<td>0.010</td>
<td>-10.</td>
<td>2.9</td>
</tr>
<tr>
<td>$S_2$</td>
<td>1.8</td>
<td>2.72</td>
<td>0.011</td>
<td>-10.</td>
<td>2.6</td>
<td>3.11</td>
<td>0.010</td>
<td>-10.</td>
<td>3.1</td>
</tr>
<tr>
<td>$S_3$</td>
<td>1.7</td>
<td>2.72</td>
<td>0.020</td>
<td>-10.</td>
<td>1.7</td>
<td>3.12</td>
<td>0.010</td>
<td>-10.</td>
<td>6.9</td>
</tr>
</tbody>
</table>

a. Average results obtained from the simulation of the EXAFS of PSII preparations poised in the $S_3$ state. In general, distances obtained for individual samples differed by less than 0.02 Å. The number of scatterers determined at a given distance was found to be within 30%.

b. Difference EXAFS of a PSII preparation poised in the $S_3$ state by the ferricyanide procedure (see section 7.3.2).

c. $F$ is the least squares difference between the Fourier isolated EXAFS and the EXAFS calculated using theoretically derived phase and amplitude functions (Teo & Lee, 1979).
Table 4-1. The second shell EXAFS of complex 2 which contains only one Mn-Mn distance is plotted (Figure 4-4) to illustrate the difference in complexity of the EXAFS obtained when two different Mn-Mn shells are present. As mentioned above, the Mn-Mn distances predicted by the EXAFS simulations are in good agreement with the distances determined by X-ray crystallography.

It is also important to note that for two of the complexes studied (e.g. complexes 1 and 9) the addition of a third component (another Mn-C or Mn-O wave) improved the simulation significantly (e.g. beyond the ratio of the number of degrees of freedom for each simulation). For complex 1 simulations using three waves yield least square residuals two orders of magnitude smaller than those obtained with two waves. The least square residual decreases from 24.4 to 0.10. Because this improvement in fit quality upon the addition of a third EXAFS component is not generally observed for the simulation of the EXAFS of the Mn complex in PSII, these complexes (e.g. 1 and 9) are not so relevant as models for the unknown site.

For comparison, the Fourier isolate and fits to the second shell EXAFS of the Mn complex in a PSII preparation poised in the $S_1$ state are contained in Figure 4-5. Also compare the simulations of the EXAFS of the Mn complex in PSII preparations poised in the $S_2$ and $S_3$ states (e.g. see Figures 7-9 and Table 7-5).

Simulation results for the fits shown in Figure 4-5 are contained in Table 4-2 and 4-1. A comparison of the simulation results obtained for the second shell EXAFS for the Mn complex in PSII with those of the model complexes, indicates that complex 1 and complex 2 have structural parameters most similar to those of the unknown. For comparison, the second shell EXAFS and fits of complex 2 are also plotted in Figure 4-5. The second shell EXAFS of this complex is strikingly similar to that of the Mn complex in PSII. Note that unlike complex 1, the envelope shape, phase of the EXAFS and frequency of the waves are nearly identical for complex 2 and the Mn complex in PSII preparations poised in the $S_1$ state. As will be shown below, correlations between parameters observed in the simulation of 2 and the Mn complex in PSII are also very similar.

**4.3.2.2 EXAFS Amplitude Effects Which Are Indicative of Valence**

Complexes 5 and 6, and 7 and 8 are pairs of isostructural, trinuclear and tetranuclear,
Figure 4-4. Fourier isolates ( ) and fits ( ) to the EXAFS due to backscattering from second shell atoms of three inorganic complexes. The EXAFS spectra and fits shown are of (a) a binuclear complex (complex 2), (b) a trinuclear complex (complex 5) and (c) a tetranuclear complex (complex 7). Note that both complexes 5 and 7 have two Mn-Mn distances which differ by 0.2 Å.
a) Mn(IV) picolinate binuclear complex

Least Square Residual = 8.6

<table>
<thead>
<tr>
<th>Atom type</th>
<th>Mn</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma(A)$</td>
<td>2.72</td>
<td>2.89</td>
</tr>
<tr>
<td>$N$</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>$\sigma^2$</td>
<td>0.010</td>
<td>0.01</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-10</td>
<td>-2</td>
</tr>
</tbody>
</table>

b) Mn(II,III,III) benzoate trimer

Least Square Residual = 6.02

<table>
<thead>
<tr>
<th>Atom type</th>
<th>Mn Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma(A)$</td>
<td>3.26</td>
</tr>
<tr>
<td>$N$</td>
<td>0.5</td>
</tr>
<tr>
<td>$\sigma^2$</td>
<td>0.005</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-10</td>
</tr>
</tbody>
</table>
c) Mn(III) acetate tetranuclear complex

<table>
<thead>
<tr>
<th>Atom type</th>
<th>Mn</th>
<th>Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>r(Å)</td>
<td>2.87</td>
<td>3.34</td>
</tr>
<tr>
<td>N</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>σ²</td>
<td>0.005</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Least Square Residual = 2.2
Figure 4-5. Plotted in (a) are the Fourier isolates ( ) and fits ( o o o o ) to the EXAFS due to backscattering from second shell atoms for the manganese complex in PSII poised in the S₁ state. For comparison the Fourier isolates and fits of the EXAFS due to second shell scatterers present in complex 2 are plotted in (b).
a) $S_1$ State

b) Mn(IV) picolinate binuclear complex

Least Square Residual=2.9

<table>
<thead>
<tr>
<th>Atom type</th>
<th>Mn</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>r(Å)</td>
<td>2.72</td>
<td>3.18</td>
</tr>
<tr>
<td>N</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>$\sigma^2$</td>
<td>0.009</td>
<td>0.010</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-10.</td>
<td>-10.</td>
</tr>
</tbody>
</table>

Least Square Residual=8.6

<table>
<thead>
<tr>
<th>Atom type</th>
<th>Mn</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>r(Å)</td>
<td>2.72</td>
<td>2.89</td>
</tr>
<tr>
<td>N</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>$\sigma^2$</td>
<td>0.010</td>
<td>0.01</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-10.</td>
<td>-2.</td>
</tr>
</tbody>
</table>
μ-oxo bridged manganese complexes which differ from one another by one unit of valence. In each case, the complex with the lower valence contains one manganese with a formal valence of II which is not present in the higher valence Mn complex. Neither complex 5 nor 6 is totally symmetric. However, the spread in Mn-Mn distances in complex 6 is about 0.02 Å, while the spread in Mn-Mn distances in complex 5 is 0.2 Å. The spread in distances indicated here is the difference between the longest Mn-Mn distance and the shortest Mn-Mn distance present in the crystal structure. This increase in static disorder in the Mn-Mn results in a dramatic reduction in the amplitude of the EXAFS observed for complex 5 relative to complex 6 (see Figure 4-6). Similar, but somewhat less pronounced, amplitude damping effects are observed for complex 8 relative to complex 7 (see Figure 4-6 b).

As described in section 2.2.1, the amplitude of the EXAFS observed for a given scattering shell depends exponentially on the magnitude of the disorder in that shell. Similar EXAFS amplitude reductions have been observed due to increases in the thermal disorder of a scattering shell which occur with increasing temperature (Brown & Eisenberger, 1979; Scott et al., 1986). Disorder within a scattering shell is parameterized in terms of a Debye-Waller like parameter. For the strong bond limit which is appropriate for all Mn-N and Mn-O bonds within the structures examined in this study, the thermal contribution to the Debye-Waller factor is given by Equation 7-1. Teo (1986b) has shown that increases in the thermal contribution to the Debye-Waller factor which occur with increasing temperature for bonds in the strong-bond limit are relatively small. Thus, the amplitude reductions observed in the Fourier transforms of the EXAFS of the pairs of isostructural complexes, shown in Figure 4-5, must be due principally to changes in static disorder associated with the change in formal valence.

As described in detail in chapter 5, each of the formal valences of Mn (e.g. Mn(II), Mn(III) and Mn(IV)) possess electronic structures which have significant different effects on both the bond lengths and distribution of bond lengths present. The electronic structure of Mn(II) is totally symmetric and, hence, interaction with the ligation sphere does not produce any net ligand field stabilization. As a result, Mn(II) complexes are relatively ionic, exhibiting almost totally symmetric first coordination sphere bond lengths. A typical Mn-O bond length for Mn(II) complexes is 2.15 Å. Because of the greater positive electrostatic potential present in the higher valences, Mn(III) and
Figure 4-6. Fourier transforms of the EXAFS of pairs of isostructural trinuclear manganese complexes which differ by one unit of valence. In (a) the Fourier transforms plotted are of the EXAFS of the trinuclear complexes 5 (___) and 6 (_____). In (b) the Fourier transforms plotted are of the EXAFS of the tetranuclear complexes 7 (____) and 8 (___). The Fourier transforms plotted are of k\(^1\)-weighted data for the k-range extending from 3.0 to 10.0 Å\(^{-1}\).
Mn(IV) complexes exhibit substantially shorter first coordination sphere bond lengths. Sites within the structure of complex 5 have been associated with each of the formal valences of Mn present (See Figure 4-2). The longer first coordination sphere bonds present in the structure of complex 5, which are due to the presence of a Mn(II) ion not present in complex 6, result not only in a greater spread in first coordination sphere bond lengths, but also in a larger spread in Mn-Mn distances as described above. The result is a significant reduction in the amplitude of Fourier peaks associated with both the first and second scattering shells.

Similar amplitude reductions are observed for the EXAFS of the Mn complex in PSII preparations which have been illuminated at 195 K in the presence of hydroxylamine relative to those PSII preparations not containing hydroxylamine (see Figure 6-5). For the sake of comparison, the Fourier transform spectra plotted for the complexes 5, 6, 7 and 8, shown in Figure 4-6, have been plotted for the same k-range and k-weighting as the spectra plotted in Figure 6-5. This similarity of the amplitude reductions observed, particularly for the EXAFS of 8 relative to 7, are indicative that generation of the \( S_0^* \) state by illumination at 195 K in the presence of hydroxylamine produces a more reduced Mn complex (relative to the Mn complex in the \( S_1 \) state) containing one Mn with a formal valence of II within the four Mn present in PSII.

4.3.2.3 Disorder in the Second Coordination Sphere

In general the structural parameters obtained from the simulation of EXAFS are limited in their accuracy by correlations between parameters in the multivariate non-linear least square fitting procedures used. A weak correlation between the energy of the ionization threshold, \( E_0 \), and the distance to a given scattering shell, \( r \), limits the accuracy in the determination of distances to ± 0.03 Å. For an unknown system the errors introduced by the correlation between \( E_0 \) and \( r \) may be reduced by constraining the variation in \( E_0 \) to a range which results in good agreement between distances obtained by simulation and distances obtained from the crystallographic characterization of inorganic models (Teo et al., 1983).

A stronger correlation between the number of atoms at a given distance and the disorder in the distance to atoms in that shell often limits the determination in the number of scatterers to ± 50 %. Methods have been developed to alleviate this parameter-
correlation limitation in fitting an unknown system based on parallel analyses of inorganic complexes of known structure. At this point the structural parameters obtained become model dependent, and as a result the selection of a "good" model becomes an important part of the analysis. Several criteria have been developed to select a good model (Cramer et al., 1978; Bunker et al., 1982; Teo et al., 1983).

According to Teo et al.(1983) a good model for the unknown system must not only possess similar structural parameters as determined from the best fits based on theory (BFBT), but must also exhibit similar parameter correlations. That is the curvature in the plot of the number of neighboring atoms versus disorder should be similar. The fine adjustment techniques developed by Teo et al. (1983) have been applied to the analysis of the iron EXAFS of the iron-molybdenum cofactor of nitrogenase (Antonio et al., 1982a), the iron EXAFS of the 3 Fe ferredoxin obtained from Desulfovibrio gigas (Antonio et al., 1982b), and the iron EXAFS of the binuclear iron centers in ribonucleotide reductase and hemerythrin (Scarrow, 1987). For the case of the 3 Fe ferredoxin, it is noteworthy to point out that the structural parameters obtained (e.g. the distances to neighboring iron and sulfur atoms and the number of iron and sulfur neighbors) are in good agreement with results obtained from an X-ray crystallographic study of this protein (Ghosh et al., 1981).

In this report we have applied the criteria established by Teo et al.(1983) to the study of the Mn complex in O₂-evolving PSII membrane preparations from spinach. It is shown that those inorganic complexes which exhibit EXAFS which are strikingly similar to the EXAFS of the Mn complex in PSII, satisfy the criteria established by Teo et al. for the selection of a good model. Further, other inorganic complexes with similar structural parameters exhibit markedly different EXAFS and are shown not to satisfy these criteria. We assert that these results indicate that the EXAFS are a sensitive function of the exact nature of the metal coordination and provide additional support for the criteria established by Teo et al.(1983).

All tables in this chapter contain the BFBT. In this section a complex which satisfies the criteria established by Teo et al. for the manganese shell observed in the EXAFS of PSII preparations is selected among ten multinuclear μ-oxo bridged complexes. In section 4.3.2.4 complexes which satisfy these criteria for the μ-oxo bridging shell of the Mn complex in PSII are selected. In section 4.4 corrected values for the number of μ-oxo
bridges and the number of manganese neighbors in the Mn complex in PSII preparations poised in the S₁, S₂ and S₃ states are calculated based on scale factors obtained from the complexes selected as described above. Finally based on these adjusted numbers the most probable structure for the Mn complex in PSII is suggested (see section 4.5).

Structural parameters obtained from the simulation of the EXAFS due to second shell scatterers present in the inorganic complex indicate that complexes 1 and 2 are the most similar to that of the Mn complex in PSII (compare Tables 4-1 and 4-2). The Mn-Mn distances in complexes 7 and 10 are somewhat longer than those determined for the Mn-complex in PSII, but are perhaps worth considering further as models for use in the refinement of the structural parameters of the unknown site. As described above Teo et al. (1983) have suggested that a good site model should not only have similar structural parameters to those exhibited by the unknown under investigation, but also exhibit similar parameter correlations in the multiparameter fits used to obtain structural information from EXAFS spectra.

Figure 4-7 a contains a plot of the apparent number of Mn neighbors present as determined by simulation of the EXAFS of complexes 1, 2, 7 and 10 versus the disorder present in the Mn-Mn distances. The curves were constructed by systematically varying the Debye-Waller factor for the short Mn-Mn distance, allowing the number of Mn neighbors, the distance to these manganese neighbors and a correction to the ionization threshold, $\Delta E_0$, (see section 2.2.1) to vary, while constraining all other parameters in the two component simulations to the best fit values contained in Table 4-1. For comparison, the same curve obtained for the Mn complex in PSII preparations poised in the S₁ and S₂ states is also plotted. The number of manganese neighbors predicted at each Debye-Waller level for complexes 2 and 10 is very similar to that observed for the Mn complex in PSII. It is important to note, however, that complex 10 contains two different Mn-Mn distances and that the shorter of the two is significantly longer (e.g. 0.09 Å) than that obtained for the Mn complex in PSII.

The Fourier isolates and fits of the second shell EXAFS of all model complexes were performed under identical conditions to those used for the Mn complex in PSII. Thus, a comparison of the quality of the fit obtained using two components and the systematic variation in the quality of that fit as a function of the Debye-Waller factor are also important considerations in selecting among possible site models. The quality
Figure 4-7. (a) The number of Mn neighbors obtained by simulation of the second shell EXAFS plotted as a function of the Debye-Waller factor for the Mn-Mn component. Curves plotted are for complexes 1, 2, 7, 10 and for the second shell EXAFS of the Mn complex in PSII preparations poised in the $S_1$ and $S_2$ states. Details of the simulation procedure used to construct these curves are described in the text.

(b) The least square residual obtained for the simulation of the second shell EXAFS plotted as a function of the magnitude of the Debye-Waller factor for complexes 1, 2, 7 and 10. Also plotted is the variation in the least square residual as a function of the magnitude of the Debye-Waller for the second shell EXAFS of PSII preparations poised in the $S_1$ and $S_2$ states.
a) Number of Mn Neighbors vs Disorder

b) Least Square Residual vs Disorder
of the fit is defined as the least-square residual between the Fourier isolate of the second shell EXAFS and the fit obtained using the theoretical phase and amplitude functions derived by Teo and Lee (1979).

Figure 4-7 b contains plots of the variation of the least-square residual for a given fit obtained by systematically varying the Debye-Waller factor of the Mn-Mn shell. These curves were constructed in a manner completely analogous to the procedure described above for the variation in the apparent number of Mn neighbors versus the disorder in the Mn-Mn distance (Figure 4-7 a). Based on this criterion and the criteria described above complex 2 is clearly the best model for the second coordination sphere of the manganese complex in PSII.

4.3.2.4 A Systematic Study of Disorder in the First Coordination Sphere

In this section, a study analogous to that presented in the previous section, but of the apparent number of μ-oxo bridges in the first coordination, is presented. Structural parameters obtained from two component fits to the EXAFS resulting from backscattering from the first coordination sphere of the ten inorganic complexes are contained in Table 4-3. Two components were used in each case for similar reasons that two components were used in simulations of the second coordination sphere. First, from the known bond lengths in the first coordination sphere, there are at least two well defined groups of bonds with distinctly different characteristics. There is a set of short bridging oxygen bonds with a small degree of static disorder. In addition, there is a longer set of bonds with a larger spread in Mn-O or N distances, corresponding to the terminal ligation sphere. Second, as was the case with the second shell EXAFS, the fit quality obtained with two waves is in general substantially better than that obtained with a single Mn-O wave. And third, the EXAFS due to the first coordination sphere of the Mn complex in PSII is best fit using two components. Thus, for the sake of comparison under identical conditions, two component fits were also used with the inorganic complexes.

It should be noted that for several of the complexes studied, most notably, complexes 1, 3, 4 and 5, the quality of the fit obtained with three components was superior to that obtained with two. For example, for complex 5, the least-square residual between the Fourier isolate and the fit decreases by a factor of more than 12 upon the addition of a third component. For complex 3, the reduction in the least-square residual is nearly a
Table 4-3
Simulation Results for the First Coordination Sphere of
Ten Inorganic Complexes

$k^3$-weighted EXAFS Data

<table>
<thead>
<tr>
<th>Complex</th>
<th>N</th>
<th>R(Å)</th>
<th>$\sigma^2$(Å$^2$)</th>
<th>$\Delta E_0$(eV)</th>
<th>N</th>
<th>R(Å)</th>
<th>$\sigma^2$(Å$^2$)</th>
<th>$\Delta E_0$(eV)</th>
<th>F$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.7(2.0)$^a$</td>
<td>1.75(1.82)</td>
<td>0.005(0.0016)</td>
<td>-10.</td>
<td>3.7(4.0)</td>
<td>1.95(2.11)</td>
<td>0.023(0.006)</td>
<td>-20.</td>
<td>13.5</td>
</tr>
<tr>
<td>2.</td>
<td>1.5(2.0)</td>
<td>1.79(1.82)</td>
<td>0.005(0.000)</td>
<td>-10.</td>
<td>2.8(4.0)</td>
<td>2.23(1.97)</td>
<td>0.010(0.006)</td>
<td>-20.</td>
<td>39.5</td>
</tr>
<tr>
<td>3.</td>
<td>2.0(1.0)</td>
<td>1.82(1.80)</td>
<td>0.005(0.000)</td>
<td>0.0</td>
<td>5.1(5.0)</td>
<td>1.98(2.10)</td>
<td>0.005(0.004)</td>
<td>-20.0</td>
<td>25.2</td>
</tr>
<tr>
<td>4.</td>
<td>1.2(1.0)</td>
<td>2.17(1.77)</td>
<td>0.005(0.000)</td>
<td>0.0</td>
<td>2.5(5.0)</td>
<td>1.97(2.11)</td>
<td>0.005(0.004)</td>
<td>-20.0</td>
<td>25.2</td>
</tr>
<tr>
<td>5.</td>
<td>2.8(0.7)</td>
<td>1.95(1.82)</td>
<td>0.007(0.000)</td>
<td>0.0</td>
<td>3.2(5.3)</td>
<td>2.07(2.10)</td>
<td>0.0013(0.009)</td>
<td>-20.</td>
<td>12.9</td>
</tr>
<tr>
<td>6.</td>
<td>0.9(1.0)</td>
<td>1.88(1.89)</td>
<td>0.0018(&lt;0.001)</td>
<td>-20.</td>
<td>1.1(5.0)</td>
<td>1.89(2.04)</td>
<td>0.0026(0.010)</td>
<td>0.0</td>
<td>19.2</td>
</tr>
<tr>
<td>7.</td>
<td>1.4(1.5)</td>
<td>1.83(1.88)</td>
<td>0.010(0.0025)</td>
<td>-10.</td>
<td>2.0(4.5)</td>
<td>2.23(2.07)</td>
<td>0.0026(0.014)</td>
<td>-20.</td>
<td>0.40</td>
</tr>
<tr>
<td>8.</td>
<td>1.2(- )</td>
<td>1.91(- )</td>
<td>0.007(- )</td>
<td>0.0</td>
<td>1.3(- )</td>
<td>2.18(- )</td>
<td>0.009(- )</td>
<td>-6.</td>
<td>9.2</td>
</tr>
<tr>
<td>9.</td>
<td>2.1(3.0)</td>
<td>1.74(1.78)</td>
<td>0.005(0.0005)</td>
<td>-10.</td>
<td>3.7(3.0)</td>
<td>2.03(2.09)</td>
<td>0.010(0.006)</td>
<td>-20.</td>
<td>27.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Complex</th>
<th>N</th>
<th>R(Å)</th>
<th>$\sigma^2$(Å$^2$)</th>
<th>$\Delta E_0$(eV)</th>
<th>N</th>
<th>R(Å)</th>
<th>$\sigma^2$(Å$^2$)</th>
<th>$\Delta E_0$(eV)</th>
<th>F$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.</td>
<td>0.8(0.8)$^e$</td>
<td>1.81(1.83)</td>
<td>0.003(0.001)</td>
<td>-18.</td>
<td>1.2(1.2)$^f$</td>
<td>2.17(2.26)</td>
<td>0.019(0.010)</td>
<td>-1.</td>
<td>2.1</td>
</tr>
</tbody>
</table>

a. The inorganic complexes examined here are referred to by the bold face number in the list of complexes in materials and methods (see section 4.2).
b. Frequently unconstrained values of the Debye-Waller factor yielded unrealistic results (e.g. 0.000 or >0.025 Å²). In such cases the Debye-Waller factor was constrained to some reasonable value (e.g. 0.005 Å² for a Mn - O shell and 0.010 Å² for the terminal ligation shell).

c. F is the least squares difference between the Fourier isolated EXAFS and the EXAFS calculated using theoretically derived phase and amplitude functions.

d. Numbers in parentheses are parameters calculated from the crystal structure of each inorganic complex. Only the static contribution to the Debye-Waller factor has been calculated here. Since the thermal contribution is often as large or larger than the static term, the simulated values are generally larger than the calculated static values.

e. Note that only the three short µ-oxo bridges to the unique apical Mn(IV) in this structure have been considered in the calculated values. All other µ-oxo bridges are anomalously long and are averaged into the terminal ligation shell.

f. Only the terminal Cl atoms have been considered in the values calculated from the crystal structure.
Table 4-4

Simulation Results for the First Coordination Sphere of
PSII Preparations Poised in the S₁, S₂ and S₃ States

<table>
<thead>
<tr>
<th>State</th>
<th>N</th>
<th>R(Å)</th>
<th>σ²(Å²)</th>
<th>ΔE₀(eV)</th>
<th>N</th>
<th>R(Å)</th>
<th>σ²(Å²)</th>
<th>ΔE₀(eV)</th>
<th>F₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₁</td>
<td>1.5</td>
<td>1.75</td>
<td>0.004</td>
<td>-20.</td>
<td>2.2</td>
<td>2.24</td>
<td>0.009</td>
<td>-20.</td>
<td>17.8</td>
</tr>
<tr>
<td>S₂</td>
<td>1.8</td>
<td>1.77</td>
<td>0.007</td>
<td>-13.</td>
<td>2.8</td>
<td>2.23</td>
<td>0.013</td>
<td>-20.</td>
<td>9.73</td>
</tr>
<tr>
<td>S₃</td>
<td>1.8</td>
<td>1.81</td>
<td>0.009</td>
<td>-11.</td>
<td>2.1</td>
<td>2.20</td>
<td>0.015</td>
<td>-20.</td>
<td>1.21</td>
</tr>
</tbody>
</table>

a. Average results obtained from the simulation of the EXAFS of PSII preparations poised in the S₂ state. In general, distances obtained for individual samples differred by less than 0.02 Å. The number of scatterers determined at a given distance was found to be within 30 %.

b. Difference EXAFS of a PSII preparation poised in the S₃ state by the ferricyanide procedure. This sample was illuminated at 240K. Difference EXAFS results obtained for a S₃ State PSII sample prepared by the PPBQ procedure were in good agreement with the results presented for samples prepared by the ferricyanide method.

c. F is the least squares difference between the Fourier isolated EXAFS and the EXAFS calculated using theoretically derived phase and amplitude functions (Teo & Lee, 1979).
factor of 4. For complex 4, the reduction in the least-square residual is nearly a factor of 100. All of the complexes that show substantial improvement in fit quality, upon addition of a third component, contain Mn ions with a formal valence of III. High spin Mn(III) complexes have a degenerate ground state electronic structure. Complexes with a degenerate ground state often exhibit significant bond length distortions due to the Jahn-Teller effect (Bersuker, 1975). For Mn(III) complexes, a tetragonal distortion due to the Jahn-Teller effect often results in an axial set of bonds with significantly longer bond lengths than the equatorial set. Thus, the three classes of bonds corresponding to the three components found in the simulation of the first coordination sphere, correspond to the short \( \mu \)-oxo bridging Mn-O bonds and the axial and equatorial bonds resulting from the Jahn-Teller distortion of Mn(III) ions.

For comparison, results obtained from fits to the EXAFS of the first coordination sphere of the Mn complex in PSII preparations poised in the \( S_1 \), \( S_2 \) and \( S_3 \) states are contained in Table 4-4. Unlike complex 1, 3, 4 and 5, addition of a third component in the simulation of the EXAFS of the Mn complex in PSII does not improve the quality of the fit for any of the S-states examined. The quality of the fit obtained for the first coordination sphere of the Mn complex in PSII preparation poised in the \( S_3 \) state is significantly better than that obtained for the \( S_1 \) or \( S_2 \) state preparations. The fit quality obtained for the \( S_3 \) state preparation is comparable to that obtained for complex 7. In fact, the EXAFS of these two complexes are strikingly similar (compare Figure 4-8 a and b). The quality of the fit obtained for complex 7 is perhaps surprising in light of the discussion regarding three component fits of Mn(III) complexes in the preceding paragraph. However, it should be noted that although complex 7 is a tetranuclear Mn(III) complex, none of the four manganese present are equivalent. The eighteen unique terminal Mn-O or Mn-N bond lengths present in this complex form a virtually continuous distribution of bond lengths, spanning the axial and equatorial bond length domains. Apparently, this continuous distribution of terminal bond lengths is adequately described by a single component with a large Debye-Waller factor.

The quality of the fit obtained for the EXAFS due to scattering from the first shell of the Mn complex in PSII preparations poised in the \( S_2 \) state is comparable to that observed for complex 2. Also, the first shell EXAFS of complex 2 and that of the Mn complex in PSII poised in the \( S_2 \) state are remarkably similar (see Figure 4-9).
Figure 4-8. Fourier isolates (________) and fits (○○○○○) of the EXAFS due to backscattering from the first coordination sphere for (a) the Mn complex in a PSII preparation poised in the S₃ state (by the ferricyanide procedure, see section 7.2.2) (b) the open tetranuclear butterfly-like complex (complex 7) (c) the binuclear manganese complex 1 and (d) the tetranuclear adamantane-like manganese complex 9.
a) $S_3$ State

\[ k^2 \chi(k) \]

Least Square Residual = 1.20

<table>
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<tr>
<th>Atom type</th>
<th>O</th>
<th>O</th>
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<tr>
<td>$r$(Å)</td>
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<tr>
<td>$N$</td>
<td>1.8</td>
<td>2.1</td>
</tr>
<tr>
<td>$\sigma^2$</td>
<td>0.009</td>
<td>0.015</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-11.</td>
<td>-20.</td>
</tr>
</tbody>
</table>

b) Mn(III) acetate tetranuclear complex

\[ k^2 \chi(k) \]

Least Square Residual = 0.40

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<tr>
<td>$N$</td>
<td>1.4</td>
<td>2.0</td>
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<tr>
<td>$\sigma^2$</td>
<td>0.013</td>
<td>0.028</td>
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<tr>
<td>$\Delta E_0$</td>
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<td>-20.</td>
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</table>
c) Mn(III,IV) bipyridyl binuclear complex

<table>
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<td>$r$ (Å)</td>
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</tr>
<tr>
<td>$N$</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>$\sigma^2$</td>
<td>0.002</td>
<td>0.005</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-20.</td>
<td>-20.</td>
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</table>

Least Square Residual = 12.0

$\chi^2(k)$

d) Mn(IV) tacn tetranuclear complex

<table>
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<tr>
<td>$r$ (Å)</td>
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<td>2.05</td>
</tr>
<tr>
<td>$N$</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>$\sigma^2$</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-13.</td>
<td>-20.</td>
</tr>
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</table>

Least Square Residual = 23.0

$\chi^2(k)$
Figure 4-9. Fourier isolates (______) and fits (ooooo) of the EXAFS due to backscattering from the first coordination sphere for (a) the Mn complex in a PSII preparation poised in the $S_2$ state (b) the binuclear Mn(IV,IV) complex 2.
Least Square Residual=10.0

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<tr>
<td>( r(\text{Å}) )</td>
<td>1.78</td>
<td>2.23</td>
</tr>
<tr>
<td>( N )</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>( \sigma^2 )</td>
<td>0.005</td>
<td>0.010</td>
</tr>
<tr>
<td>( \Delta E_0 )</td>
<td>-12.</td>
<td>-20.</td>
</tr>
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Least Square Residual=6.0

<table>
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<tbody>
<tr>
<td>( r(\text{Å}) )</td>
<td>1.79</td>
<td>2.23</td>
</tr>
<tr>
<td>( N )</td>
<td>1.4</td>
<td>2.8</td>
</tr>
<tr>
<td>( \sigma^2 )</td>
<td>0.005</td>
<td>0.006</td>
</tr>
<tr>
<td>( \Delta E_0 )</td>
<td>-10.</td>
<td>-20.</td>
</tr>
</tbody>
</table>
Similarities in the correlation of parameters in the multiparameter, non-linear, least-square fitting procedure, described below, largely confirm the fact that the structure of the first coordination sphere of complex 2 most closely resembles that of the Mn complex poised in the $S_2$ state, and that the first coordination sphere structure of complex 7 most closely resembles that of the Mn complex in PSII preparations poised in the $S_3$ state.

An analysis of the variation in the apparent number of $\mu$-oxo bridges present in these ten inorganic complexes as a function of the disorder in the bridging Mn-O bond has been performed in a manner completely analogous to the analysis of the number of Mn neighbors in the second coordination sphere. Figure 4-10 a contains a set of curves obtained from this analysis. Also, for comparison, the variation in the apparent number of $\mu$-oxo bridges observed in the simulation of the EXAFS of the first coordination sphere of the Mn complex in PSII preparations poised in the $S_2$ and $S_3$ states is included. Best fit values for the second component in the simulation, corresponding to the terminal ligation are contained in Tables 4-3 and 4-4. The absolute number of $\mu$-oxo bridges present in the Mn complex in PSII poised in the $S_2$ and $S_3$ states predicted from the simulation and the variation in this number as a function of the magnitude of the Debye-Waller factor most closely resemble those predicted for complexes 1 and 2. Note that the absolute number of $\mu$-oxo bridges predicted for the Mn complex is the same at each Debye-Waller level for the $S_2$ and $S_3$ state preparations.

Figure 4-10 b contains a plot of the least-square residual for the fits to the EXAFS due to the first coordination sphere for the ten inorganic complexes and the Mn complex in PSII poised in the $S_2$ and $S_3$ states as a function of the disorder in the Mn $\mu$-oxo bond length. Based on the similarity both in absolute fit quality and the variation in fit quality observed with the Debye-Waller factor, complex 2 is the best model for the Mn complex in preparation poised in the $S_2$ state. For the $S_3$ state, it would appear that complexes 7 and 10 exhibit very similar simulation behavior. Both complexes 7 and 10 are tetranuclear complexes with four non-equivalent manganese centers.

4.4 Fine Adjustments Based on Models

Once models for each scattering shell have been selected, based on the criteria described above, adjustments of the structural parameters of the unknown system may
Figure 4-10. (a) The apparent number of $\mu$-oxo bridges obtained by simulation of the EXAFS due to the first coordination sphere as a function of the magnitude of the Debye-Waller factor for the $\mu$-oxo shell.

(b) A plot of the least square residual between the Fourier isolate of the EXAFS due to backscatter from the first coordination sphere and the fits obtained by systematically varying the Debye-Waller factor for the Mn $\mu$-oxo bridging shell. Exact details of the procedure used to construct these curves is described in the text.
a) Number of Oxo Bridges vs Disorder

```
Number of Oxo Bridges

Debye-Waller

Complex 9
Complex 1
S2-State
Complex 2
Complex 6
Complex 7
Complex 10
```

b) Least Square Residual vs Disorder

```
Least Square Residual

Debye-Waller

Complex 9
Complex 1
Complex 6
Complex 2
S2-State
S3-State
Complex 10
Complex 7
```

be made. The distances to each scattering shell in the unknown have been refined by constraining the range of variation in the energy of the ionization threshold to a range with which simulations of the inorganic complexes yielded good agreement with the X-ray crystallographic data. For the distances to Mn neighbors, the average uncertainty is ± 0.02 Å. For the μ-oxo bridging shell, the average uncertainty is somewhat higher (e.g. ± 0.05 Å).

Improvements in the estimate of the number of atoms within a scattering shell may be made by applying correction or scale factors obtained from the best models to the numbers obtained for the unknown. Scale factors are calculated as the ratio of the number of scatterers determined from the crystal structures, divided by the number of scatterers determined by simulation of the EXAFS. Based on the criteria described by Teo et al. (1983) the best model for the Mn complex in the S_1 and S_2 states is complex 2 (see section 4.3.2.3 and 4.3.2.4). The scale factor for the μ-oxo bridging shell calculated at the Debye-Waller value which corresponds to the best fit values for the S_1 and S_2 states is 1.44 and 1.26, respectively. Thus, the number of μ-oxo bridges in the S_1 and S_2 states is 2.2 ± 0.6 and 2.3 ± 0.6. Uncertainties are calculated based on simulations in which all parameters except the number of atoms of interest and the Debye-Waller value were constrained to their best fit values. Limits were determined based on the number of scatterers in simulations in which the least square residual was equal to twice the minimum value (Teo et al., 1983). For the S_3 state, complex 7 is a better model for the first shell (see section 4.3.2.4). The scale factor obtained from complex 7 is 1.07. Applying this correction to the simulation of the S_3 state, the number of μ-oxo bridges present is 1.9 ± 0.4. Applying the scale factor for the number of Mn neighbors obtained from complex 2 to the simulation results obtained for the Mn complex in the S_1 and S_2 states, one obtains 1.0 ± 0.3 and 1.22 ± 0.4 respectively.

4.5 Conclusions: Relevance of Various Inorganic Complexes as Models for the Manganese Complex in the OEC

1. Based on fine adjustments based on models, the most probable structure for the Mn complex in the S_1, S_2 and S_3 states is a pair of bi-nuclear, di-μ-oxo bridged manganese complexes.

2. In all cases, when a shell of Mn atoms exists within a given complex in the range of
2.72 to 3.41 Å, an analysis of the EXAFS of that complex yields an accurate estimate of the distance to that shell. An analysis of the EXAFS of the Mn complex in PSII yields only one Mn shell at 2.72 ± 0.03 Å. Thus, all multinuclear, \( \mu \)-oxo bridged manganese complexes that contain more than one Mn-Mn distance, such as complex 7, or that have Mn-Mn distances significantly different from 2.72 Å are not relevant models for the active site. This clearly rules out structures such as the adamantane-like complex (complex 9), the trinuclear Mn-Mn complexes (e.g. complexes 5 and 6), the distorted cubane-like structure (complex 10) and the mono-\( \mu \)-oxo bridged structures, all of which possess Mn-Mn distances in the range of 3.1 - 3.3 Å.
Chapter 5
Light-Induced Changes in the Electronic Structure of the Manganese Complex Involved in Photosynthetic Oxygen Evolution: A Manganese K-Edge X-Ray Absorption Study

5.1 Introduction

An abundance of physical and biochemical evidence indicates that a cluster of manganese atoms located within a membrane-bound protein chlorophyll complex is the site where the light-driven oxidation of water to molecular oxygen takes place (see section 1.1.3). EPR and X-ray absorption spectroscopy have clearly demonstrated the involvement of a manganese aggregate in the stabilization and storage of oxidizing equivalents (For a review see Dismukes, 1986; Babcock, 1987).

The first evidence that manganese atoms associate as a binuclear or tetranuclear center in the \( \text{O}_2 \)-evolving complex came from a low temperature EPR study of the \( S_2 \) state. Dismukes and Siderer (1981) found that a complex EPR signal, commonly referred to as the multiline signal (MLS) and exhibiting an abundance of hyperfine structure resulted after the OEC was advanced to the \( S_2 \) state by a single flash of light. The periodicity of four in the maximum amplitude of this EPR signal confirmed its \( S_2 \) state assignment. The striking similarity of this EPR signal to those exhibited by mixed valence binuclear manganese complexes characterized only three years earlier led to the implication of manganese (Cooper et al., 1978). Simulations of the EPR signal by assuming a binuclear \( \text{Mn}_2(\text{III,IV}) \) (Dismukes & Siderer, 1981), \( \text{Mn}_2(\text{II,III}) \) (Hansson & Andréasson, 1982) or a mixed valence tetranuclear manganese cluster (Dismukes et al., 1982) were also based in part on the generally accepted stoichiometry of four manganese per reaction center. However, the formal valence of the manganese complex yielding the multiline EPR signal remains controversial (Hansson & Andréasson, 1982; Dismukes et al., 1982; Mabad et al., 1985, Dismukes et al., 1987). UV absorption changes of the OEC during the \( S_1 \) to \( S_2 \) state transition have been suggested to arise from a formal valence change of \( \text{Mn(III)} \) to \( \text{Mn(IV)} \) (Dekker et al., 1984 a,b). However, the interpretation of the optical difference data is a subject of some controversy (Lavergne, 1985, 1987) and, the valence changes suggested have recently been challenged (Vincent & Christou, 1986).
Brudvig et al. (1983) found that the $S_2$ state characterized by the multiline EPR signal could be generated and cryogenically trapped in higher yield by a low temperature continuous illumination procedure. We have stabilized the OEC in various states of the catalytic cycle and examined changes in the physical and electronic structure of the manganese present through the use of X-ray absorption spectroscopy. The MLS has been the principal spectroscopic evidence for the state composition of a given PSII preparation.

The use of X-ray absorption edge spectroscopy in the study of metal complexes has been reviewed by Srivastava and Nigam (1972). Although as yet there is no quantitative theory which adequately describes the X-ray absorption edge spectrum, it is clear that it contains a potential wealth of information about the oxidation state and site symmetry of a metal center. Many of the assignments of transitions in the edge region remain controversial (Cotton & Hanson, 1958; Shulman et al., 1976; Bair & Goodard, 1980). However, most theorists agree on the assignment of the low intensity transition below the edge jump to a 1s to 3d transition of principally metal character, although the dominant mechanism of this transition is still a subject of controversy (Shulman et al., 1976; Bair & Goodard, 1980; Kutzler et al., 1980; Hahn et al., 1982). In this report, we examine in detail light-induced changes in 1s $\rightarrow$ 3d transition of the Mn K-edge spectrum of PSII preparations poised in the $S_1$ and $S_2$ states.

With the advent of synchrotron radiation and the development of a fluorescence detection scheme (Jaklevic et al., 1977), the sensitivity of the technique has been extended to allow application to systems dilute in the metal of interest such as metalloenzymes. Despite its potential utility in probing the structure of a metal center, there have been relatively few examples where X-ray absorption spectroscopy (XAS) has been used to demonstrate significant changes in the electronic structure of a metal center of a metalloenzyme associated with enzymatic activity (Hu et al., 1977; Blumberg et al., 1978; Tullius et al., 1978; Brown et al., 1980; Elam et al., 1982; Goodin et al., 1984).

Our previous X-ray edge studies indicated that the higher valences of manganese (Mn(III) and Mn(IV)) are present in the Mn complex of PSII preparations poised in the $S_1$ and $S_2$ states (Goodin et al., 1984, Yachandra et al., 1987). In this report, we extend the analysis of the absolute energy of the Mn K-edge inflection to a much broader range of inorganic complexes confirming our original conclusions.
We have previously reported light-induced shifts in the energy of the Mn K-edge of dark adapted PSII preparations (Goodin et al., 1984; Yachandra et al., 1987). In this report the magnitude of the shift to higher energy in the observed in the position of the Mn K-edge inflection upon advancement to the $S_2$ state is examined with respect to similar differences in edge inflection energy observed between isostructural multinuclear manganese complexes which differ in their formal valence by a single equivalent.

Upon illumination we have observed (Sauer et al., 1987) a dramatic light induced change in the structure of the $1s \rightarrow 3d$ transition of the Mn K-edge X-ray absorption spectrum of active $O_2$-evolving PSII subchloroplast membrane preparations from spinach and the cyanobacterium, *Synechococcus* (McDermott et al., 1987a). A comparison of the structure of the $1s \rightarrow 3d$ transition of the Mn K-edges of PSII preparations poised in the $S_1$ and $S_2$ states to an extensive list of inorganic manganese complexes covering a relevant range of formal oxidation states and nuclearities is presented, in an attempt to elucidate the nature of the changes in the electronic structure of the Mn-OEC occurring in the $S_1 \rightarrow S_2$ state transition. A similar qualitative comparison of other less clearly assigned regions of the X-ray absorption edge spectrum (e.g. $1s \rightarrow 4p$ transitions and multiple scattering transitions) is also included.

5.2 Material and Methods

5.2.1 PSII Membrane Preparations

Active $O_2$-evolving PSII subchloroplast membrane preparations from spinach and *Synechococcus* were prepared as previously described (Yachandra et al., 1986; McDermott et al., 1987). Optically dense samples (25-30 mg/ml chlorophyll) containing $\sim$30% glycerol were mounted in mylar backed lucite sample holders. Both EPR and XAS measurements were performed directly on samples mounted in this manner. The MLS which is the principal spectroscopic indicator of state composition was recorded at 8K and 32 mW microwave power. EPR spectra were recorded on a Varian E-9 EPR spectrometer equipped with an E-102 microwave bridge and an Air Products LTR liquid helium cryostat. Sample temperature was monitored with a gold-chromel thermocouple. EPR spectra were digitally acquired using an Explorer III storage oscilloscope interfaced to a VAX 11/785 computer. Long term dark-adapted PSII preparations (> 1 hr) were advanced to the $S_2$ state by continuous illumination at 190 K. Samples were illuminated
for 2 min using a 400 watt tungsten light source filtered with a 5cm water filter. Samples were maintained within 2 K of 190 K by a stream of cooled dry nitrogen monitored with a copper-constantan thermocouple.

5.2.2 Inorganic Manganese Complexes

Twenty-five structurally characterized inorganic complexes spanning a relevant range of oxidation states and nuclearities were examined in this study. Table 5-1 contains a list of the models examined and the synthesis and crystal structure reference for each complex. Models will be referred to in the text by the boldface number in the leftmost column of Table 5-1. Samples were diluted into an inert light element matrix (LiBF₄ or LiCO₃) and mounted in lucite sample holders previously described (Yachandra et al., 1986a).

5.2.3 X-Ray Absorption Measurements

X-ray absorption spectra were recorded at the Stanford Synchrotron Radiation Laboratory on beam lines IV-2 and IV-1 using Si(400) and Si(111) double crystal monochromators. The energy resolution of these crystals is approximately 1 eV. The intrinsic linewidth of features in the 1s → 3d region is approximately 1.5 eV. Spectra were recorded using a fluorescence detection system similar to that described by Powers et al. (1981). It consisted of an array of NE104 plastic scintillators coupled to EMI 9813 B photomultiplier tubes. The fluorescence detector was equipped with a chromium filter and Soller slit assembly (Stern & Heald, 1979). The fluorescence signal was ratioed to a measure of the incident flux which was monitored by an additional scintillator. This scintillator viewed the flux of X-rays scattered from a thin mylar film which was placed directly in the incident X-ray beam. It was found that this method of ratioing yielded a much more linear response than the ionization chamber method we have previously employed.

All samples were maintained at or below 190 K in a home built double Kapton walled cryostat cooled by a liquid nitrogen blow-off jet. To ensure sample integrity and a stable state composition, EPR spectra of the PSII samples were monitored before and after exposure. Energy calibration in the X-ray absorption experiments was maintained by simultaneously monitoring the narrow 1s → 3d transition (centered at 6543.3 eV) of a KMnO₄ standard (Goodin et al., 1979). Typically, one scan (3sec per point) yielded
a satisfactory signal-to-noise ratio for the inorganic complexes. To achieve the signal-to-noise ratio presented here for the PSII preparations, 10 to 30 scans were averaged.

5.2.4 Data analysis

X-ray absorption spectra were digitally acquired on an LSI 11/34 microcomputer interfaced with a CAMAC controller system. The data were transferred to a VAX 11/785 for analysis. Software originally written by Kirby (1981) and modified extensively by Goodin (1984) was used in the analysis. The X-ray absorption cross-section prior to the onset of Mn K-edge absorption was set equal to zero by subtracting a linear fit to the spectra below the edge. Spectra were normalized to a unit edge jump determined by an extrapolation of a quadratic fit of the X-ray absorption a few hundred eV above the edge to the maximum absorption peak.

To facilitate the determination of the edge inflection energy or the position of unresolved peak maxima in the pre-edge region, first and second derivatives with respect to energy were taken. Linear smoothing fits were performed prior to taking each derivative. Edge inflection energies were determined from the first major maximum in the first derivative following a 3ev smooth. Position and or separation of unresolved peaks in the pre-edge transition were determined from the minima in the second derivative of the absorption spectrum following 1 eV running smooths. Uncertainties are estimated to be ± 0.1 eV. The amplitude of 1s → 3d transitions were determined at the point of the first minimum in the second derivative. This was done in order to minimize the contribution of intensity from much more intense absorptions due to allowed 1s → 4p and continuum transitions.

5.3 Results and Discussion

This section contains an analysis of the light-induced changes in the Mn K-edges of the Mn complex in PSII preparations occurring during the S₁ → S₂ state transition. An essential component of this analysis has been a comparative study of twenty-five structurally characterized inorganic complexes spanning a relevant range of valences and nuclearities. Four aspects of the changes in Mn K-edge structure are examined. In the first section (section 5.3.1) the energy of the Mn K-edge of PSII preparations poised in the S₁ and S₂ states is compared to those of the set of inorganic complexes to determine the formal valence of the manganese present in each S-state. In the
second section (section 5.3.2) specific transitions of the Mn K-edges of PSII preparations poised in the $S_1$ and $S_2$ states are compared to those of the inorganic complexes. It is important to note that an analysis of this detail has been possible due to significant improvements in the signal-to-noise ratio of recently acquired Mn K-edge spectra of PSII preparations poised in the $S_1$ and $S_2$ states (see Figure 5-1). Differences in a low intensity transition at low energy associated with $1s \rightarrow 3d$ transitions are examined in an attempt to associate specific valence changes in the manganese complex in PSII preparations occurring during the $S_1 \rightarrow S_2$ state transition. In addition, other regions of the X-ray absorption spectrum (e.g. the $1s \rightarrow 4p$ region and the multiple scattering or XANES region) are examined. Conclusions drawn from these less clearly assigned regions of the spectrum are compared to assignments based on the analysis of the $1s \rightarrow 3d$ region.

In the third section (section 5.3.3) the light-induced shift to higher energy observed for the PSII preparations during the $S_1 \rightarrow S_2$ state transition is compared to differences in edge inflection energy observed for isostructural multinuclear $\mu$-oxo bridged manganese complexes which differ by one unit of formal valence (e.g. one oxidative equivalent per complex). It is important to note that a comparison of isostructural complexes is particularly relevant to the evaluation of the number of oxidative equivalents stored within the Mn-containing $O_2$-evolving complex in PSII in light of the EXAFS analysis of these two states which indicates that the structure of the complex does not change during the $S_1 \rightarrow S_2$ state transition.

In the final section (section 5.3.4) complications in the interpretation of the edge results are considered. The EXAFS of the Mn complex within PSII preparations indicates that the four Mn present are organised as binuclear complexes (see chapter 4). The EPR signal associated with the $S_2$ state indicates that the manganese present in this state must be a mixture of the higher valences (e.g. Mn(III) and Mn(IV)). Based on a review of the properties (e.g. optical, magnetic and crystallographic) of multinuclear mixed valence manganese complexes, it is suggested that the assumption of valence trapped behavior in the previous sections is in fact valid.

**5.3.1 Manganese K-edge Inflection Energies**

A comparison of the Mn K-edge inflection energy of the PSII preparations poised in
Figure 5-1. Mn X-ray absorption K-edge spectra of PSII preparations from spinach (a) and *Synechococcus* (b) poised in the $S_1$ (---) and $S_2$ (-----) states. The $S_1$ state is obtained upon long term (> 1hr) dark adaptation at 277 K. Samples were poised in the $S_2$ state by illumination at 195 K. Advancement to the $S_2$ state was monitored by the formation of the multiline EPR signal (Spectra not shown). Spectra were smoothed for presentation by applying a 1 eV sliding fit to the raw data. A linear pre-edge background was subtracted from the data to set the pre-edge absorption to zero. The inset in each spectrum is a 5 × magnification of the $1s \rightarrow 3d$ transition of the X-ray absorption spectrum. The $S_2$ state in both preparations exhibits a much more pronounced pre-edge peak relative to the intensity of the absorption in the intervening region prior to the edge inflection.
the $S_1$ and $S_2$ states with those of the inorganic complexes demonstrates that the higher valences of manganese (Mn(III) and Mn(IV)) are involved in these two states (Table 5-1 and Figure 5-2). The Mn K-edge inflection energy of the manganese complex in PSII preparations poised in the $S_2$ state is high, in the range of energies associated with Mn(IV) complexes; and the inflection energy for the Mn complex in PSII preparations poised in the $S_1$ state is low, in the range of inflection energies associated with Mn(IV) complexes. Ranges for the energy of the Mn K-edge inflection energy for each valence are indicated on the left side of Figure 5-2. As we have previously reported (Kirby et al., 1981a), the Mn K-edge inflection energy is sensitive not simply to the valence of the metal ion but to the effective potential of the ion, which is influenced by the electron donating character of the ligating atoms. The coordination charge used in the correlation plot shown in Figure 5-2 is an attempt to correct for the differences in the electronegativity of the ligating atoms and hence more accurately describe the effective potential of the complex (Kirby et al., 1981a). This treatment neglects numerous important effects which have been demonstrated to affect the energy of the edge inflection, such as covalency, symmetry of the metal center (Shulman et al., 1976) and the nuclearity of the complex (Cartier et al., 1986).

Despite these limitations, we conclude that the manganese atoms present in PSII preparations poised in the $S_1$ and $S_2$ states possess a mixture of valences containing only Mn(III) and Mn(IV). Note that the $S_2$ state must be a mixed valence state because its EPR spectrum indicates that it is a Kramers spin system. Given the energy of the Mn K-edge inflection of the $S_2$ state, it is suggested that the most probable formal valence of the four manganese present is (III, IV, IV, IV). The coordination charge calculated for the $S_2$ state of spinach (1.4), using the regression line plotted in Figure 5-2, indicates that all four manganese present have a formal valence of IV unless the coordination is virtually all by relatively electronegative donor groups, such as carboxylates derived from acidic side chains of the membrane bound proteins. Based on the coordination charge versus edge inflection energy correlation line, a change in the donor set from $O_6$ to $O_5N$ for each manganese present is predicted to shift the edge energy down by 0.4 eV. This suggests a possible explanation for the difference between the absolute edge inflection energies of the spinach and those of the Synechococcus PSII preparations. The ligation set of the Mn complex in the Synechococcus preparations may contain on
Table 5-1

Mn K-edge Inflection Energies and Calculated Coordination Charges for PSII Preparations and Model Complexes

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Donor Coordination</th>
<th>Edge inflection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Set*</td>
<td>Energy (eV)</td>
</tr>
<tr>
<td>PSII Preparations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S₁</td>
<td>(1.05)*</td>
<td>6551.4</td>
</tr>
<tr>
<td></td>
<td>S₂</td>
<td>(1.40)*</td>
<td>6552.2</td>
</tr>
<tr>
<td></td>
<td>Synechococcus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S₁</td>
<td>(0.70)*</td>
<td>6550.6</td>
</tr>
<tr>
<td></td>
<td>S₂</td>
<td>(0.96)*</td>
<td>6551.2</td>
</tr>
<tr>
<td>Model Complexes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn(II) monomers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Mn(acac)₂·2H₂O</td>
<td>O₆</td>
<td>-0.456</td>
</tr>
<tr>
<td>2</td>
<td>Mn(pyr)$_4$Br₂</td>
<td>N₄Br₂</td>
<td>-1.513</td>
</tr>
<tr>
<td>3</td>
<td>Mn(MIm)$_3$Cl₂</td>
<td>N₃Cl₂</td>
<td>-0.768</td>
</tr>
<tr>
<td>4</td>
<td>Mn(H₂O)₆$^{2+}$</td>
<td>O₆</td>
<td>-0.456</td>
</tr>
<tr>
<td>Mn(III) monomers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Mn(acac)$_3$</td>
<td>O₆</td>
<td>0.544</td>
</tr>
<tr>
<td>6</td>
<td>Mn(salen)Cl</td>
<td>O₂N₂Cl</td>
<td>0.511</td>
</tr>
<tr>
<td>Mn(III) binuclear complexes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Mn₂O(OAc)$_2$(HBPz)$_3$</td>
<td>O$_3$N$_3$</td>
<td>0.051</td>
</tr>
<tr>
<td>8</td>
<td>Mn₂O(OAc)$_2$(tmtacn)$_2$(ClO$_4$)$_2$</td>
<td>O$_3$N$_3$</td>
<td>0.051</td>
</tr>
<tr>
<td>9</td>
<td>Mn₂(OH)$_2$(Salpn)$_2$</td>
<td>O$_4$N$_2$</td>
<td>0.216</td>
</tr>
<tr>
<td>10</td>
<td>Mn₂(OAc)$_2$(spa)$_2$</td>
<td>O$_3$N</td>
<td>0.381</td>
</tr>
<tr>
<td>Mn(III) trinuclear complexes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Mn₃(benzoate)$_6$(Im)$_3$(ClO$_4$)$_2$[NBu$_4$]</td>
<td>O$_3$N</td>
<td>0.381</td>
</tr>
<tr>
<td>12</td>
<td>Mn₃O(OAc)$_6$(py)$_3$(ClO$_4$)</td>
<td>O$_3$N</td>
<td>0.381</td>
</tr>
<tr>
<td>13</td>
<td>Mn₃O(OAc)$_3$HOAc</td>
<td>O₆</td>
<td>0.544</td>
</tr>
<tr>
<td>Mn(III) tetranuclear complex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Mn₄O$_2$(OAc)$_7$(bipy)$_2$ClO$_4$</td>
<td>2(O₆),2(O₄N$_2$)</td>
<td>0.378</td>
</tr>
</tbody>
</table>
Mn(IV) monomers

15 Mn(salicylate)₂bipy  O₂N₂  1.216  6552.5
16 MnO₂  O₆  1.540  6552.1

Mn(IV) binuclear complexes

17 Mn₂O₂(phen)₄(ClO₄)₄  O₂N₄  0.886  6551.0

Mn(IV) tetranuclear complex

18 Mn₄O₆(tacn)₄(ClO₄)₄  O₃N₃  1.051  6552.6

Mixed valence multinuclear complexes

19 Mn₃(III,IV)O(OAc)₂(tacn)₂(ClO₄)₃  O₃N₃  0.551  6550.5
20 Mn₃(III,IV)O₂(bipy)₄(ClO₄)₃  O₂N₄  0.386  6549.9
21 Mn₃(III,IV)O₂(phen)₄(PF₆)₃  O₂N₄  0.386  6549.7
22 Mn₃(II,III,III)O(benzoate)₆(py)₂(H₂O)  2(O₂N)O₆  0.242  6549.2
23 Mn₃(II,III,III)O(OAc)₆(py)₃py  O₅N  0.045  6548.6
24 Mn₄(II,III,III,III)O₂(benzoate)₇(bipy)₂  2(O₂N)₂(O₄N₂)O₆  0.130  6548.6
25 Mn₁₂(OAc)₁₀(H₂O)₄O₁₂ 2·HOAc  O₈  0.877  6550.1

where acac=acetylacetonate, bipy=1,10-phenanthroline, Im=imidazole, MIm=2-methylimidazole, NBu₄=tetra-N-butylammonium ion, py=pyridine pyr=pyrazole, salen=ethane-1,2-diylbis(salicylideneiminate), salpn=the schiff base prepared from salicylaldehyde and 1,2-diamino propane, H₂spa=3-salicylideneamino-1-propanol, tacn=1,4,7-triazacyclononane and tmtacn=N',N",N"'-trimethyl-1,4,7-triazacyclononane.

a. Elements in the first coordination sphere

b. Calculated coordination charges for the PSII preparations shown in parentheses were determined from the measured edge inflection energy and the linear least square fit to the model complexes shown in Fig 2.

c. References: 1, Onuma & Shibata (1970); 2, Reedijk et al. (1971); 3, Phillips et al. (1976); 4, No crystal structure exists for a solution of Mn(II) chloride, however an EXAFS analysis indicates an octahedron of oxygens at 2.15 Å; 5, Morosin & Brathovde (1964); 6, Pecoraro & Butler (1986); 7, Sheats et al. (1987); 8, Wieghardt et al. (1985); 9, Malsen & Waters (1973); 10, Mikuriya et al. (1981); 11, 12, G. Christou, personal communication; 13, Hessel & Romers (1969); 14, Vincent et al. (1987); 15, Pavacik et al. (1986); 16, Náray-Szabó (1969); 17, Stebler et al. (1986); 18, Wieghardt & Bossek (1983); 19, Wieghardt et al. (1986); 20, Plaksin et al. (1972); 21, Stebler et al. (1986); 22, 23, Vincent et al. (1987b); 24, Vincent et al. (1987a); 25, Lis (1980).

d. MnO₂ has an extended lattice structure in the solid state. The lattice is like that of rutile.
Figure 5-2. A plot of the Mn K-edge inflection energy versus coordination charge for a set of twenty five manganese complexes. Calculated values for the coordination charge of each complex and the Mn K-edge inflection energies are listed in Table 5-1. The coordination charge is an attempt to correct the formal valence of the metal for differences in electron donating characteristics of the ligands (see text for details). The line drawn is a linear least squares fit to the data. (Edge energy (eV) = 6549.0 + 2.23 $\eta$, $r$=0.88 where $\eta$ is the coordination charge as defined by Kirby, 1981 and $r$ is the correlation coefficient for the linear least squares fit). The position of the $S_1$ and $S_2$ states of spinach are indicated (+) on the best fit line. Mn K-edge energy ranges for each valence of Mn are indicated.
Coordination Charge vs. Mn K-edge Inflection Energy

![Graph showing the relationship between coordination charge and Mn K-edge inflection energy. The graph includes data points and labels for Mn(II), Mn(III), and Mn(IV).]
the average a greater number of nitrogen donors derived from histidine or amino side chains of the membrane bound protein.

5.3.2 A Comparison of Regions of the X-ray Absorption Spectrum

Weaker peaks 5 to 10 eV below the edge inflection have been assigned to a 1s → 3d transition for the first row transition elements (Shulman et al., 1976; Bair & Goodard, 1980). Changes in the amplitude of the 1s → 3d transition reflect changes in the symmetry of the metal center (Srivastava & Nigam, 1972).

A comparison of the structure of the 1s → 3d transition for the S₁ and S₂ state PSII preparations to those of a set of crystallographically characterized inorganic complexes reveals a striking similarity in the appearance of this transition in S₁ state preparations to those of Mn(III) complexes and S₂ state preparations to those of Mn(IV) complexes (see Figure 5-3). The Mn(IV) complexes and the S₂ state PSII preparations all have a much more pronounced peak at low energy than do the Mn(III) complexes and the S₁ state preparations. This indicates that the S₁ → S₂ transition corresponds to a formal valence change of Mn(III) to Mn(IV). The similarity of the structure of the 1s → 3d transition of the S₂ state preparations to that of Mn(IV) complexes provides further support of the valence assignment of the S₂ state based on the absolute energy of the Mn K-edge inflection of this state (see section 5.3.1).

Differences observed between Mn(III) and Mn(IV) complexes in the structure of the 1s → 3d transitions are attributable to a number of factors. The energy of the 1s → 3d transition is known to be less sensitive to the valence of the metal than is the edge inflection (Shulman et al., 1976). For the complexes examined in this study, the position of the first absorption maximum in the pre-edge, as determined from the first minimum in the second derivative, ranges from 6539.9 for Mn(II) complexes to 6541.4 for Mn(IV) complexes while the edge inflection energy ranges from 6546.6 for Mn(II) complexes to 6552.6 for Mn(IV) complexes (See Table 5-2). As a result, the energy separation between the edge inflection and the 1s → 3d in Mn(II) complexes ranges from 5.1 to 6.6 eV; while for Mn(III) complexes, this separation ranges from 8.0 to 10.7 eV and for Mn(IV) complexes, this separation ranges from 10.7 to 12.0 eV. Thus, the separation between the first maximum in the 1s → 3d region and the edge inflection (ΔE = E₁s−₃d, see Table 5-2) is another indication of valence which, unlike the absolute energy of the edge inflection does not require an external energy reference.
Figure 5-3. The 1s → 3d absorption profile of the Mn K-edge spectrum of O₂ evolving PSII preparations from spinach and *Synechococcus* poised in the $S_1$ and $S_2$ states as described in the text. To emphasize the similarity of the structure of the 1s → 3d absorption of the $S_1$ state to that of Mn(III) complexes, a series of spectra of Mn(III) complexes of different nuclearity has been plotted for comparison. Similarly for comparison with the $S_2$ state, the 1s → 3d transition for number of Mn(IV) complexes has been plotted. (a) From top to bottom, the spectra are: 1. a spinach PSII preparation poised in the $S_1$ state, 2. a *Synechococcus* preparation poised in the $S_1$ state, 3. the trinuclear complex 13, Mn$_3$O(OAc)$_7$HOAc, 4. the binuclear Mn(III) complex 7, Mn$_2$O(OAc)$_3$(HBPz)$_3$ and 5. the Mn(III) mononuclear complex 6, Mn salen Cl. (b) The pre-edge spectra of: 1. a spinach PSII preparation poised in the $S_1$ state, 2. the tetrinuclear Mn(IV) complex 18, [Mn$_4$O$_6$(tacn)$_4$](ClO)$_4$, 3. the binuclear Mn(IV) complex 17, [Mn$_3$O$_5$(phen)$_4$(ClO))$_4$ and the mononuclear Mn(IV) complex 15, Mn(salicylate)$_2$-bipyridine.
For the manganese complex in PSII preparations poised in the $S_2$ state, this energy difference indicates that the manganese present have a formal valence of IV.

The contribution of absorption intensity from allowed continuum or bound state transitions to molecular orbitals of principally p-character to the intervening region between the $1s \rightarrow 3d$, and the first major inflection is greater for Mn(III) than for Mn(IV). However, this does not explain the more pronounced peak observed in the pre-edges of the Mn(II) complexes. The peak in the $1s \rightarrow 3d$ region of the Mn(II) complexes is more prominent despite its even closer proximity to the much more intense transitions in the edge inflection. The lack of ligand field stabilization in the high spin $d^5$ configuration of Mn(II) leads to much weaker bonding and, as a result longer bond lengths, a smaller value of $10D_q$ and a much more symmetric coordination. The similarity of the Mn(IV) edges to those of Mn(II) complexes indicates that similarly symmetric coordination may be significant factor determining the pre-edge absorption profile. This is in sharp contrast to the broad absorption characteristic of the Mn(III) $1s \rightarrow 3d$ transitions. See, for example, the edges of a set of six-coordinate Mn(II), Mn(III) and Mn(IV) complexes shown in Figure 5-4. All of these complexes have roughly octahedral oxygen ligation spheres.

The amplitudes of the $1s \rightarrow 3d$ transitions contained in Table 5-2 are determined as a percentage of the edge jump (see section 2.2.2) at the position of the first minimum in the second derivative with respect to energy. The lowest energy peak was chosen as the point of measure so as to minimize contributions from the onset of much more intense allowed transitions in the edge inflection. Although there is substantial overlap, it would appear that the Mn(IV) complexes exhibit significantly more intense $1s \rightarrow 3d$ transitions than Mn(III) complexes. Changes in the intensity of this feature have previously been assigned to differences in the symmetry of the metal center which relate to the amount of p-orbital mixing in these molecular orbitals (Shulman et al., 1976). Here we are observing significant variation within a set of six coordinate complexes.

Differences in the structure of the $1s \rightarrow 3d$ transition region of the Mn K-edges of Mn(III) and Mn(IV) complexes must originate from significant differences in their electronic structure. It has been shown previously that the magnitude of observed splittings in the $1s \rightarrow 3d$ transitions in certain iron complexes is comparable to crystal field splittings observed in the d-d transitions of optical spectra (Shulman et al., 1976).
Table 5-2
Amplitude and Position of 1s → 3d Transitions
of PSII Preparations and Model Complexes

<table>
<thead>
<tr>
<th>Complex No.</th>
<th>1s → 3d Energy (eV)</th>
<th>ΔE</th>
<th>1s → 3d Amplitude</th>
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</thead>
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<tr>
<td><strong>PSII Preparations</strong></td>
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<td></td>
<td></td>
</tr>
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<td></td>
</tr>
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<td>Energy (eV) 1s - 3d</td>
<td>Energy (eV) Mn K-edge</td>
<td>Amplitude (%)</td>
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*Mn(IV) monomers*

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*Mn(IV) binuclear complex*

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<th>Energy (eV) 1s - 3d</th>
<th>Energy (eV) Mn K-edge</th>
<th>Amplitude (%)</th>
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<td>7.3</td>
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*Mn(IV) tetranuclear complex*

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<th>Energy (eV) 1s - 3d</th>
<th>Energy (eV) Mn K-edge</th>
<th>Amplitude (%)</th>
</tr>
</thead>
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</table>

*Mixed valence multinuclear clusters*

<table>
<thead>
<tr>
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<th>Energy (eV) Mn K-edge</th>
<th>Amplitude (%)</th>
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<tr>
<td>25</td>
<td>6540.2</td>
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<td>12.5</td>
</tr>
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a. The 1s $\rightarrow$ 3d transition energy defined here is the energy determined from the first minimum in the second derivative with respect to energy (See Fig 6).

b. The energy difference between the lowest energy peak in the 1s $\rightarrow$ 3d transition E$_{1s-3d}$ region and the Mn K-edge inflection energy (E$_{mn}$).

c. The amplitude calculated is determined at the position of the lowest energy 1s $\rightarrow$ 3d transition, determined from the second derivative of the spectrum with respect to energy. The amplitudes are reported as a percent of the edge jump (See text).
The 1s → 3d region of the PSII preparations and a number of the complexes examined in this study exhibits what appears to be underlying substructure due to the presence of three or more overlapping bands (see Figure 5-3). Measurements of room temperature magnetic susceptibilities for the complexes studied here indicate high spin configurations in all cases. High spin Mn(III) has a degenerate ground state in an octahedral field and is thus subject to Jahn-Teller distortions (Bersuker, 1975). Extensive optical studies of Mn(III) ions in solution and in the solid state are indicative that the splitting of the $e_g$ orbitals (in octahedral symmetry) is substantial (Davis et al., 1968; Figgis et al., 1978). The low energy d-d band assigned to $^5B_{1g} \rightarrow ^5A_{1g}$ (in a tetragonal field) ranges from 9000 cm$^{-1}$ (1.1 eV) to 15,380 cm$^{-1}$ (1.9 eV) (Davis et al., 1968). Two other spin allowed d-d transitions at progressively higher energy are also observed; a band at 13,580 cm$^{-1}$ (1.6 eV) to 21,500 cm$^{-1}$ (2.2 eV) which has been assigned to $^5B_{1g} \rightarrow ^5B_{2g}$ (10 Dq) and a band at 14,800 cm$^{-1}$ (1.8 eV) to 21,500 cm$^{-1}$ (2.7 eV) which has been assigned to $^5B_{1g} \rightarrow ^5E_g$ in a tetragonal field (Dingle, 1965). The $e_g$ orbitals have substantial $\sigma$-antibonding character. Thus, the Jahn-Teller effects in this system manifest themselves not only in the optical spectra of these ions but also in significant bond length distortions are frequently observed. Static (Figgis et al., 1978), dynamic (Davis et al., 1968) and pseudo Jahn-Teller (Bersuker, 1975) distortions of six-coordinate Mn(III) complexes have been reported.

In principle the crystal field splitting for Mn(IV) should be as much as 50% greater than that observed for Mn(III); however, in practice the differences are much smaller. Reported values for 10 Dq range from 15,900 cm$^{-1}$ (1.97 eV) to 21,800 cm$^{-1}$ (2.7 eV) (Lever, 1984). Because of the instability of Mn(IV) complexes, few have been characterized crystallographically (Wieghardt & Bossek, 1983; Hartman et al., 1984; Kessissoglou et al., 1986; Pavacik et al., 1986; Stebler et al., 1986). However, within this limited data set, it is clear that Mn(IV) has a preference for a more symmetrical octahedral coordination while Mn(III) complexes always exhibit substantial distortions (typically tetragonal) from octahedral coordination (Bersuker, 1975). Thus both the optical and crystallographic data available for Mn(III) and Mn(IV) complexes indicates that the significant differences in the 1s → 3d region of the X-ray absorption spectrum are due to substantial Jahn-Teller splittings of the d-orbital energy levels of high-spin d$^4$ Mn(III) ions.
The implicit assumption being made here in comparing the optical data is that the ground state properties largely determine the observed ordering of the d-orbitals due to crystal field effects. It has been suggested that the excited state properties may play a dominant role for purely ionic systems and that the excited state energy levels more closely resemble those of an ion of the same valence but of the next element in the X-ray transitions (Shulman et al., 1976). A one-electron approximation is reasonable for the optical transitions; however, it is clear that multi-electron effects and the effects of core polarization are significant effects which cannot be neglected in the X-ray spectra (Bair & Goodard, 1980; Kutzler et al., 1980). Thus care must be exercised in directly comparing splittings in the 1s → 3d region of the X-ray absorption spectrum to the energy of observed crystal field transitions. Finally, there is some evidence from single-crystal polarized X-ray absorption measurements that quadrupolar transitions constitute an important mechanism in 1s → 3d transitions of centrosymmetric complexes (Hahn et al., 1982), but quadrupolar transitions are not generally significant in optical spectroscopy (Lever, 1984). Thus the more intense 1s → 3d transitions of the Mn(II) and Mn(IV) complexes may be due partially to enhanced quadrupolar transition probabilities in these more symmetrical complexes.

Similar structure to that observed in the 1s → 3d region is also observed in the 1s → 4p region (See Figure 5-4). Shoulders on the low energy side of the 1s → 4p transition (such as that exhibited in Figure 5-5b) have been assigned to splittings in the 1s → 4p transition (Cotton & Hanson, 1958), 1s → 4s transitions (Shulman et al., 1976) or ligand to metal shakedown transitions coupled to the 1s → 4p transition (Bair & Goodard, 1980). A shakedown transition results when an optical transition (in this case presumably a ligand to metal charge transfer band) couples to an X-ray transition. Transitions in the 1s → 4p region for Mn(III) ions are much broader and less intense transitions than those observed for Mn(II) and Mn(IV) ions. This may be due to crystal field splitting of the p-levels. It has also been suggested that the shakedown transitions of high-spin d⁴ ions would be split due to the presence of a single electron in the E₉ orbitals (in octahedral symmetry) which results in singlet or triplet final states separated by the spin pairing energy (~ 1.9 eV) (Bair & Goodard, 1980). The 1s → 4p transitions in the Mn(IV) complexes are generally more intense and narrower than those of the Mn(III) complexes. As was suggested for the pre-edge transitions, this may reflect the
more symmetric coordination which Mn(IV) complexes tend to exhibit.

Transitions at higher energy, above the $1s \rightarrow 4p$ transitions in the X-ray Near Edge Structure (XANES) region, were at first thought to be higher Rydberg transitions (Shulman et al., 1976; Bair & Goodard, 1980); however, more recent calculations indicate that these higher energy transitions are due to multiple scattering “shape resonances” (Penner-Hahn et al., 1986). These broader absorptions or shape resonances are particularly sensitive to the geometry of the molecule – the shorter the metal-ligand bond length, the higher the energy of the absorption. Thus, as the series of edges in Figure 5-4 indicates, the broad shape resonances, higher in energy than the intense $1s \rightarrow 3d$ transitions, move to higher energy with increasing valence and decreasing bond length. The broad featureless post-edge absorptions of the PSII preparations in both the $S_1$ and $S_2$ states suggest the superposition of shape resonances due to both Mn(III) and Mn(IV).

5.3.3 Changes in the K-edges of Isostructural Multinuclear Complexes which Differ by one Unit of Valence

The magnitude of the difference between the $S_1$ Mn K-edge inflection energy and the $S_2$ edge inflection (see Figure 5-1) suggests that one or two equivalents have been stored within the manganese complex during the $S_1 \rightarrow S_2$ state transition. This is indicated by a comparison of observed differences between isostructural multinuclear clusters which differ by one oxidative equivalent. Figure 5-5 contains the Mn K-edges of a set of five pairs of isostructural binuclear, trinuclear and tetranuclear manganese complexes which differ by one formal unit of valence. The differences in edge inflection energy observed for the isostructural complexes (e.g. 1.0 to 1.8 eV) is larger than the difference in edge inflection energy observed for the Mn in the OEC poised in the $S_1$ and $S_2$ states. Note also that the magnitude of the shifts observed here is somewhat smaller than the 2.23 eV shift obtained for a unit valence change for a valence trapped monomer (e.g. the slope of the correlation line shown in Figure 5-2). This may reflect the effect of delocalization of the electron density within these multinuclear clusters, which tends to be greater for the higher oxidation state complexes than for the Mn(II,III) complexes, which exhibit shifts closer to the 2.23 eV value predicted for a valence-trapped monomer (e.g. per unit of coordination charge).

The absolute energy of K-edge inflection of the PSII preparations indicates that it
Figure 5-4. Mn K-edge X-ray absorption spectra of a set of Mn(II), Mn(III) and Mn(IV) complexes with roughly octahedral oxygen coordination spheres. The spectra illustrate significant differences in the edge structure associated with valence. From top to bottom the spectra are of complex 1 Mn(acac)$_2$·2H$_2$O, 5 Mn(acac)$_3$ and 16 MnO$_2$. Ranges for 1s → 3d transitions, 1s → 4p and multiple scattering transitions in the XANES region are indicated.
XANES Region

- Mn(acac)$_2$·2H$_2$O
- Mn(acac)$_3$
- MnO$_2$

1s → 4p
1s → 3d

Normalized F/I$_0$

X-ray Energy (eV)

6525. to 6600.
Figure 5-5. Mn K-edges of five pairs of isostructural multinuclear manganese complexes which differ by one formal unit of valence have been plotted. The spectra shown are (a) 8, Mn$_2$(III,III)O(OAc)$_2$(tmtacn)$_2$(ClO$_4$)$_2$ and 19, Mn$_2$(III,IV)O(OAc)$_2$(tacn)$_2$(ClO$_4$)$_3$, (b) 21, Mn$_2$(III,IV)O$_2$-(phen)$_4$(BF$_4$)$_3$ and 17, Mn$_2$(IV,IV)O$_2$(phen)$_4$(ClO)$_4$, (c) 24, Mn$_4$(II,III,III,III)O$_2$(benzoate)$_7$(bipy)$_2$ and 24, Mn$_4$(III,III,III,III)O$_2$, (d) 22, Mn$_3$(II,III,III)O(benzoate)$_7$(py)$_2$H$_2$O and 11, Mn$_3$(III,III,III)(benzoate)$_8$(Im)$_3$(ClO$_4$)$_2$[NBu$_4$] and (e) 23 Mn$_3$(II,III,III)O(OAc)$_6$(py)$_3$py and 12 Mn$_3$(III,III,III)O(OAc)$_6$(py)$_3$(ClO$_4$). In each case the first complex cited above is the dashed spectrum, with a lower edge inflection energy. The solid lines are the corresponding edges of the higher valence complex.
is more appropriate to compare the shifts observed for complexes which have a formal valence change from Mn(III) to Mn(IV), although shifts observed for lower S-state transitions may involve other formal valence changes (see section 6.3). The magnitude of the change in the edge inflection energy that we observe for the isostructural pairs of binuclear complexes which involve a formal valence change from Mn(III) to Mn(IV) (1.0 - 1.3 eV) is closer to the magnitude of light-induced edge inflection energy shifts that we observe for the PSII preparations (compare Figure 5-1 and 5-5). As we have previously suggested, this indicates the storage of one oxidative equivalent within a dimeric complex or, as the stoichiometry of four requires, two equivalents within the manganese present in PSII (Goodin et al., 1984). Comparisons of edge properties of isostructural pairs of complexes which differ in formal valence are particularly relevant to the interpretation of the observed light-induced changes in the PSII preparations, for it is unlikely that major changes in the geometry or ligation sphere of the manganese complex are occurring at the low temperature (195 K) at which state advancement is maximized and cryogenically stabilized by continuous illumination. Also EXAFS analyses of the Mn complex poised in these two states indicate that the geometry of the complex is unchanged during this transition (Yachandra et al., 1987). Note, however, that the changes in the 1s → 3d transition region of the K-edge of the PSII preparations are unlike any of the changes observed in the K-edges of the pairs of isostructural models, which suggests that the simple picture of one equivalent removed from a dimeric structure is inadequate.

5.3.4 Mixed Valence Multinuclear Manganese Complexes

Although it would appear that the structure of the 1s → 3d region of the X-ray absorption spectrum of the S₁ state of PSII preparations from spinach and Synechococcus resembles that of Mn(III) complexes and this region of the X-ray absorption spectrum of the S₂ preparations resembles that of Mn(IV) complexes, considering other known complicating factors, these arguments are too simplistic. As stated earlier, it is now generally accepted that there are four manganese atoms per PSII reaction center. The fact that the manganese complex in PSII preparations in the S₂ state exhibits an EPR signal (the multiline EPR signal) due to a Kramers spin system indicates that the Mn present do not have a uniform valence, nor is it possible that the storage of one or two oxidative equivalents would result in a change from all Mn(III) to all Mn(IV). As
described in detail in chapter 4, an extensive parallel analysis of a set of \( \mu \)-oxo bridged multinuclear manganese complexes indicates that the Mn present in PSII preparations poised in the \( S_2 \) state are most probably associated as a pair of binuclear di-\( \mu \)-oxo bridged complexes. These facts necessitate the consideration of significant electronic effects associated with mixed valence multinuclear systems. Since the early reviews of Robin and Day (1967), Hush and Allen (1967) and with the formulation of a theory of intervalence electron transfer by Hush (1967), a tremendous amount of interest and work has been devoted to the study of mixed valence metal complexes; most notable and extensive has been the work of Taube and Creutz (Creutz, 1983).

The spectral properties of a mixed valence complex can deviate substantially from those expected for the sum of constituent ions, depending on the extent of resonance stabilization within the complex. Based on the properties exhibited by a mixed valence complex, Robin and Day (1967) developed a practical classification scheme. Class I complexes are valence trapped, and the spectral properties are just the sum of the spectra of the individual ions present. Class III ions are fully delocalized and the spectral properties do not resemble those exhibited by the individual ions. Class II complexes exhibit optical properties similar to the fragment ions but also exhibit some additional properties (e.g. intervalence transitions).

Recently, a number of extensive physical characterizations of multinuclear mixed valence manganese complexes have appeared (Cooper & Calvin, 1977; Cooper et al., 1978; Stebler et al., 1986; Sheats et al., 1987; Vincent et al., 1987b). The optical, magnetic and crystallographic data available for this set of mixed valence, multinuclear complexes indicate that manganese exhibits a tendency toward valence trapped behavior. All of the complexes examined in this study fall into class I or class II of Robin and Day's scheme. This is indicated by the following facts.

Generally the magnitude of the coupling between manganese centers, determined by fitting variable temperature susceptibility data, assuming an isotropic Heisenberg exchange operator, is substantially smaller for \( \mu \)-oxo bridged manganese complexes than for isostructural iron complexes. For example the calculated value for exchange coupling in the binuclear mono \( \mu \)-oxo bridged manganese complex, 8, is \( < 1 \) cm\(^{-1} \), while the analogous Fe(III) dimer exhibits an exchange coupling of \( J=-121 \) cm\(^{-1} \) (Sheats et al., 1987). Similarly the trinuclear manganese complexes (11,12,22,23) all exhibit
exchange coupling constants $\lesssim -10 \text{ cm}^{-1}$ while the isomorphous iron analogs exhibit antiferromagnetic exchange couplings in the range of -20 to -30 cm$^{-1}$ (Vincent et al., 1987b). Those complexes which exhibit substantial couplings like the binuclear $\mu$-oxo bridged complexes 20 ($J=-150 \text{ cm}^{-1}$)(Cooper et al., 1978), 17 ($J=-144 \text{ cm}^{-1}$) and 21 ($J=-148 \text{ cm}^{-1}$) (Stebler et al, 1986) are substantially smaller than one would expect for two metal centers as close as they are (2.7 Å) in these complexes. This anomalously low coupling is even more marked in the case of the tetranuclear butterfly-like complex, 14 ($J=-22.5 \text{ cm}^{-1}$ for the two nearest Mn centers (2.85 Å) and $J=-7.6 \text{ cm}^{-1}$ for the more distant pairs). For mono $\mu$-oxo bridged binuclear complexes (e.g. like 7, 8 and 19), it has been suggested that the lesser number of electrons in the $E_1$ antibonding orbitals of these manganese complexes eliminates important exchange coupling paths available in the corresponding iron complexes (Sheats et al., 1987; Wieghardt et al., 1986).

The position and linewidth of intervalence bands in the near IR spectrum of the binuclear mixed valence complexes 19,20,21 and the Mn$_2$(III,IV) mixed valence analog of 7 also support the assignment of weakly interacting class II ions. Calculation of the delocalization coefficient (Cooper & Calvin, 1977) ($\alpha^2=0.01$) for 20 indicated that the electron is only 1% delocalized, which is fairly small compared to the extent of delocalization in Creutz-Taube ions (e.g. [(NH$_3$)$_5$Ruhpz]$^2+$, $\alpha^2=0.0026$) considering the $r^{-2}$ dependence of the delocalization coefficient. The metal-metal distance in 20 is 2.7 Å while the corresponding distance in the Creutz-Taube ion is 6.8 Å(Creutz, 1983). Note that the corresponding mixed valence ruthenium pentammine complex bridged by a single intervening oxygen is a fully delocalized class III ion.

Finally, first coordination sphere bond lengths determined from the X-ray crystal structure which are characteristic of each valence are observed for three of the mixed valence ions studied; the di-$\mu$-oxo bridged Mn$_2$(III,IV) complex (20), the triangular trinuclear $\mu_3$-oxo bridged Mn$_3$(II,III,III) complex (22) and the dodecanuclear manganese complex (25) which contains a mixture of Mn(III) and Mn(IV) centers. An extensive analysis of the temperature dependence of the diffraction data of 21 indicated the presence of static or dynamic disorder, again consistent with the assignment of a valence trapped system (Stebler et al., 1986). The equivalence of the metal centers in 23 has been attributed to rapid thermal electron transfer (Vincent et al., 1987b) However, this points to a limitation of the crystallographic method in that it is unable to discriminate
between static and dynamic electron delocalization due to the time scale of the technique. Experiments using a faster time scale (IR measurements of Fe-N stretching frequencies and Mossbauer measurements) in the analogous mixed valence iron trinuclear system indicated a substantial amount of static delocalization (Meesuk et al., 1987). This also suggests that the X-ray absorption measurements of bound state transitions may provide a useful tool for discriminating between static and dynamic delocalization in these mixed valence complexes. The level of disorder in the structure of 19 was such that it was not possible to tell unequivocally whether the two sites are inequivalent. Thus for those complexes for which sufficient quality diffraction data exist, the tendency of manganese toward valence trapped behavior is evident.

This tendency of manganese in its higher valences to exhibit valence trapped behavior supports the contention that the changes in the $1s \rightarrow 3d$ region of the PSII preparations can be ascribed to differences associated with a change in the valence of one of the ions present from Mn(III) to Mn(IV). However, it should be noted that this dramatic difference in the structure of the $1s \rightarrow 3d$ transition is generally not observed in isostructural multinuclear clusters which differ by a single equivalent (examine the $1s \rightarrow 3d$ transition region of the Mn K-edges in Figure 5-3). This indicates that more than one equivalent may be involved in the $S_1 \rightarrow S_2$ state transition.

It has been shown in a study of iron binuclear complexes that the amplitude of the $1s \rightarrow 3d$ transition increases with large exchange couplings between metal centers. This amplitude enhancement reportedly becomes significant only when the magnitude of the exchange coupling exceeds $80 \text{ cm}^{-1}$ (Roe et al., 1984). There have been reports that the magnitude of the coupling between the manganese centers in the OEC is on the order of 50 to $130 \text{ cm}^{-1}$, (de Paula et al., 1985, 1986), however recently the basis of this model has been challenged (Hansson et al., 1987). When more highly purified $O_2$-evolving core preparations become available, it may be possible to determine the magnitude of the exchange coupling between the manganese centers in the OEC by performing variable temperature magnetic susceptibility measurements. It should be noted that it is probable that the $1s \rightarrow 3d$ transition enhancement observed for the binuclear iron complexes with increasing exchange coupling is probably not manifested by multinuclear manganese complexes. For example, the amplitude of the $1s \rightarrow 3d$ transition in the tetranuclear adamantane-like complex (18), in which the coupling between centers
is known to be small, is significantly larger than the amplitude of the 1s → 3d transition in di-μ-oxo bridged binuclear complex (17) in which the coupling between centers is large. Thus, at least for these two complexes (17 and 18) the variation in the amplitude of the 1s → 3d transition is independent of the exchange coupling between manganese centers. Also, as described above the tendency of the higher valences of manganese toward valence trapped behavior is much more pronounced than that observed for the higher valences of iron. Thus, it is suggested that the amplitude enhancement of the 1s → 3d transitions observed for the binuclear iron complexes is a consequence of direct exchange coupling caused by electron delocalization, an effect which is not significant for multinuclear mixed valence μ-oxo bridged manganese complexes. Exchange coupling between manganese centers is mediated through superexchange pathways and does not involve electron delocalization.

5.4 Conclusions

1. A comparison of the absolute edge energy of PSII preparations from spinach and Synechococcus poised in the S₁ and S₂ states with the edge inflection energies of an extensive set of inorganic complexes demonstrates that the higher valences of manganese are present in these states. Considering the EPR properties and the Mn K-edge energy of the S₂ state, it is suggested that the formal valence of the four manganese present in PSII in the S₂ state is most probably (III,N,N,N).

2. An examination of edge inflection energies of a set of five pairs of isostructural multinuclear manganese complexes indicates that the light-induced edge inflection energy shift observed in the PSII preparations could be explained by the storage of one or two oxidative equivalents as we have previously suggested.

3. The dramatic light-induced change in the structure of the 1s → 3d pre-edge transition of PSII preparations from spinach and Synechococcus indicates a formal valence change of Mn(III) → Mn(IV) during the S₁ → S₂ state transition. Differences in the pre-edge absorption profile of Mn(III) and Mn(IV) complexes are suggested to arise from significant Jahn-Teller splittings of the d-orbitals which occur in the high spin d⁴ configuration of Mn(III) complexes. The tendency of manganese toward valence trapped behavior in its higher valences supports the assignment of the oxidation state change occurring in PSII during the S₁ → S₂ transition.
Chapter 6

The $S_0$ State of the Oxygen Evolving Complex of Photosystem II
Induced by Incubation with Hydroxylamine: A Mn X-ray Absorption Study
Comparing the Structure of the Manganese Complex in Photosystem II Preparations Cryogenically Stabilized in the $S_0$ and $S_1$ States

6.1 Introduction

The mechanism of photosynthetic water oxidation is a complex light-driven four electron transfer process involving a membrane-bound chlorophyll-protein complex. The current phenomenological model of photosynthetic water oxidation involves five intermediate states, commonly called $S_0$ - $S_4$ (Kok et al, 1970). This is based on a remarkable phenomenon first observed by Joliot et al.(1969); they found that the oxygen evolved when dark-adapted chloroplasts are excited by a series of short saturating flashes of light occurs in discrete pulses which exhibit a periodicity of four in amplitude. The first maximum flash yield of oxygen was observed on the third flash and thereafter on every fourth flash. Thus, the $S_1$ state is the dominant state present in dark-adapted chloroplasts. The $S_4$ state is a transient state which decays to produce the $S_0$ state concurrent with the release of $O_2$. Absorption of a photon by the Photosystem II (PSII) reaction center produces a single oxidative equivalent. The coupling of four sequential photo-events to the oxidation of two water molecules in the production of a molecule of oxygen is now known to involve a cluster of manganese atoms (for a review, see Babcock, 1987). The first spectroscopic evidence of the involvement of manganese came from the discovery of a multiline EPR signal with hyperfine structure characteristic of manganese (Dismukes & Siderer, 1981). Based on its flash dependence, the multiline EPR signal was associated with the $S_2$ state.

It has been suggested that the manganese cluster is the binding site of the substrate water. There is some indication of direct ligation of water to manganese from studies of line broadening of the multiline EPR signal upon $H_2^{17}O$ exchange (Hansson et al., 1986) and from line narrowing which occurs upon $^2H_2O$ exchange (Nugent, 1987). However, the assertion of direct binding is based principally on studies involving "water analogs" which inhibit or alter the normal cycle of oxygen evolution. Ammonia and a few low molecular weight amines competitively inhibit water oxidation (Sandusky & Yocum,
1983, 1984, 1986). Ammonia has been shown to alter the properties of the multiline EPR signal (Beck et al., 1986). This has been taken as evidence of direct ligation of a "water analog" to the manganese cluster.

Another class of low molecular weight "water analogs" includes hydroxylamine, hydrogen peroxide and hydrazine. At low concentration these mild reducing agents do not inhibit steady state oxygen evolution. However, they alter the normal light driven cycle. Low concentration hydroxylamine (Bouges, 1971), hydrogen peroxide (Velthuys & Kok, 1978) and hydrazine (Kok & Velthuys, 1977) cause a two-flash delay in the occurrence of the first maximum flash yield of oxygen (e.g., the first maximum flash yield of oxygen is delayed until the fifth flash). Of these three molecules, the effect of hydroxylamine has been studied most extensively. Several mechanisms have been proposed to explain the effect of hydroxylamine. These mechanisms differ in the extent to which the oxygen evolving complex is purportedly reduced in the dark (Bouges-Bocquet, 1973). The details of three mechanisms described below are summarized in Scheme 6-1. In dark-adapted chloroplast suspensions incubated with low concentrations of hydroxylamine, a two-electron reduction of the oxygen evolving complex (OEC) has been proposed to occur in the dark, resulting in a super-reduced state that has been designated $S_{-1}$ (mechanism 1 in Scheme 6-1). Alternatively, it has been suggested that a one-electron reduction occurs in the dark (generating the $S_0$ state) and that a second one-electron reduction occurs after one turnover of PSII (mechanism 2 in Scheme 6-1). Finally, it has been suggested that two molecules of hydroxylamine bind to the OEC in the dark and that a single-electron reduction occurs during each of two successive turnovers of PSII (mechanism 3 in Scheme 6-1). This proposed mechanism leaves the OEC poised in the $S_1$ state throughout this process.

In this report, we present the results of an Mn K-edge X-ray absorption spectroscopy study of the effect of hydroxylamine on the manganese complex in PSII. At high concentrations of hydroxylamine (e.g. 1 - 2 mM) the manganese in chloroplast (Cheniae & Martin, 1970) or PSII membrane preparations (Tamura & Cheniae, 1985) is irreversibly released as Mn$^{2+}$. The principal focus of this report has been to investigate the reversible effects occurring at low concentrations of hydroxylamine. We will refer to the state of the OEC generated by incubating dark-adapted PSII membrane preparations with low concentrations of hydroxylamine in the dark as the $S_{-1}$ state. The
**Scheme 6-1**

<table>
<thead>
<tr>
<th>Dark reactions</th>
<th>Light induced reactions</th>
</tr>
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<tbody>
<tr>
<td>$S_1 + 2 \text{NH}<em>2\text{OH} \rightarrow S</em>{-1}$</td>
<td>$h\nu \rightarrow S_0 \rightarrow S_1$</td>
</tr>
<tr>
<td>$N_2 + 2 \text{H}^+$</td>
<td>$h\nu \rightarrow S_0 \rightarrow S_1$</td>
</tr>
<tr>
<td>$S_1 + 2 \text{NH}_2\text{OH} \rightarrow S_0 \cdot \text{NH}_2\text{OH}$</td>
<td>$h\nu \rightarrow S_0 \rightarrow S_1$</td>
</tr>
<tr>
<td>$\frac{1}{2} \text{N}_2 + \text{H}^+$</td>
<td>$h\nu \rightarrow \frac{1}{2} \text{N}_2 + \text{H}^+$</td>
</tr>
<tr>
<td>$S_1 + 2 \text{NH}_2\text{OH} \rightarrow S_1 \cdot 2\text{NH}_2\text{OH}$</td>
<td>$h\nu \rightarrow S_1 \cdot \text{NH}_2\text{OH} \rightarrow S_1$</td>
</tr>
<tr>
<td>$\frac{1}{2} \text{N}_2 + \text{H}^+$</td>
<td>$\frac{1}{2} \text{N}_2 + \text{H}^+$</td>
</tr>
</tbody>
</table>
state of the OEC generated by continuous illumination of S_{-1} preparations at 195 K will be referred to as the S_0 state. X-ray absorption spectroscopy is an element-specific technique capable of yielding information directly about the oxidation state (Kirby et al., 1981a, Sauer et al., 1987, also see chapter 5) and immediate ligation sphere of the absorbing element (for a review see Powers, 1982). We have used this technique to address three aspects of the effect of hydroxylamine: 1) We have examined the extent of reduction of the manganese in PSII occurring in the dark upon addition of hydroxylamine. Changes in the oxidation state of manganese were also examined after continuous illumination at 195 K; 2) Extended X-ray absorption fine structure analysis provides a means of determining the number, the identity and, the distances of ligating atoms from the absorbing atom. Assuming then that the hydroxylamine-induced S_0 (S_0) state is the same as the normal S_0 state, an examination of differences between the structure of the hydroxylamine-induced S_0 state and the S_1 state may reflect structural rearrangements important to an understanding of the mechanism of oxygen evolution; and 3) Do the changes in the structure and oxidation state of manganese induced by hydroxylamine treatment provide evidence of the binding of a "water analog" to the manganese at the water oxidation site? Changes in the structure of the absorption edge can yield information regarding the changes in site symmetry or ligation of the metal absorber. By examining the Mn K-edges of dark adapted PSII preparations with and without hydroxylamine present, effects caused by direct binding of hydroxylamine to manganese are addressed.

6.2 Experimental Procedures:

6.2.1 Preparation of PSII membranes for X-ray absorption measurements.

Oxygen evolving PSII subchloroplast membranes were prepared as previously described (Yachandra et al., 1986a). Because of the instability of hydroxylamine solutions, hydroxylamine treated samples were prepared by diluting volumes of freshly prepared 1mM stock solutions into 20 ml volumes of PSII subchloroplast membranes suspended at 3.5 mg/mL chlorophyll. Samples containing final concentrations of 40μM, 60μM and 100μM hydroxylamine were prepared. Samples were allowed to stand in the dark for 30 min to ensure complete reaction. After 30 min an ethanolic solution of DCMU was added to the PSII membrane suspension to bring the final concentration of DCMU to 50μM.
Samples for X-ray absorption spectroscopy were prepared by pelleting PSII sub-chloroplast membranes at 35,000×g for 1 h. Tightly packed pellets containing ~30% glycerol were transferred to lucite sample holders. Samples were supported on the distal side by 0.025 mm thick mylar tape. The size of the sample holders was chosen so that they could be inserted directly into an Air Products Helitrans cryostat for monitoring the EPR spectrum of the sample. All illuminations, EPR measurements and X-ray absorption measurements were performed directly on samples mounted in these holders. EPR spectra of PSII preparations were recorded both before and after X-ray exposure to ensure sample integrity and a stable S-state composition.

Samples were dark adapted for 1 h prior to illumination. The samples were then illuminated with a 400 W tungsten lamp through a 5 cm water filter for 90 sec at 195 K in a dry-ice/methanol bath contained within an optical Dewar. Sample temperature was monitored throughout with a copper-constantan thermocouple.

EPR spectra were recorded using a Varian E109 spectrometer equipped with a model 102 microwave bridge. Sample temperature was maintained at 8 K using an Air Products liquid He cryostat. Spectra were recorded at 9.21 GHz with a field modulation of 32 G at 100 kHz, using a microwave power of 50 mW. The amplitude of the multiline EPR signal was quantitated by adding peak-to-trough amplitudes of four of the hyperfine lines on the low field side of g=2 measured from the illuminated-minus-dark difference spectra.

X-ray absorption spectra collected in fluorescence mode (Jaklevic et al., 1977) were recorded at the Stanford Synchrotron Radiation Laboratory, Stanford, California during dedicated operation of the SPEAR storage ring which provides 40 - 80 mA electron beams at 3.0 GeV. The Mn K-edge absorption spectra were obtained on the wiggler beam line IV-2 using a Si(111) double crystal monochromator. Energy calibration was maintained by simultaneously measuring the strong narrow pre-edge feature of KMnO₄ at 6543.3 eV (Goodin et al., 1983). Despite a loss of a factor of about four in flux, EXAFS scans were recorded using Si(400) monochromator crystals due to the presence of variable diffraction “glitches” (see section 2.3.1) in the EXAFS spectra obtained with Si(111) crystals. EXAFS spectra were recorded using a solid state lithium-drifted silicon X-ray detector as previously described (Jaklevic et al., 1977; Goulding et al., 1983a). The use of a triangular pulse shaping amplifier substantially improved the
photon counting rate of this solid state detection system (Goulding et al., 1983b).

X-ray absorption edge spectra were recorded using a plastic scintillation array. Details of the design of the plastic scintillation array used in fluorescence detection of Mn K-edge absorption spectra have been described in detail previously (Yachandra et al., 1986). Samples were maintained in liquid nitrogen in the dark prior to and immediately following X-ray absorption measurements. To insure functional integrity and confirm a stable S-state composition, EPR spectra were recorded as described above, before and after X-ray measurements. During X-ray measurements, the PSII samples mounted in lucite sample holders were inserted into a double wall Kapton cryostat maintained at 170 K by a liquid N$_2$ cooled N$_2$ gas flow system.

6.2.2 X-ray Data Analysis

Data analysis procedures have been described in detail elsewhere (Kirby, 1981c; Goodin, 1983; Yachandra et al., 1986a, also see section 2.2). As a result, only a brief outline of data analysis procedures will be presented here. Mn K-edge spectra of PSII preparations analyzed are the sum of 3-4 individual scans. A linear pre-edge background was subtracted. The Mn K-edge energy was taken as the first major edge inflection point. This was calculated by determining the maximum of the first derivative of the smoothed edge. A linear 4 eV smoothing fit was calculated for each edge. The derivative of the smoothed edge was taken as an approximation to the true first derivative. Uncertainties in the determination of the first major inflection are typically ± 0.1 eV.

For EXAFS analysis, 30 - 40 individual scans were added after they had been examined for satisfactory signal-to-noise ratio and were determined to be free of anomalies. The EXAFS oscillations, $\chi(k)$, in the absorption cross-section were obtained from the data by subtraction of a cubic spline fit to remove the free atom absorption and any background contributions to the spectrum. Because the concentration of manganese in PSII preparations is exceedingly small (approximately 500 - 800 $\mu$M) and as a result our signal-to-noise ratio is relatively small, we choose to multiply our data by $k^1$ to compensate for the gradual decay in the amplitude of EXAFS oscillations. Usually EXAFS oscillations are multiplied by $k^3$ to compensate for the approximately $1/k^3$ dependence of EXAFS oscillations beyond $k=4$ Å$^{-1}$. Where possible, individual scattering shells were isolated by applying a window function to the Fourier transform of the EXAFS.
The back transformed data were then fit using phase and amplitude functions which have been calculated by Teo and Lee (1979). The use of Fourier filtered data greatly improves convergence of the non-linear least squares fitting procedure which yields the structural information. Four parameters are used in the simulation of each component. The distance information contained in the frequency of the EXAFS waves is determined by simulation and is in general very accurate. The accuracy is limited only by a weak correlation between the distance parameter and the assignment of the threshold for ionization. The uncertainty is typically ±0.03 Å. The number of scatterers in a given shell determines the amplitude of that wave. However, the uncertainty in the number of scatterers determined by simulation is greater than that for the distances obtained, due to a strong correlation between the degree of disorder and the number of atoms within a shell. The disorder within a scattering shell is parameterized by a Debye-Waller like term. The error in the determination of the number of scatterers can be as large as 50%, and is typically ±20% (Teo & Lee, 1979). When two unresolved components are present in a given scattering shell, additional correlations can occur between the amplitude of one component and the distance to the other which limit the accuracy of a given parameter (Eisenberger & Lengeler, 1980).

Fits to the EXAFS of the PSII preparations were performed using constraints on the range of variation in the ionization threshold (E₀) which we have found yield the best agreement with the internuclear distances obtained from the crystal structures of inorganic complexes. These constraints reduced systematic errors associated with the correlation between the energy of the ionization threshold (which determines the phase and to a lesser extent the frequency of a given EXAFS wave) and the distance to a scattering shell. For example, for the Mn-Mn distances in multinuclear μ-oxo bridged complexes, agreement to within ±0.02 Å is obtained by constraining E₀ to the range between 6550 and 6560 eV (see chapter 4). In all simulations an initial edge threshold of 6560 eV is assumed. The energy of the edge threshold was varied in the non-linear least square minimization procedure by allowing a correction term to the initial estimate of the ionization threshold (ΔE₀) to vary between 0 and -10 eV. This approach using fine adjustments based on models was developed by Teo et al. (1983).

6.3 Results

6.3.1 EPR Spectral Characterization
Continuous illumination at 195 K of dark adapted subchloroplast PSII membranes generates and traps a species which exhibits a multiline EPR signal (Brudvig et al., 1983). The decrease in amplitude of this signal following illumination at 195 K of PSII membranes containing low concentrations of hydroxylamine is the criterion that was used in this study to determine the fraction of centers delayed by two flashes in the S-state cycle. Centers affected in this manner are now poised in a state resembling $S_0$.

Changes in the EPR spectrum in the region around $g=1.9$ were monitored as an indication of reduction of the primary acceptor in PSII preparations (Rutherford & Zimmermann, 1984). This signal is analogous to the reduced primary acceptor signal which has been extensively characterized in reaction center preparations from purple non-sulfur bacteria (Butler et al., 1984). dePaula et al. (1985) have used the amplitude of this signal to quantitate the relative number of stable charge separations.

Figure 6-1 contains the illuminated-minus-dark X-band EPR spectra recorded at 8 K of the following preparations: (a) a dark-adapted PSII sample which was illuminated at 195 K for 90 s and (b) a PSII sample which was treated in the same manner but also contained 60 $\mu$M hydroxylamine. The amplitude of the residual multiline EPR signal exhibited by the hydroxylamine-treated sample is < 5% of the control $S_2$ sample. The amplitude of the reduced primary acceptor signal shown in Figure 6-1a was estimated by comparing the amplitude of the second manganese hyperfine feature on the high field side of $g=2$ in the multiline spectrum presented here with a multiline spectrum which is not convolved with the reduced quinone acceptor signals (e.g. the 8K EPR spectrum of a PSII preparation containing 1mM PPBQ which was illuminated at 195 K and warmed for 30 s at 293 K, data not shown). Based on this estimate, the amplitude of the $g=1.9$ feature in the difference EPR spectra of both samples is approximately the same. This was taken as an indication of stable charge separation and the storage of an oxidative equivalent on the donor side of PSII in both cases. As a byproduct of the oxidation of $Q_{400}$, an extremely stable charge separated state (in this case the $S_2$ state) is generated by illumination at 195 K of PSII preparations containing 1mM PPBQ followed by warming for 30 s at 293 K (Zimmermann & Rutherford, 1986). The warming step results not only in the oxidation of $Q_{400}$ but also in the complete oxidation of the reduced primary quinone acceptor.

6.3.2 Mn K-edge X-ray Absorption Spectra
Figure 6-1. Illuminated-minus-dark difference spectra of PSII preparations illuminated for 2 min at 195 K without hydroxylamine (upper trace) and containing 60 μM hydroxylamine (lower trace). Spectrometer conditions are described in Experimental procedures, section 6.2.1.
Mn K-edge X-ray absorption studies were performed on samples poised predominantly in the S\textsuperscript{−1}, S\textsuperscript{0}, S\textsuperscript{1} and S\textsuperscript{2} states. Signal averaged Mn K-edge X-ray absorption spectra of PSII samples poised in the S\textsuperscript{−1} and the S\textsuperscript{1} states are shown in Figure 6-2. The S\textsuperscript{−1} spectrum, shown in Figure 6-2, is that of a dark-adapted PSII preparation incubated with 40\textmu M hydroxylamine. Within the signal-to-noise and the uncertainty of the measured edge inflection energies, these two spectra are identical.

Changes in the edge position would reflect oxidation state changes. Changes in the symmetry of the site accompanying a conformational change within the manganese cluster would be indicated by changes in the shape and/or position of the edge (for a review see Srivasta & Nigam, 1972). The absence of such changes indicates that PSII preparations poised in the the S\textsuperscript{1} state and the dark-adapted preparations treated with hydroxylamine contain manganese complexes at the same oxidation level and that the site symmetry is unaltered. This is direct spectroscopic evidence that hydroxylamine does not cause reduction of the manganese center in the dark at the concentrations which induce a two-state delay in the catalytic cycle.

Signal-averaged Mn K-edge spectra of the S\textsuperscript{0}, S\textsuperscript{1} and S\textsuperscript{2} states are shown in Figure 6-3. The Mn K-edge of the S\textsuperscript{0} preparation shown is that of a PSII sample treated with 40 \textmu M hydroxylamine and illuminated at 195 K. A dramatic shift to lower energy upon illumination of hydroxylamine-containing samples is observed. This shift is comparable in magnitude but opposite in sign to the light-induced shift that we have previously reported for the S\textsuperscript{1} \rightarrow S\textsuperscript{2} state transition (Yachandra et al., 1986a). The shifts that we observed for the S\textsuperscript{1} \rightarrow S\textsuperscript{2} and S\textsuperscript{1} \rightarrow S\textsuperscript{0} states are comparable in magnitude to the shift observed when one equivalent is removed from a binuclear manganese complex; however, similar shifts are also observed when a single equivalent is removed from a tetranuclear manganese complex (see section 5.3.1).

Table 6-1 contains the multiline EPR signal amplitudes, the g=1.9 EPR signal amplitudes, the estimated state composition and Mn K-edge inflection energies for a set of hydroxylamine treated PSII preparations. Three hydroxylamine concentrations were examined: 40 \textmu M, 60\textmu M and 100 \textmu M. All PSII preparations containing hydroxylamine exhibited a light-induced shift to lower X-ray edge energy. The most straightforward interpretation of this result is that upon illumination reduction of the manganese center occurs.
Figure 6-2. A comparison of the Mn K-edge absorption spectra of dark-adapted PSII samples with \((S^*_{1})\) and without \((S_{1})\) hydroxylamine. Within the signal-to-noise and the uncertainty of the edge measurement, the spectra of the PSII samples poised in the \(S_{1}\) and \(S^*_{1}\) states are identical. A linear fit to the spectrum below the onset of Mn absorption has been removed. The data were collected in fluorescence mode and were ratioed to the incident X-ray photon intensity, \(I_0\). Normalized fluorescence counts, \(F/I_0\), are plotted as a function of X-ray photon energy in eV. Smoothed curves have been drawn through the data points. A running quadratic fit with a 2eV smoothing domain was used to smooth the data for presentation.
Qualitatively, the manganese K-edge energy increases with increasing oxidation state. However, the position of the K-edge is actually an indication of the positive potential of the metal center and is, thus, also affected by the electron donating character of ligand atoms. The manganese K-edge energy of the S₀ state is roughly in the range of edge energies associated with a formal valence of Mn(III) (Kirby et al., 1981a; Sauer et al., 1987; see also section 5.3.1).

In addition to the light-induced shift, a shift to lower X-ray edge energy occurs in dark-adapted preparations containing higher concentrations of hydroxylamine, relative to dark-adapted samples which do not contain hydroxylamine. The magnitude of this shift increases with increasing hydroxylamine concentration. Also contained in Table 6-1 is the fraction of the total manganese present as Mn²⁺ as determined by the amplitude of the six line EPR spectrum recorded at 8K. Total manganese present was determined by atomic absorption. The magnitude of the dark shift in the Mn K-edge inflection energy correlates with the amount of Mn²⁺ released. A shift of ~ 0.5 eV occurs for each increment of 10% of the total manganese released as Mn²⁺. This suggests that, at higher concentrations of hydroxylamine, a dark reduction of the manganese complex occurs which results in the release of Mn²⁺. Simulations performed by adding a fraction of the X-ray absorption edge of hexa-aquo Mn²⁺ to the S₁ edge as indicated in Table 6-1, yield decreases in the X-ray edge energy, relative to S₁, consistent with the observed behavior. Hydroxylamine is known to release manganese in the form of Mn²⁺ at higher concentrations (Cheniae & Martin, 1970, Tamura & Cheniae, 1985). We and others (Cheniae & Martin, 1970; Tamura & Cheniae, 1986) have found that the release of Mn²⁺ is strongly correlated with the loss of oxygen evolving activity. The lowest concentration of hydroxylamine, which induces a light-induced edge shift, does not result in a shift to lower edge energy in the dark, relative to controls which do not contain hydroxylamine. This indicates that the dominant reaction leading to the two state delay in the S-state cycle, which occurs at low concentrations of hydroxylamine, is due to a two-electron, light-induced reduction of the manganese center. The dark reactions which increase with increasing hydroxylamine concentration reflect only a small fraction of the centers present. The manganese released is in the form of non-functional Mn²⁺.

6.3.3 Mn EXAFS Analyses

The k¹-weighted EXAFS modulations of a S₀ sample are shown in Figure 6-4
Figure 6-3. A comparison of the Mn K-edge absorption spectra of PSII preparations poised in the $S_0$, $S_1$ and $S_2$ states of the oxygen evolving complex. Spectra shown are: the Mn X-ray absorption edges of a dark-adapted PSII preparation (---); a PSII preparation illuminated at 195 K (---) and a PSII preparation containing 40μM hydroxylamine illuminated at 195K (-----). Spectra are plotted as described in Figure 6-2.
Table 6-1
Mn K-edge Infection Energies of PSII Preparations
Treated with Hydroxylamine

<table>
<thead>
<tr>
<th>Hydroxylamine Concentration (µM)</th>
<th>Sample Treatment</th>
<th>Multiline Signal Amplitude</th>
<th>State Composition</th>
<th>Mn²⁺ Conc.</th>
<th>Mn K-edge Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Dark Adapted</td>
<td>0</td>
<td>100% S₁</td>
<td>5%</td>
<td>6551.4</td>
</tr>
<tr>
<td>0</td>
<td>Illum. 195K</td>
<td>100</td>
<td>100% S₂</td>
<td>5%</td>
<td>6552.3</td>
</tr>
<tr>
<td>40</td>
<td>Dark Adapted</td>
<td>0</td>
<td>35% S₁, 65% Sₐ₋₁</td>
<td>5%</td>
<td>6551.5</td>
</tr>
<tr>
<td>40</td>
<td>Illum. 195K</td>
<td>35</td>
<td>35% S₂, 65% S₈</td>
<td>5%</td>
<td>6550.2</td>
</tr>
<tr>
<td>60</td>
<td>Dark Adapted</td>
<td>0</td>
<td>93% Sₐ₋₁</td>
<td>12%</td>
<td>6550.6</td>
</tr>
<tr>
<td>60</td>
<td>Illum. 195K</td>
<td>0</td>
<td>93% S₈</td>
<td>12%</td>
<td>6549.9</td>
</tr>
<tr>
<td>100</td>
<td>Dark Adapted</td>
<td>0</td>
<td>84% Sₐ₋₁</td>
<td>21%</td>
<td>6549.9</td>
</tr>
<tr>
<td>100</td>
<td>Illum. 195K</td>
<td>0</td>
<td>84% S₈</td>
<td>21%</td>
<td>6549.5</td>
</tr>
</tbody>
</table>

a. Multiline signal amplitudes are expressed as percentages of the control S₂ state preparation which was generated by illumination at 195K. Uncertainties in the measurement of the amplitude of the multiline EPR signal are estimated to be ± 5% of maximum amplitude.

b. Mn²⁺ concentrations are expressed as a percentage of the total manganese present as determined by atomic absorption. Uncertainties in the Mn²⁺ concentration are estimated to be ± 3% of the total Mn present. Mn²⁺ concentrations were determined by measuring the amplitude of the six line spectrum observed at 8K relative to solutions of MnCl₂ solutions of known concentration.

c. Reductions in the state composition from 100% are based on the release of non-functional Mn²⁺ above the control levels.
Figure 6-4. $k^1$-weighted EXAFS region of the Mn X-ray absorption spectrum of (a) a dark adapted PSII preparation (e.g. a $S_1$ state preparation) and (b) a PSII preparation poised in the $S_0$ state. The data are indicated with a dashed line and the solid trace is the sum of the Fourier components due to the two main peaks in the Fourier transform of the EXAFS spectra shown in Figure 6-5.
Wavevector $k(\text{Å}^{-1})$ vs. $k\chi(k)$
Figure 6-5. Fourier transforms of the $k^1$-weighted Mn EXAFS of an $S_1$ state preparation (-----) and an $S_0$ state preparation (-- -- --). Spectra plotted are the Fourier transforms of the Mn EXAFS spectra shown in Figure 6-4. The significantly lower amplitude of the $S_0$ state preparation Mn EXAFS peaks indicates a much more disordered system. The distances to each scattering shell are shorter than the true bond lengths due to an average phase shift, $\langle \frac{d_{el}(h)}{dh} \rangle_k$, which is characteristic of the given absorber-scatterer pair.
together with the $k^1$-weighted EXAFS spectrum of a $S_1$ sample. Differences in the EXAFS waves are evident. Also, the amplitude of the oscillations in the EXAFS of the PSII preparation in the $S_1$ state are significantly greater than those of the $S_0$ state. Figure 6-5 contains the Fourier transforms of the $k^1$-weighted EXAFS of Mn in the $S_0$ and $S_1$ states. Two major peaks are evident in the Fourier transforms of both PSII preparations. The amplitude of the peaks in the Fourier transform of the $S_0$ Mn EXAFS are substantially lower than the corresponding peaks in the $S_1$ state EXAFS Fourier transform. This change is characteristic of an increase in the disorder present in the distances to both scattering shells. Similar behavior has been observed with increasing temperature in other systems (Brown & Eisenberger, 1979, Scott et al., 1986). Note that the apparent distances to the scattering shells in Figure 6-5 are shorter than the actual distances to neighboring atoms. This is due to the fact that the radial distribution function, $R'$, obtained by Fourier transforming an EXAFS spectrum yields distances which are convolved with an averaged phase shift. This phase shift is characteristic of the absorbing-atom scattering-atom pair. This complication, which tends to reduce the apparent distance, is actually useful in that it is indicative of the nature of the scattering atom.

Figure 6-6 contains the EXAFS oscillations due to back-scattering from the first coordination sphere of the manganese complex in $S_0$ and $S_1$ state preparations together with simulations of the spectra based on theoretical phase and amplitude functions calculated by Teo and Lee (1979). The $S_0$ preparation EXAFS spectra shown are of a sample prepared by incubation with 60 $\mu$M hydroxylamine. The illuminated-minus-dark EPR spectrum of this sample is shown in Figure 6-1b. The Fourier filtered data shown are a composite of the Fourier components which yield the peak labeled I in the Fourier transform. Table 6-2 contains a summary of the results obtained from simulations of the first coordination sphere of the manganese EXAFS of the $S_0$ and the $S_1$ state PSII preparations. The presence of two unresolved components is clearly revealed. The least squares residual, $F$, improves by more than an order of magnitude upon the addition of a second EXAFS component. The two components obtained are quite reasonable in light of what is known about the first coordination sphere of $\mu$-oxo bridged multinuclear manganese complexes. The shorter, less disordered oxygen shell at 1.8 Å corresponds to the bridging oxygens, while the longer more disordered shell corresponds to the
terminal ligation shell which is typically more disordered in Mn(III) complexes due to Jahn-Teller distortions (Plaksin et al., 1972; Stebler et al., 1986; Sheats et al., 1987; Vincent et al., 1987).

The quality of the simulation of the first shell EXAFS of \( S_0 \) data is significantly better than that obtained for the first shell of the \( S_1 \) data. This may indicate that the distribution of first coordination sphere distances in the \( S_0 \) is a more continuous distribution of bond lengths which is more accurately modeled by a Gaussian Debye-Waller term than is the distribution of distances present in the \( S_1 \) state (See section 4.3.3).

Figure 6-7 shows the EXAFS oscillations due to the second shell scatterers in PSII preparations poised in the \( S_0 \) and \( S_1 \) states. We have previously shown that the k-weighting behavior of the second shell indicates the presence of a heavy scatterer like manganese (Kirby et al., 1981b, Yachandra et al., 1986a). Simulations of the second shell scatterers also indicate that this shell is heterogeneous. Simulations using two components, a manganese scatterer and a carbon shell or two different manganese neighbors, improve the fit quality by a factor of as much as three. Unlike the first shell, the origin of the heterogeneity in the second shell is less clear. An examination of second shell distances in the crystal structures of multinuclear oxo-bridged manganese complexes with biomimetic ligands, such as carboxylates or heterocyclic rings, indicates that the second shell scatterers of the manganese in PSII must also contain some carbon atoms. In addition, it is generally accepted that there are four manganese per PSII reaction center (Kuwabara & Murata, 1984, Govindjee et al., 1985); thus, it is reasonable to consider heterogeneity in the Mn-Mn distance arising from two inequivalent dimers or due to distortions in a higher nuclearity complex. Thus, two models of the heterogeneity in the second shell were assumed, each containing two components as described. Table 6-3 contains the best fit parameters for the second shell of PSII preparations poised in the \( S_0 \) and \( S_1 \) states, assuming the following scattering shell models: 1) one manganese neighbor and a carbon shell and 2) two different manganese neighbors.

It is important to note that the apparent spread in Mn-Mn distances determined from the two manganese neighbor simulation is greater in the \( S_0 \) state preparation (e.g. 0.18 Å for \( S_0 \) compared to 0.12 Å for the \( S_1 \)). The Mn-Mn, Mn-C simulations yield lower values for the apparent number of manganese neighbors for the \( S_0 \) preparations relative.
### Table 6-2
Simulation Results for the First Coordination Sphere of PSII Preparations Poised in the $S_1$ and $S_0$ States

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$N$</th>
<th>$R$ (Å)</th>
<th>$\sigma^2$ (Å$^2$)</th>
<th>$\Delta E_0$</th>
<th>$N$</th>
<th>$R$ (Å)</th>
<th>$\sigma^2$ (Å$^2$)</th>
<th>$\Delta E_0$</th>
<th>$F^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_1$</td>
<td>1.5</td>
<td>1.76</td>
<td>0.005</td>
<td>-20.</td>
<td>4.4</td>
<td>2.25</td>
<td>0.028</td>
<td>-20.</td>
<td>0.0066</td>
</tr>
<tr>
<td>$S_0$</td>
<td>1.6</td>
<td>1.79</td>
<td>0.004</td>
<td>-16.</td>
<td>2.3</td>
<td>2.20</td>
<td>0.023</td>
<td>-20.</td>
<td>0.0026</td>
</tr>
</tbody>
</table>

a. Simulations were performed using theoretically calculated phase shift and backscattering amplitude functions calculated by Teo & Lee, 1979.

b. The goodness of fit criterion is a least square residual, $F$, between the Fourier isolated waves (See caption of Figure 6) and the simulation of these waves.
Figure 6-6. The lowest frequency component, or first shell, of the $k^1$-weighted Mn EXAFS of PSII preparations poised in the $S_1$ and $S_0$ states. This low frequency component is obtained by multiplying the Fourier transformed data by a function which isolates the peak in the Fourier transform which occurs at the shortest distance. This spectrum is then back transformed to yield the solid traces which are simulated using theoretically calculated amplitude and phase functions. The best fits to the “Fourier isolated” EXAFS are indicated by the dashed traces. The spectra shown in (a) are of the EXAFS of an $S_1$ state preparation and the spectra in (b) are the EXAFS of an $S_0^*$ state PSII preparation. Simulation parameters for the fits shown are contained in Table 6-2.
3.0 Wavevector $k(\text{Å}^{-1})$ 10.0

$\chi(k)$

$\chi(k)$
Figure 6-7. Fourier isolates (______) and fits (______) to the second shell of the k^4-weighted EXAFS of PSII preparations poised in the S_1 (a) and S_0 (b) states. The simulations plotted are two component simulations assuming a Mn-Mn wave and a Mn-C wave. The results obtained from the simulations shown are contained in Table 6-3.
Wavevector $k \left( \text{Å}^{-1} \right)$

(a) $k_x(k)$

(b) $k_x(k)$
Table 6-3

Simulation Results for the Second Shell of PSII Preparations Poised in the $S_1$ and $S_0$ States

$k^1$-weighted EXAFS Data

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Single Component Simulations</th>
<th>Two Component Simulations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mn - Mn</td>
<td>Mn - C</td>
</tr>
<tr>
<td>R(\AA)</td>
<td>2.73</td>
<td>3.06</td>
</tr>
<tr>
<td>N</td>
<td>2.0</td>
<td>1.2</td>
</tr>
<tr>
<td>$\sigma^2$(\AA$^2$)</td>
<td>0.015</td>
<td>0.010c</td>
</tr>
<tr>
<td>F</td>
<td>0.0016</td>
<td></td>
</tr>
<tr>
<td>R(\AA)</td>
<td>2.72</td>
<td>3.26</td>
</tr>
<tr>
<td>N</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>$\sigma^2$(\AA$^2$)</td>
<td>0.017</td>
<td>0.010c</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-10.</td>
<td>-20.</td>
</tr>
<tr>
<td>F</td>
<td>0.0068</td>
<td></td>
</tr>
</tbody>
</table>

a. Simulations were performed using theoretically calculated phase shift and backscattering amplitude functions calculated by Teo & Lee, 1979.

b. The quality of a given fit is indicated by a least square residual, $F$, between the Fourier isolated waves (See caption of Figure 6) and the curve obtained by simulation of these waves.

c. The Debye-Waller factors used in these simulations were constrained to reasonable values (see sections 7.3.6 and 7.3.7). Left unconstrained these parameters floated to unreasonably low or high values.
to the $S_1$ preparation. This is consistent with the supposition that the $S_5$ state has a larger spread in the Mn-Mn distances. It has been noted previously, that EXAFS waves resulting from the scatter from neighboring atoms at distances which differ by $\sim 0.2$ Å tend to interfere destructively. The result is an erroneously low apparent number of scatterers at the given mean distance (Kirby et al., 1981b).

In summary, the simulation results agree with the apparent increase in disorder indicated by the dramatic reduction in the amplitude of the Fourier transform peaks of the EXAFS of the $S_5$ state relative to the $S_1$ state.

6.4 Discussion

6.4.1 The Mechanism of Action of Hydroxylamine The striking similarity of the Mn K-edge of dark-adapted PSII samples containing low concentrations of hydroxylamine, state $S_{i-1}$, and without hydroxylamine, state $S_i$, is spectroscopic evidence that manganese is not reduced by hydroxylamine in the dark. Thus, the first two mechanisms described in the Scheme 6-1 may be ruled out. This striking similarity of the $S_1$ and $S_{i-1}$ Mn K-edges also indicates that hydroxylamine does not bind to manganese in the dark.

Most mechanisms which have been proposed for the action of hydroxylamine suggest that it competitively binds to the “water oxidizing site” in the dark ( Förster & Junge, 1985, Radmer & Ollinger, 1982). However, recently, Beck & Brudvig (1987) have suggested that the hydroxylamine binds at a site competitive with the chloride binding site. Previous EPR and EXAFS studies indicate that the chloride binding site does not involve direct ligation to the manganese complex (Yachandra et al., 1986b, 1987). The binding of hydroxylamine, as a water analog to manganese would involve, at the very least replacement of one oxygen ligand by one nitrogen ligand in the coordination sphere of manganese. This would result in a change in the effective potential (see coordination charge discussion in section 5.3.2) of the manganese which should result in a detectable change in the position of the X-ray absorption edge. Based on an analysis of the edges of an extensive list of model complexes, the magnitude of this shift can be estimated to be. The absence of a change between the edges of the $S_{i-1}$ and $S_i$ samples indicates that hydroxylamine does not bind to manganese in the dark.

The simplest interpretation of the light-induced shift to lower energy observed in
samples treated with hydroxylamine is that concurrent with advancement of PSII, hydroxylamine induces a two-electron reduction of the oxygen evolving complex. This is indicated by the following facts. The inflection point of the Mn K-edge of the dark-adapted PSII membrane suspensions with and without hydroxylamine present are at the same energy, indicating that the oxidation state of the manganese complex in the OEC is the same for $S_{2}$ and $S_{1}$. In the absence of hydroxylamine, the Mn K-edge inflection of preparations which have been illuminated at 195 K, shifts to higher energy by approximately 1 eV (Goodin et al., 1984, Yachandra et al., 1987). The magnitude of the shift in edge inflection energy observed upon illumination of PSII preparations containing hydroxylamine is comparable in magnitude but opposite in sign to that we observed for samples that do not contain hydroxylamine. Thus, the edge inflection energy of an $S_{0}$ preparation is nearly 2 eV lower than that of the state (the $S_{2}$ state) that would have been generated by continuous illumination at 195 K of samples which do not contain hydroxylamine. This implies that the reduction induced by illumination of PSII preparations containing hydroxylamine is at least a two-electron reduction of the manganese complex within the OEC. The simplest reaction consistent with these observations is a two-electron reduction of the manganese complex occurring upon illumination at 195 K resulting in the generation of the $S_{0}$ state. Concurrent with the reduction of the OEC a two-electron oxidation of two molecules of hydroxylamine produces dinitrogen, two protons, and two water molecules as depicted in Equation 6-1 below.

$$2\text{NH}_2\text{OH} \rightarrow 2\text{e}^- + \text{N}_2 + 2\text{H}^+ + 2\text{H}_2\text{O}$$

The net reaction describing this proposed mechanism is depicted in Scheme 6-2. Note that it seems probable that at least transient generation of the $S_{2}$ state initiates the proposed two-electron reduction of the manganese complex in the presence of hydroxylamine.

Förster and Junge (1985a) examined the effect of hydroxylamine on the flash induced proton release pattern observed in whole chloroplasts using absorption changes of methyl red. The flash pattern of protons released in the presence of hydroxylamine matches the normal pattern but is delayed by two flashes. The amplitude of the absorption change on the first flash was consistent with the release of two protons. Junge and Förster (1985b) speculated that these two protons are the product of the oxidation of two molecules of hydroxylamine. Alternatively, it has been suggested that these two
protons are the result of the subsequent binding and hydrolysis of two water molecules at the water oxidation site (Andréasson et al., 1983). More recently, it has been suggested, based on mass spectrometric measurements with $^{18}$O labeled water, that the substrate water does not bind irreversibly to the OEC until the $S_3 \rightarrow S_4$ state transition (Radmer & Ollinger, 1986). This lends more credence to the assertion that two protons released after the first flash in the presence of hydroxylamine are the result of oxidation products of two molecules of hydroxylamine. Based on this assertion, Förster and Junge (1986) proposed a model in which hydroxylamine causes a two-electron reduction of the manganese complex upon advancement to the $S_2$ state (see Scheme 6-2 and Eqn. 6-1). These results are consistent with our assertion of a light-induced hydroxylamine-mediated two-equivalent reduction of the manganese cluster.

<table>
<thead>
<tr>
<th>dark reaction</th>
<th>light induced reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_1 + 2\text{NH}_2\text{OH} \rightarrow S_1\text{2NH}_2\text{OH}$</td>
<td>$h\nu$</td>
</tr>
<tr>
<td>$\downarrow$</td>
<td>$S_0$</td>
</tr>
<tr>
<td>$N_2 + 2\text{H}^+$</td>
<td></td>
</tr>
</tbody>
</table>

Förster and Junge (1985c) also found that the effect of hydroxylamine was reversible in the dark with a half-time to maximum effect on the order of one minute. This reversibility of hydroxylamine binding would seem to argue against dark reduction of the oxygen evolving complex. Based on an observed sigmoidicity in the methyl red absorption changes occurring on the first flash with increasing hydroxylamine concentration, Förster and Junge (1985c) concluded that as many as four molecules of hydroxylamine bind cooperatively to the oxygen evolving complex. Based on this observation, Förster and Junge (1986) proposed a model in which serial bridged binding of hydroxylamine to a binuclear manganese center induced a significant increase in the manganese-manganese distance. The small changes that we have observed within the binuclear manganese core
structure rule out the possibility of a large conformational change due to bridged binding of hydroxylamine at manganese. The observed cooperativity and reversibility of the effect have been independently confirmed by Diedrich-Glaubitz et al. (1987) using inside out thylakoids and bromocresol-purple absorption changes.

Recently, Beck & Brudvig (1987) suggested that a two-electron reduction of the oxygen evolving complex to an S_1 state occurs at higher concentrations of hydroxylamine in the dark. At these concentrations of hydroxylamine (≥ 200μM) reversible inhibition of oxygen evolution occurs. These authors also reported a slower reaction, presumably involving a four-electron reduction of the oxygen evolving complex to a "S_3" state, and subsequently resulted in release of Mn(II). We have observed a reaction which results in release of Mn(II) which increases with increasing hydroxylamine concentration. This is, perhaps, consistent with the four-electron reduction scheme proposed by Beck & Brudvig (1987). We see no evidence for an intermediate two-electron reduction process occurring in the dark at the concentrations examined. However, the differences may be due to the differences in hydroxylamine concentration used and the duration of incubation.

Using a mass spectrometer equipped with a gas permeable silicone membrane, Radmer and Ollinger (1982) investigated the flash-induced evolution of volatile molecules from chloroplasts in the presence of hydroxylamine at concentrations which alter the normal cycle of water oxidation. They found nitrogen to be the only stable oxidation product other than oxygen. The nitrogen evolved upon a series of flashes was found to be maximum on the first flash. This surplus of nitrogen evolved on the first flash was assumed to be the product of hydroxylamine oxidation at the water oxidation site. The simplest explanation of the evolution of nitrogen from active centers, consistent with the proton release pattern and our Mn K-edge results indicating that manganese is not reduced in the dark, is a concerted irreversible oxidation of two molecules of hydroxylamine occurring upon illumination as depicted in Scheme 6-2. Presumably, this process would leave the oxygen evolving complex in a state indistinguishable from the S_0 state.

6.4.2 Relevance of the Properties of S_0 to the native S_0

X-ray absorption spectroscopy cannot be used to determine whether the hydroxy-
Lamine induced $S_0$ state, $S_0^*$, is the same as the native $S_0$ state. By examining the effect of hydroxylamine on the UV absorption changes accompanying S-state transitions, Witt et al. (1987) have attempted to address this question. Their observations concur with those of Dekker et al. (1984) in that UV absorption changes which they assign to a change in the oxidation state of manganese occur during the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions and are reversed on the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition. Unlike the difference spectra reported by Dekker et al. (1985), the spectrum reported by Witt et al. (1987) for the hydroxylamine-induced $S_0$ to $S_1$ transition has a maximum at 300 nm, significantly shifted from the maximum observed in the difference spectrum for the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ state transitions. Witt et al. (1987) speculate that this difference may reflect an oxidation state change corresponding to Mn(II) to Mn(III) for the $S_0 \rightarrow S_1$ state transition and a Mn(III) to Mn(IV) oxidation state change corresponding to the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ state transitions. Although they do not report a complete spectrum for the calculated four flash-induced $S_0 \rightarrow S_1$ difference spectrum, they claim the calculated changes at 300 nm agree with the difference spectrum of the hydroxylamine-induced $S_0 \rightarrow S_1$ state transition. However, it should be noted that the assignment of the optical difference spectra to specific oxidation state changes of manganese has recently been questioned (Vincent & Christou, 1986).

The similarity of the hydroxylamine-induced $S_0$ state and the three flash-induced $S_0$ state has also been examined by observing the flash-induced changes in proton relaxation properties (Srinivasan et al., 1986). The generation of a paramagnetic center with a high proton relaxation efficiency after the first flash in the presence of hydroxylamine correlated well with the generation of this species after three flashes in the absence of hydroxylamine. Based on this observation, Srinivasan et al. (1986) speculated that the proton relaxation properties of this center are what one might expect for a flash-generated Mn(II) center.

Recently Styring & Rutherford (1987) have suggested that the equilibrium between $S_0$ and $D^+$, which results in the slow conversion of $S_0$ to $S_1$ in the dark is a process which prevents the loss of manganese present in the $S_0$ state. Based on the fact that Mn(II) exhibits faster ligand exchange rates, and, thus, is more labile, Styring and Rutherford suggested that the $S_0$ state contains at least one Mn(II) ion. If we assume that $S_0$ and $S_0^*$ are the same, then our proposed irreversible mechanism of hydroxylamine oxidation
indicates that the $S_0 \rightarrow S_1$ transition involves the stabilization and storage of a single oxidative equivalent at the manganese cluster. We have previously shown that there are no significant structural changes occurring in the $S_1 \rightarrow S_2$ state transition despite the clear change in oxidation state (Yachandra et al., 1987). Based on our edge and EXAFS results for the $S_0$ and $S_1$ states the $S_0 \rightarrow S_1$ state transition appears to involve both an oxidation state change from Mn(II) to Mn(III) and a structural rearrangement. This difference probably reflects a different oxidation state change occurring during this transition as Witt et al. (1987), Srinivasan & Sharp (1986) and Styring & Rutherford (1987) have suggested.

The energy of the Mn K-edge inflection of the $S_0$ preparations is roughly in the range of Mn(III) complexes. However, the $S_0$ edge energy is less than 1eV above multinuclear μ-oxo bridged manganese complexes that contain one manganese with a formal valence of II. This difference in edge inflection energy could be due to a larger percentage of oxygen donor groups, such as carboxylates, in the coordination sphere of the manganese cluster in PSII. Note that the energy of the Mn K-edge inflection of the $S_2$ state is also higher than one would expect for the mixed valence state that the EPR properties indicate it must be (see section 5.3.1).

Very recently, Vincent et al. (1987b) have synthesized and obtained X-ray crystal structures for a set of triangular trinuclear manganese complexes which have a formal valence of Mn$_3$(II,III,III) and Mn$_3$(III,III,III). They found a Mn-Mn distance in the Mn$_3$(II,III,III) complex 0.1 Å longer than the corresponding distance in the Mn$_3$(III,III,III) complex. Similar changes in the static disorder in the Mn-Mn distances have also been observed for isostructural pairs of tetranuclear Mn complexes (Vincent et al., 1987a). Differences in the amplitude of peaks in the Fourier transforms of these isostructural models are remarkably similar to the differences that we have observed between the Fourier transforms of the EXAFS of $S_0$ and $S_1$ state preparations (see section 4.3.2.2). Conversely, differences in the EXAFS of isostructural binuclear complexes which differ by one oxidative equivalent but involve as much as only the higher valences (e.g. Mn(III) and Mn(IV)) have been found to be negligibly small (Kirby et al., 1981b). Recently, an X-ray crystallographic study of this pair of binuclear complexes has confirmed the structural homology (Stebler et al., 1986). Wieghardt et al. (1986) have observed similarly small changes in structure between a pair of binuclear structures.
which differ by one unit of valence and involve only Mn(III) and Mn(IV). This suggests that the larger apparent spread in the Mn-Mn distances, indicated by the two Mn shell simulations of the $S_0$ state Mn EXAFS, may be due the presence of one manganese with a formal valence of Mn(II).

6.5 Conclusions

1. Hydroxylamine does not reduce the manganese complex in PSII in the dark at the concentrations which cause a two-state delay in the S-state cycle.

2. The reduction of the manganese complex following a single turnover of PSII is consistent with a model of the action of hydroxylamine in which irreversible oxidation of two molecules of hydroxylamine results in the production of di-nitrogen, the release of two protons and the generation of the $S_0$ state of the oxygen evolving complex.

3. The lower Mn K-edge of the oxygen evolving complex and the higher disorder in the bond lengths in the $S_0$ state together imply a heterogeneous mixture of valences including one Mn(II).
Chapter 7

The $S_3$ State of Photosystem II: A Mn X-Ray Absorption Study
Comparing the Structure of the Manganese Complex in Photosystem II
Preparations Cryogenically Stabilized in the $S_2$ and $S_3$ States

7.1 Introduction

The current model of photosynthetic oxygen evolution carried out by higher plants and cyanobacteria involves five intermediate oxidation states, commonly referred to as $S$-states; $S_0$ - $S_4$ (Kok et al., 1970). This model is based on the remarkable observation made by Joliot et al. (1969) that the production of $O_2$ by dark-adapted chloroplasts subjected to a series of short saturating flashes of light occurs in distinct pulses with a periodicity of four. The states $S_0$, $S_1$, $S_2$ and $S_3$ are relatively stable intermediate oxidation states of the oxygen evolving complex (OEC). The $S_4$ state is a transient intermediate and decays to produce the $S_0$ state, concurrent with the release of $O_2$. The $S_1$ state is predominant in long-term dark-adapted PSII preparations. Significant progress has been made toward identifying the chemical nature of these intermediates (for a recent review, see Babcock, 1987). An abundance of physical and biochemical evidence indicates that a cluster of manganese located within a membrane-bound protein-chlorophyll complex is the site where the light-driven oxidation of water to molecular oxygen takes place.

The first physical evidence implicating a manganese cluster in the water oxidation process in catalytically active chloroplast preparations, came from the discovery of a light-induced multiline EPR signal assigned to a manganese complex because of its characteristic hyperfine structure. The multiline EPR signal was associated with the $S_2$ state based on the variation in its amplitude observed with flash number (Dismukes & Siderer, 1981). A large number of studies have now been performed, extensively characterizing the $S_2$ state using the multiline EPR signal. On the other hand, there have been relatively few reports of attempts to stabilize active $O_2$-evolving preparations cryogenically in the $S_3$-state (Brudvig et al., 1983; Goodin et al., 1984). As yet, no spectroscopic signal uniquely characteristic of the $S_3$ state has been discovered. As a result, previous studies relied upon decreases in the amplitude of the multiline EPR signal as an indication of advancement to the $S_3$ state following a single turnover by
Here, we report experiments poising samples predominantly in the S₃ state by a double turnover, low temperature illumination of samples where Q₄₀₀ was initially present in the oxidized form. The chemical identity of the high potential (Eₘₚ = 370mV at pH 7.5) acceptor, Q₄₀₀, has recently been established as the the high spin iron (Fe³⁺) of the iron-quinone acceptor complex of PSII (Petrouleas & Diner, 1986). We produced Q₄₀₀ either by chemical oxidation using ferricyanide (Petrouleas & Diner, 1986) or by photo-reductant induced oxidation through the use of phenyl-p-benzoquinone (PPBQ) (Zimmermann & Rutherford, 1986). Low temperature illumination of PSII preparations in which Q₄₀₀ was produced by either of these procedures was found to yield substantial S₃ state compositions. The photochemical events occurring during the procedure used to accumulate the S₃ state were monitored by following the EPR signals associated with Q₄Fe²⁺ and the high spin Fe³⁺. The final S state composition was deduced from these measurements, and from measurements of the amplitude of the multiline signal relative to controls limited to a single turnover of PSII, as well as from quantitations of EPR signals arising from alternate donors such as cyt b₅₆₅ and radical species.

X-ray absorption spectroscopy has been demonstrated to yield detailed structural information about the local structure of a metalloenzyme and does not require a crystalline sample (Powers, 1982; Teo, 1986). Further, the accuracy of the distance information obtained from multiparameter fitting of extended X-ray absorption fine structure (EXAFS) is significantly better than that obtainable from X-ray diffraction studies of proteins, and the sensitivity of the technique to changes in the local structure of a metalloenzyme has been demonstrated to be of utility in deducing important details of the mechanism of enzyme activity (Eisenberger & Kincaid, 1978). With the multiline EPR signal as a monitor of state composition, the direct involvement of manganese in the storage of oxidative equivalents on the donor side of photosystem II has been demonstrated using Mn X-ray absorption edge spectroscopy (Goodin et al., 1984). The ligation sphere of manganese in the S₁ and S₂ states has also been probed using EXAFS (Yachandra et al., 1986a, 1987). The objective of the EPR studies presented in this report was the development of a protocol for the generation of a sample poised predominantly in the S₃ state so that X-ray absorption studies of the manganese cluster within the oxygen evolving complex could be extended to this state. Samples extensively characterized by EPR
were then examined using X-ray absorption spectroscopy. Unlike our previous studies of the S₁ → S₂ state transition (Goodin et al., 1984, Yachandra et al., 1987), no change was observed in the edge position or structure of the Mn X-ray absorption K-edge upon state advancement from S₂ to S₃. EXAFS analyses of PSII preparations cryogenically stabilized in the S₂ and S₃ states indicate that a small structural change occurs at the manganese site in PSII during the S₂ → S₃ state transition. The X-ray absorption data presented here are of significantly improved signal-to-noise ratio compared to those we have previously published for the S₁ and S₂ states, due principally to improvements in fluorescence detection. The present analysis largely confirms our previous reports (Yachandra et al., 1986a, 1987). Conclusions are drawn from a detailed comparison of the simulation behavior of four inorganic site models with results obtained for the PSII preparations and the significance of these analyses to other results and models of the water oxidation mechanism already published in the literature is discussed.

7.2 Materials and Methods

7.2.1 PSII Subchloroplast Membrane Preparation and Handling

The procedure for isolation of active O₂-evolving PSII subchloroplast membranes from market spinach has been described by Yachandra et al. (1986). Single or double turnover experiments using DCBQ (Eastman), PPBQ (Eastman) or DCMU (Sigma) were performed by adding a calculated volume of DMSO stock solutions of the given reagent to PSII membranes suspended at 2 mg/mL chlorophyll. Chemical oxidation of cyt b₅₅₉ and Q₄₀₀ was accomplished by incubating PSII membranes suspended at 3 mg/mL chlorophyll in the dark for 10 min in a buffer containing 5mM K₃Fe(CN)₆, 15 mM NaCl, 5mM MgCl₂ and 50 mM MES (pH=6.5). To obtain the manganese concentrations required for the X-ray absorption experiments, the PSII membrane suspensions were centrifuged at 30,000 × g for 1hr. Pellets containing approximately 30 % glycerol were transferred to lucite sample holders. Chlorophyll concentrations in samples prepared for X-ray absorption experiments ranged from 25 - 30 mg/mL.

Low temperature illumination or equilibration was accomplished in an optical Dewar cooled by a N₂ gas stream, maintained at a constant temperature by a Varian V6040 temperature controller. Samples were mounted directly into lucite sample holders that were designed to fit into the liquid He cryostat associated with the EPR spectrometer.
as well as the cooled N₂ gas flow cryostat used for the X-ray absorption experiments. All illuminations, constant temperature equilibrations, EPR measurements and X-ray absorption measurements were performed on samples mounted in this manner.

7.2.2 EPR Spectral Characterization

Low temperature X-band EPR spectra were recorded using a Varian E109 EPR spectrometer equipped with an E-102 microwave bridge and an Air Products LTR liquid helium cryostat. The temperature of the sample was monitored using a gold-chromel thermocouple junction. EPR spectra were digitized using an Explorer III storage oscilloscope and then transferred to a VAX 11-785 for further analysis. Separate EPR spectra were recorded for each electron transfer component of interest under conditions that maximized the EPR signal amplitude. Exact spectrometer conditions for each EPR signal are indicated in the figure captions. The magnitude of the g=4.3 EPR signal, associated with adventitiously bound iron, was used as an internal calibration reference when comparing two different samples. The amplitude of this feature in dark-adapted samples was found to differ less than 5 % between samples from the same preparation. Multiline EPR signal amplitudes were quantitated by adding peak-to-trough amplitudes of four of the low field hyperfine lines from illuminated-minus-dark difference spectra. The \( Q_{400} \) signal was quantitated by measuring the peak-to-baseline amplitudes of the lowest field component near \( g=8 \). The amplitude of the reduced quinone acceptor, \( Q^-Fe^{2+} \), was determined from the peak-to-trough difference of the \( g=1.9 \) EPR signal observed in the illuminated samples, for it was found that in the PSII preparations used in this study the dominant EPR signal characteristic of the \( Q^-Fe^{2+} \) complex was the \( g=1.9 \) feature (Rutherford & Zimmermann, 1984). Signal II₅, which is present in the dark and associated with D⁺, a donor to PSII with unknown function, was quantitated by measuring peak-to-trough amplitudes between the two outermost hyperfine lines. The amplitude of oxidized cyt \( b_{659} \) was determined by measuring the peak-to-baseline amplitudes of the \( g=3.0 \) feature.

7.2.3 Manganese Model Complexes

Four structurally characterized inorganic model complexes were examined in this study. The three inorganic models examined were 1 Mn₂(III,IV)(μ-O)₂(2,2'-bipyridine) (Plaksin et al., 1972); 2 Mn(IV)₂(μ-O)₂(picolinate); 3 Mn(IV)O₆(tacn)₄(ClO₄)₄
(Wieghardt & Bossek, 1983) and 4 Mn_{4}(III)(OAc)_{4}(2,2'-bipyridine)_{4}ClO_{4} (Vincent et al., 1987). Complex 2 was synthesized and characterized in the laboratory of Dr. G. Christou. Models will be referred to in the text by the boldface number indicated above. Samples were diluted into an inert light element matrix (LiBF_{4} or LiCO_{3}) and mounted in lucite sample holders.

7.2.4 X-ray Absorption Measurements

X-ray absorption spectra were recorded at the Stanford Synchrotron Radiation Laboratory on wiggler beam lines IV-2 and VI-2 using a Si<400> and double crystal monochromator. The energy resolution of these crystals is approximately 1 eV. X-ray absorption edge spectra were recorded using a fluorescence detection system (Jaklevic et al., 1977) similar to that described by Powers et al. (1981). It consisted of an array of NE104 plastic scintillators coupled to EMI 9813 B photomultiplier tubes. The fluorescence detector was equipped with a chromium filter and Soller slit assembly (Stern & Heald, 1979). The fluorescence signal was ratioed to a measure of the incident flux which was monitored by an additional scintillator. This scintillator viewed the flux of X-rays scattered from a thin mylar film which was placed directly in the incident X-ray beam. This method of ratioing yielded a much more linear response than the ionization chamber method previously employed (B. Chance, personal communication).

EXAFS spectra of the PSII preparations were collected using a lithium-drifted silicon solid state detector as previously described (Jaklevic et al., 1977). Count rate capabilities of this detection system were substantially enhanced through the use of a triangular pulse shaping amplifier (Goulding et al., 1983). The use of an energy-resolving detector capable of efficient background rejection significantly improved the signal-to-noise ratio of these spectra over previously published results (Yachandra et al., 1987). All samples were maintained at or below 190 K in a home built double Kapton walled cryostat, cooled by a nitrogen gas flow system. To assure sample integrity and a stable state composition, EPR spectra of the PSII samples were monitored before and after exposure to the X-ray beam. Energy calibration in the X-ray absorption experiments was maintained by simultaneously monitoring the narrow pre-edge feature of a KMnO_{4} standard (Goodin et al., 1979). Typically, one scan (3 sec per point) yielded satisfactory signal-to-noise ratios for the model complexes. To achieve the signal-to-noise ratio presented here for the PSII preparations, 10 - 30 scans were averaged.
7.2.5 Data Analysis

All analyses of the X-ray absorption spectra were performed on a VAX 11/785. The region below the onset of manganese X-ray absorption was set equal to zero by subtracting a linear fit to the spectrum below the edge. Spectra were normalized to a unit edge jump determined by extrapolation of a quadratic fit to the post edge absorption to the energy of the first major absorption peak. The point of intersection is defined as the unit edge jump. Details of the analysis of the EXAFS data have been described previously (Yachandra et al., 1986, 1987).

Detailed structural information regarding the number of ligands and the distances to these ligands is obtained by fitting the EXAFS data to the known functional form of these waves. Commonly either theoretically calculated phase and amplitude functions (Teo & Lee, 1979) or empirical phase and amplitude functions derived from structurally similar model complexes (Cramer et al., 1976) were used in these simulations. Here, we have taken the former approach; however, adjustments based on a set of crystallographically characterized inorganic site models were used to refine the information obtained from the fits (Teo et al., 1983).

Under favorable conditions, distances accurate to ± 0.03 Å and determinations of the number of scatterers to within 20% of crystallographically determined values are obtained. The energy of the absorption edge is allowed to vary in simulations using theoretically derived phase functions to allow better phase matching to the experimental waves. In this study, the variation of this parameter, ΔE₀, was constrained to values between 0 to -20eV for the light scatterers and to between 0 to -10eV for the manganese neighbor; for it was found that simulations in closest agreement with the crystallographic distances were obtained with ΔE₀ values in these ranges. Manganese-manganese distances obtained from an EXAFS analysis of multinuclear μ-oxo bridged manganese complexes were found to be within ± 0.02 Å of the crystallographically determined distances when these constraints on the range of variation in the edge threshold were applied. Based on calculated Debye-Waller factors for inorganic models exhibiting similar parameter correlations, the Debye-Waller factors for each scattering shell of the PSII preparations were constrained to a reasonable range to reduce the uncertainty in the number of scatterers. Also, scale factors which improve the accuracy of the determination of the number of atoms in a given shell of the PSII preparation were
determined from these inorganic models. Scale factors were calculated from the ratio of the simulated number of scatterers in a given shell to the number of atoms at a given distance from the absorbing atom determined from the crystal structure of the inorganic model.

7.3 Results

O₂-evolving PSII samples suitable for X-ray spectroscopy and poised in the S₃ state were prepared by a low temperature illumination of dark-adapted PSII preparations in which Q₄₀₀ has been oxidized (i.e. the high spin iron of the acceptor complex has been oxidized to Fe³⁺). Two protocols based on recently published results concerning the generation of oxidized Q₄₀₀ and its subsequent photo-reduction were used in this study: Petrouleas and Diner (1986) oxidized Q₄₀₀ using a ferricyanide treatment of PSII membranes in the dark. EPR signals associated with oxidized Q₄₀₀ were identified in the region near g=8 and g=5.5. Zimmermann and Rutherford (1986) found that addition of 1mM PPBQ to a PSII subchloroplast membrane suspension can also result in the oxidation of Q₄₀₀ following a low temperature illumination and subsequent warming. We have employed both the ferricyanide oxidation and the PPBQ-mediated oxidation procedure for the generation of oxidized Q₄₀₀. Each procedure was found to have certain advantages in the double-turnover illumination procedure used to generate samples poised predominantly in the S₃ state.

7.3.1 PPBQ Experiments

The steps in the experiment in which PPBQ is used to oxidize Q₄₀₀ and leading to a PSII preparation containing a substantial S₃ state composition are as follows: 1) Long term (> 1h) dark adaptation followed by addition of a calculated volume of a PPBQ stock solution which brought the suspension to 1mM, in the dark; 2) Illumination at 77K for a period of 1h; 3) Warming samples for 1 min at 293 K; and 4) Illumination at 240 K for 10 min. The electron transfer events described below were monitored by examining changes in the amplitudes of EPR signals associated with each electron transfer component.

Illumination of dark-adapted PSII membranes at 77 K results in the photo-accumulation of Q₄⁻Fe²⁺ largely at the expense of cyt b₅₅₉ oxidation. These events were monitored by EPR spectroscopy. Figure 7-1 shows the EPR spectra of cyt b₅₅₉⁺
Figure 7-1. Changes in the EPR spectra of a) Q_{400} b) cyt b_{560} and c) Q_{4}Fe^{2+}, which are observed for PSII preparations during the course of the S_3 state preparation procedure using PPBQ. In each panel the EPR spectra shown from top to bottom are as follows: a dark adapted PSII preparation, the same preparation following illumination at 77 K for 1h, the same preparation following warming for 1 min at 293 K and the bottom spectrum is that of this preparation following illumination at 240 K for 10 min. Spectrometer conditions: a) and c) 32mW microwave power at 9.21 GHz, 32 Gauss field modulation at 100 kHz, recorded at 5K and for b) 0.2mW microwave power at 9.21 GHz, 20 Gauss field modulation at 100 kHz, recorded at 18 K.
Figure 7-2. Illuminated-minus-dark EPR spectra of the multiline signal in the control $S_2$ state preparation (top trace) and a PSII preparation poised partially in the $S_3$ state by the procedure employing PPBQ. The $S_3$ state preparation was generated by illumination at 240 K. Spectrometer conditions: 32 mW power at 9.21 GHz, 32 Gauss field modulation at 100 kHz, recorded at 8 K.
Table 7-1
Changes in the EPR Signal Amplitudes\(^a\) of Electron Transfer Components of PSII Observed During the S\(_3\) Preparation Procedure Using PPBQ

<table>
<thead>
<tr>
<th>Sample Preparation</th>
<th>MLS</th>
<th>(Q_\Delta\text{Fe}^{2+})</th>
<th>(Q_{400})</th>
<th>Cyt b(_{559})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1. Dark adapt 1h</td>
<td>&lt;5%</td>
<td>&lt;10%</td>
<td>&lt;2</td>
<td>6.5</td>
</tr>
<tr>
<td>Step 2. Illuminate at 77K</td>
<td>&lt;5%</td>
<td>90%</td>
<td>&lt;2</td>
<td>11.0</td>
</tr>
<tr>
<td>Step 3. Warm to 293 K</td>
<td>12%(^b)</td>
<td>&lt;10%</td>
<td>19</td>
<td>10.0</td>
</tr>
<tr>
<td>Step 4. Illuminate at 240 K 54%</td>
<td>110%</td>
<td>&lt;2</td>
<td>16.0</td>
<td></td>
</tr>
</tbody>
</table>

\(a\). All amplitudes reported here are in arbitrary units. Percentages reported are of the MLS and \(g=1.9\) feature of the EPR signal associated with \(Q_\Delta\text{Fe}^{2+}\), observed in \(S_2\) control preparations. Exact details of the quantitation are described in materials and methods.

\(b\). The small amount of MLS generated during the warming step is presumably due to the conversion of the amount of the \(g=4.1\) species (formed at 77K) to the \(S_2\) state characterized by the MLS (see Casey & Sauer, 1984).
and $Q_A^{-}$-$Fe^{2+}$ which were induced by this process. Warming in the dark of such a sample containing PPBQ results in the reoxidation of $Q_A^{-}$ and oxidation of the ferrous ion in a process that was first demonstrated by Zimmermann and Rutherford (1986). These results were largely confirmed in this study and Figure 7-1 shows that following the warming step, the $Q_A^{-}$-$Fe^{2+}$ signal decays to its original amplitude, demonstrating the complete reoxidation of the primary quinone acceptor. The oxidation of Fe$^{2+}$ (i.e. the appearance of oxidized $Q_{400}$) is indicated by well-resolved features near $g=8$ and $g=5.5$ (Figure 7-1a).

Zimmermann and Rutherford (1986) showed that significant amounts of oxidized $Q_{400}$ could be induced by the addition of PPBQ to dark-adapted PSII membranes - (presumably by oxidizing the amount of $Q_B^{-}$ present in the dark). To avoid this side reaction which would affect the final S-state composition, $Q_B^{-}$ was oxidized before the PPBQ treatment by the addition of 50 μM DCBQ. As observed by Zimmermann and Rutherford (1986), DCBQ was found not to induce the oxidation of Fe$^{2+}$, and Figure 7-1a shows that the addition of PPBQ after this treatment effectively suppressed the generation of $Q_{400}$ in the dark.

Several changes in the EPR spectra of the PSII preparations following the final period of illumination at 240 K are indicative of a double turnover of PSII: 1) the $Q_A^{-}$-$Fe^{2+}$ signal at $g=1.9$ is induced to virtually the same extent as after the illumination at 77 K, indicating one complete charge separation (Figure 7-1c) and 2) the EPR signals at $g=8$ and $g=5.5$ disappear (Figure 7-1a), indicating the reduction of Fe$^{3+}$ to Fe$^{2+}$ and the stabilization of a second oxidative equivalent on the donor side of Photosystem II.

As shown in Figure 7-1b, the amplitude of the EPR signal of cyt b$_{559}$ increases by 50 % during the illumination at 240 K. Because cyt b$_{559}$ does not compete with S$_1$ state donation at this temperature, it is concluded that cyt b$_{559}$ competed with S$_2$ during the second turnover. Quantitation of the EPR signal indicates that 0.5 cyt was oxidized. Thus, the remaining 50 % of the centers underwent the double oxidation resulting in the accumulation of the S$_3$ state. This interpretation is in agreement with the amount of multiline signal photo-induced during the last step, which corresponds to the remaining 50 %, when compared to a similar sample poised in the S$_2$ state by illumination at 200K (Figure 7-2). The results are summarized in Table 7-1.
7.3.2 Ferricyanide Experiments

In the second procedure used to generate PSII preparations poised in the $S_3$ state, $Q_{400}$ was oxidized by ferricyanide before the double turnover illumination. The protocol used is summarized as follows: 1) Long term (> 1 h) dark adaptation; 2) Ferricyanide oxidation of $Q_{400}$ in the dark with concurrent oxidation of cytochrome $b_{569}$; and 3) Illumination for 10 min at a temperature above the threshold (235 K) for advancement to the $S_3$ state (Brudvig et al., 1983).

An additional step was incorporated into the procedure to insure that PSII was limited to two turnovers. DCMU, which blocks electron transfer between $Q_A$ and $Q_B$, was added to the preparation after the treatment with 5mM ferricyanide. PSII suspensions were washed in the dark with a MES buffer (pH=6.5) containing 200 $\mu$M DCMU, effectively washing out the ferricyanide used in the previous step. This step eliminated concerns about possible leakage of electrons from $Q_A^-\text{Fe}^{2+}$ through the DCMU block to the ferricyanide in the aqueous phase and removed the interference due to the broad and intense ferricyanide signal centered at $g=2$, which obscures the reduced quinone signals at $g=1.9$ and 1.82. Samples treated in this manner were used in the X-ray absorption experiments.

Control experiments were also performed in which the 5mM ferricyanide was retained during the DCMU wash step. These controls were important in evaluating whether $Q_{400}$ and high potential cyt $b_{569}$ remain oxidized at the ambient potential attained after the ferricyanide is removed. Table 7-2 contains the amplitudes of the EPR signals associated with $Q_{400}$ and oxidized cyt $b_{569}$ at each step of the process leading to a PSII sample poised in the $S_3$ state using the ferricyanide procedure. Note that the amplitudes of the $g=8$ feature in the EPR spectrum of $Q_{400}$ observed in samples in which the ferricyanide was washed out is at the same level, or somewhat higher than the amplitude of this feature in samples in which the ferricyanide was retained. This indicates that the re-reduction of $Q_{400}$ at the ambient potential achieved after the ferricyanide is removed is kinetically limited. Table 7-2 shows also that the amplitude of the $g=3.0$ feature associated with oxidized cyt $b_{569}$ in the 18K spectra of the samples retaining ferricyanide is significantly higher than the amplitude of this feature in the samples in which the ferricyanide was washed out.

However, it is important to note that the amplitude of the cytochrome EPR signal
Table 7-2

<table>
<thead>
<tr>
<th>Sample Preparation Procedure</th>
<th>MLS</th>
<th>Q&lt;sub&gt;400&lt;/sub&gt;</th>
<th>Cyt b&lt;sub&gt;559&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1. Dark adapt 1h</td>
<td>&lt;5%</td>
<td>&lt;2</td>
<td>&lt;2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Step 2. Ferricyanide oxidation</td>
<td>&lt;5%</td>
<td>15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Step 3. DCMU washed</td>
<td>&lt;5%</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>Step 4. Illuminate at 240 K</td>
<td>34%</td>
<td>&lt;2</td>
<td>12</td>
</tr>
</tbody>
</table>

a. All amplitudes reported here are in arbitrary units. Percentages reported for the MLS are relative to the control S<sub>2</sub> state preparation. Exact details of the quantitation are described in Materials and Methods.

b. The amplitude reported for the dark sample is that of low potential cytochrome b<sub>559</sub> which is normally oxidized at the ambient potential of the preparation. The amplitude of this signal is near the uncertainty in the measurement of the g=3.0 feature (e.g. ± 1)

c. EPR signal amplitudes of Q<sub>400</sub> and cyt b<sub>569</sub> found in controls in which ferricyanide was retained.
Figure 7-3: EPR signals associated with the PSII acceptors, $Q_7^{Fe^{2+}}$ and $Q_{400}$. The spectra shown in a) are as follows: The top trace is the spectrum of a PSII preparation dark adapted for 1h, treated with 5mM ferricyanide in the dark for 10 min and then subsequently washed free of ferricyanide with a pH=6.5 MES buffer containing 200µM DCMU. The second trace from the top is the same preparation following illumination at 240 K. The lower trace is the spectrum of the control $S_2$ state sample prepared by illumination at 195 K. Spectrometer conditions are the same as for the spectra shown in Figure 7-1c. The spectra shown in b) are of the low field region of the EPR spectrum of PSII preparations recorded at 5K. The solid trace is the spectrum of a dark adapted PSII preparation treated with 5mM ferricyanide in the dark. The dashed line is the spectrum of the same PSII preparation following illumination at 240 K. Spectrometer conditions are the same as for Figure 1a.
Figure 7-4. Illuminated-minus-dark EPR spectra of the multiline signal in the control $S_2$ state preparation (top trace) and a PSII preparation poised partially in the $S_3$ state by the ferricyanide procedure. The $S_3$ state preparation was generated by illumination at 240 K. The state composition indicated by the fraction of the residual multiline in the lower spectrum is: 66% $S_3$ and 34% $S_2$. EPR spectrometer conditions are the same as for Figure 7-2.
remained relatively constant during the final illumination step. This demonstrates that photo-induced oxidation of cyt b\textsubscript{569} was not a significant side reaction at the temperatures utilized, presumably because the cyt b\textsubscript{569} that would have been photo-oxidized under these conditions had been chemically oxidized.

The effect of illumination following the dark oxidation was examined at three different temperatures: 250 K, 245 K and 240 K. The reduction in the amplitude of the multiline signal relative to the control 195 K illumination is relatively constant (57 - 66 %) across the range of temperatures of illumination examined. The maximum S\textsubscript{3} state composition was achieved at the lowest temperature of illumination. Also the magnitude of the reduction of the MLS relative to the controls was consistently higher than that obtained in the PPBQ experiments. This is due principally to the elimination of competitive donation by cytochrome b\textsubscript{569}.

Figure 7-3 shows the 5 K EPR spectra of the ferricyanide-oxidized PSII sample before and after illumination at 240 K. The salient spectral changes associated with the transfer of two equivalents to the acceptor side and the generation of a PSII preparation poised predominantly in the S\textsubscript{3} state are evident from these two spectra. The large increase in the g=1.9 region and the disappearance of signals in the g=8 and g=5.5 regions of the EPR spectra recorded at 5K indicate near quantitative reduction of QA and Q\textsubscript{400}, respectively (See Figure 7-3). The amplitude of the multiline signal formed indicates a state composition of 66 % S\textsubscript{3} and 34 % S\textsubscript{2} (See Figure 7-4).

The amplitude of the semiquinone signals was based on the 5K EPR spectra recorded following illumination at low temperature (See Figure 7-3). The amplitude of the g=1.9 feature was found to be 75 - 90 % of the amplitude of the control value. The decreasing amplitude of this feature with increasing temperature may indicate an increase in the contribution of the back reaction (charge recombination) at higher temperatures.

7.3.3 X-ray Absorption Edge Studies

Figure 7-5 contains a representative Mn K-edge spectrum of a S\textsubscript{3} sample together with that of a control S\textsubscript{2} state preparation. The Mn K-edge shown is that of a S\textsubscript{3} state preparation generated by illumination at 240 K using the ferricyanide procedure. In addition to the ferricyanide samples, a sample poised in the S\textsubscript{3} state by the PPBQ procedure and subsequently warmed to room temperature for 30 s to allow thermal
Figure 7-5. The Mn K-edge X-ray absorption spectrum of a PSII preparation poised in the $S_2$ state (---) and a preparation poised predominantly in the $S_3$ state (______) using the ferricyanide procedure. Spectra were smoothed using a quadratic running fit with a smoothing domain of 2 eV. The inset is a $\times5$ magnification of the $1s \rightarrow 3d$ transition of the X-ray absorption spectrum.
equilibration was examined. The energy of the Mn K-edge inflection for all of the $S_2$ and $S_3$ samples studied here was found to be within 0.2 eV of 6552.1 eV. The edge shape and the energy of the Mn K-edge inflection of the $S_2$ and $S_3$ state preparations are remarkably similar. The energy of the Mn K-edge inflection and the similarity of the $1s \rightarrow 3d$ transition both are indicative of the presence of Mn(IV) (Sauer et al., 1987). The low intensity feature at energy lower than the edge inflection is due to X-ray transitions of principally $1s \rightarrow 3d$ character. The intensity of this feature has been shown to be a function of the symmetry of the metal complex (Shulman et al., 1976). The inset in Figure 7-5 is an expanded view of this transition. The intensity and shape of this feature in the $S_3$ spectrum are similar to the $1s \rightarrow 3d$ transitions we recently reported for the $S_2$ state (Sauer et al., 1987).

7.3.4 X-ray Absorption Fine Structure Analysis

EXAFS spectra for PSII preparations poised predominantly in the $S_3$ state using the ferricyanide procedure and for $S_2$ state preparations were recorded. A "thermally relaxed" $S_3$ sample prepared by warming a sample prepared by the PPBQ method to 293 K in the dark for 30 s was also examined. EXAFS of the $S_3$ state preparations were analysed by difference spectroscopy. A fraction of the EXAFS of a control $S_2$ state preparation was subtracted from the EXAFS of the $S_3$ state preparations that contained a residual $S_2$ state composition indicated by the fraction of the multiline signal amplitude present in the $S_3$ samples (See Tables 7-1 and 7-2). Figure 7-6 contains the EXAFS spectrum of a PSII preparation poised in the $S_2$ state and the difference EXAFS spectrum of a PSII preparation poised in the $S_3$ state by illumination at 240 K following the ferricyanide procedure. The solid line in Figure 7-6 is the Fourier isolate of the two peaks evident in the Fourier transforms of the $k^3$-weighted EXAFS spectrum shown in Figure 7-7.

Two major peaks are evident in the Fourier transforms of the EXAFS spectra in both the $S_2$ and $S_3$ preparations (Figure 7-7). These peaks represent maxima in the backscattering of outgoing photoelectron waves and appear as a function of the distance between the central absorbing atom and neighboring atoms. The apparent distance ($R'$) to a given scattering shell is shorter than the internuclear distance between the central absorbing atom and the ligating atoms owing to a contribution of the effect of an average phase shift ($\left(\frac{\partial a(k)}{\partial k}\right)_k$). The phase shift ($a(k)$) is induced by the effect of the potentials
Figure 7-6. EXAFS region of the Mn X-ray absorption spectrum of PSII preparations poised (a) in the S₂ state and (b) in the S₃ state. The spectrum in (b) is the difference EXAFS spectrum of a PSII preparation poised predominantly in the S₃ state by the ferricyanide procedure from which a fraction of the control S₂ state preparation EXAFS has been subtracted. The data are indicated with a dashed line and the solid line represents the contribution from the two main peaks in the Fourier transform of the EXAFS shown in Figure 7-7.
Wavevector $k(A^{-1})$

$k^2x(k)$

3.0

10.5

Wavevector $k(A^{-1})$
of the metal absorber and its ligands on the photoelectron.

7.3.5 k-Weighting Behavior

By multiplying EXAFS spectra by increasing powers of $k$ (the photoelectron wave vector amplitude), EXAFS waves whose envelopes peak at higher $k$ values are selectively enhanced. These waves are due to backscattering from heavier elements. Thus, the Fourier transforms of peaks containing contributions from heavier elements increase more with $k$-weighting than do peaks associated with scattering from light elements. This $k$-weighting behavior has been used previously to determine whether a transition metal is present in a given shell, as seen in the transform of the EXAFS of a metalloenzyme (Kirby et al., 1981; Woolery et al., 1984; Yachandra et al., 1986). In the Fourier transforms of the EXAFS of the $S_2$ preparation, the second peak increases in a manner consistent with significant contributions from heavy scatterers, such as a neighboring transition metal in a multinuclear cluster (See Figure 7-7). The details of the $k$-weighting behavior of the Fourier transform peaks in the two states would appear to differ substantially. As is described below, this is probably due to differences in the static disorder in the Mn-Mn distances present in each state.

7.3.6 Simulations of the First Shell Indicate a Di-$\mu$-oxo Bridged Structure

The best fits to the Fourier isolates of the first shell of the EXAFS of an $S_2$ state PSII preparation and that of the difference EXAFS of an $S_3$ state preparation are shown in Figure 7-8. In both cases, simulations including two $O$ or $N$ waves were found to be significantly better than single wave fits. The difference between the two distances obtained in the two wave fits is consistent with the proposition that the static disorder present in the EXAFS waves, which yields this broad peak in the transforms of Figure 7-7, is larger than can be adequately described with a single Debye-Waller factor (Brown & Eisenberger, 1979). Simulation results for the first shell of the EXAFS of the $S_2$ state preparations and the difference EXAFS of the $S_3$ state preparations are contained in Table 7-3.

In addition to the quality of the fits obtained, the parameters obtained for the two unresolved shells are eminently reasonable in light of what is known about the bonding in multinuclear oxo-bridged manganese complexes. The simulation yields a short Mn - $O$ or Mn - $N$ bond that displays a relatively small degree of disorder, and a set of
Figure 7-7. Fourier transforms of (a) the EXAFS of an S$_2$-state PSII preparation and (b) of the difference EXAFS of an S$_3$-state preparation, prepared by the ferricyanide procedure. The apparent distance $R'$ is shorter than the actual distance to a given neighboring atom due to the effect of the averaged phase, $\left(\frac{\omega \phi}{\omega k}\right)_k$, of the EXAFS wave. The three Fourier transforms shown in (a) and (b) were obtained by multiplying the data by $k$ (the photo-electron wave vector amplitude), $k^2$ and $k^3$. 
Relative Amplitudes

Apparent Distance $R'$ (Å)

- $k^1$-weighted
- $k^2$-weighted
- $k^3$-weighted
Figure 7-8. The lowest frequency component, or first shell, of the Mn EXAFS of PSII preparations poised in the $S_2$ and $S_3$ states. This low frequency component is obtained by multiplying the Fourier transformed data by a function which isolates the peak in the Fourier transform occurring at the shortest effective distance. This spectrum is then back transformed and simulated using theoretically calculated amplitude and phase functions. The best fits to the "Fourier isolated" first shell spectra are indicated by the dashed traces. The simulations shown are best fits assuming two different Mn-O distances. The spectra shown are of (a) the EXAFS of an $S_2$ state preparation and (b) the difference EXAFS of an $S_3$ state preparation. Simulation parameters for the fits shown are contained in Table 7-3.
\[ k^2 \chi(k) \]

**Wavevector** 
\[ k(\text{Å}^{-1}) \]

**Graphs:**

- **a**
- **b**
Table 7-3
Simulation Results for the First Coordination Sphere of PSII Preparations Poised in the S2 and S3 States

<table>
<thead>
<tr>
<th>State</th>
<th>N</th>
<th>R(Å)</th>
<th>σ²(Å²)</th>
<th>ΔE₀(eV)</th>
<th>N</th>
<th>R(Å)</th>
<th>σ²(Å²)</th>
<th>ΔE₀(eV)</th>
<th>F^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₂</td>
<td>1.8</td>
<td>1.77</td>
<td>0.007</td>
<td>-13.</td>
<td>2.8</td>
<td>2.23</td>
<td>0.013</td>
<td>-20.</td>
<td>9.73</td>
</tr>
<tr>
<td>S₃</td>
<td>1.8</td>
<td>1.81</td>
<td>0.009</td>
<td>-11.</td>
<td>2.1</td>
<td>2.20</td>
<td>0.015</td>
<td>-20.</td>
<td>1.21</td>
</tr>
</tbody>
</table>

a. Average results obtained from the simulation of the EXAFS of PSII preparations poised in the S₂ state. In general, distances obtained for individual samples differed by less than 0.02 Å. The number of scatterers determined at a given distance was found to be within 30% of the average number.

b. Difference EXAFS of a PSII preparation poised in the S₃ state by the ferricyanide procedure. This sample was illuminated at 240K. Difference EXAFS results obtained for a S₃ State PSII sample prepared by the PPBQ procedure were in good agreement with the results presented for samples prepared by the ferricyanide method.

c. F is the least squares difference between the Fourier isolated EXAFS and the EXAFS calculated using theoretically derived phase and amplitude functions (Teo & Lee, 1979).
longer bonds spread over a greater range of distances. These two sets of first shell ligands correspond remarkably well to the bridging and terminal ligands observed in multinuclear oxo-bridged manganese complexes (Plaksin et al. 1972; Wieghardt & Bossek, 1983; Sheats et al, 1987; Vincent et al, 1987).

The shorter Mn-O distance to the "bridging" shell is on the average ~ 0.04 Å longer in the S₃ preparations than in the S₂ preparations. Although this systematic difference appears to be real, care must be exercised in attempting to assign the nature of this change to a specific structural rearrangement. The uncertainties in the distances obtained from the simulation of two unresolved components are often higher (e.g. ± 0.05 Å) due to the correlation between the amplitude of one component and the separation to the other (Brown & Eisenberger, 1979).

To refine the simulation parameters obtained from the Mn EXAFS of the PSII preparations, four structurally characterized site models were examined in detail and their EXAFS simulation parameters compared to that of the PSII preparations poised in the S₂ and S₃ states. Three of these four inorganic complexes: 1 Mn₂(III,IV)(µ-O)₉-(2,2'-bipyridine)₄(ClO₄)₉ (Plaksin et al., 1972); 3 Mn₄(IV)O₆(tacn)₄(ClO₄)₄ (Wieghardt & Bossek, 1983); and 4 Mn₄(III)(OAc)₇(2,2'-bipyridine)₂(ClO₄) (Vincent et al, 1987) have been proposed as site models for the manganese cluster within the OEC of PSII (Kirby et al., 1981, Brudvig & Crabtree, 1986, Vincent et al., 1987). The similarity of the 16 line EPR spectrum exhibited by complex 1 to the multiline EPR spectrum exhibited by PSII membrane suspension poised in the S₂ state was the first evidence implicating a functional role for Mn in photosynthetic O₂-evolution (Dismukes & Siderer, 1981). Also Mn-Mn and Mn-O distances obtained by simulation of the EXAFS of the Mn complex in PSII are similar to distances obtained from the simulation of the EXAFS of 1 (Kirby et al., 1981, Yachandra et al., 1986a, 1987). Complex 2 is similar structurally to 1; however, unlike 1 the terminal ligation is a mixture of carboxylates and heterocyclic rings (See Figures 4-1 and 4-2). Complex 3 is a tetranuclear Mn(IV) adamantane-like structure which has been proposed to be a model for the Mn complex of PSII poised in the S₃ state (Brudvig & Crabtree, 1986). Complex 4 is a tetranuclear Mn(III) complex which consists essentially of a di-µ-oxo bridged core structure with two additional Mn atoms mono-µ-oxo bridged to the dimeric core via three-centered oxygen bridges. Complex 4 is unique among the complexes examined in this study
in that it contains two different Mn-Mn distances. Like complex 2 this complex has a heterogeneous terminal ligation containing both carboxylates and heterocyclic rings.

The static Debye-Waller factor, the mean distance to each scattering shell, and the number of scatterers in each shell were calculated for each model using the crystallographic coordinates (See Table 7-4). The magnitude of the thermal contribution to the Debye-Waller factor can be estimated from measured symmetric stretching frequencies in the IR (Teo, 1986). For strong bonds, this thermal contribution to the Debye-Waller factor is approximated by:

\[ \sigma = 4.106 \left( \frac{1}{\mu \nu} \right)^{1/4} \]

where \( \nu \) is the vibrational frequency in cm\(^{-1} \) and \( \mu \) is the reduced mass.

Based on \(^{18}\text{O}\) substitution studies of the bipyridine binuclear complex (complex 1), the 688 cm\(^{-1} \) band in the IR was assigned to the Mn-O bridging mode (Cooper & Calvin, 1977). From this value, the magnitude of the vibrational contribution to the Debye-Waller factor is estimated to be \( \sigma^2 = 0.0020 \) Å\(^2 \).

The correlation between unresolved components was more of a problem for most of the models than for the PSII preparations. Unconstrained Debye-Waller factors for the \( \mu \)-oxo bridging shell approached zero for all inorganic complexes except 4. To decrease this effect in the model complex simulations a reasonable value containing approximately equal static and dynamic contributions was assumed (e.g. 0.005 Å\(^2 \)). The best fit parameters for the simulation of the first shell of each model complex are contained in Table 7-4.

Refinement of the simulations of the first coordination sphere of the PSII EXAFS based on inorganic model data indicates that the manganese present in PSII is principally in the form of binuclear units (e.g. probably a pair of binuclear complexes). For the \( S_2 \) state these simulation results are remarkably similar to those of 2. Further, the variation in the apparent number of scatterers in the first shell obtained by systematically varying the Debye-Waller factor most closely resembles the behavior observed in the PSII preparations poised in the \( S_2 \) state (See section 4.3.2.4). These criteria have been suggested by Teo et al (1983) for the selection of a model for use in determining a scale factor (e.g., the number of scatterers determined by simulation divided by the number calculated based on the crystal structure) to refine the number of atoms in a
Table 7-4

Simulation Results for the First Coordination Sphere of Four Inorganic Complexes

<table>
<thead>
<tr>
<th>Model No.</th>
<th>N</th>
<th>R(A)</th>
<th>(\sigma^2(Å^2))</th>
<th>(\Delta E_0(eV))</th>
<th>N</th>
<th>R(A)</th>
<th>(\sigma^2(Å^2))</th>
<th>(\Delta E_0(eV))</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.7(2.0)</td>
<td>1.75(1.82)</td>
<td>0.005(0.0016)</td>
<td>-10.</td>
<td>3.7(4.0)</td>
<td>1.95(2.11)</td>
<td>0.023(0.006)</td>
<td>-20.</td>
<td>13.5</td>
</tr>
<tr>
<td>2.</td>
<td>1.5(2.0)</td>
<td>1.79(1.82)</td>
<td>0.005(0.0000)</td>
<td>-10.</td>
<td>2.8(4.0)</td>
<td>2.23(1.97)</td>
<td>0.006(0.010)</td>
<td>-20.</td>
<td>6.0</td>
</tr>
<tr>
<td>3.</td>
<td>2.1(3.0)</td>
<td>1.74(1.78)</td>
<td>0.005(0.0005)</td>
<td>-10.</td>
<td>3.7(3.0)</td>
<td>2.03(2.09)</td>
<td>0.010(0.006)</td>
<td>-20.</td>
<td>27.8</td>
</tr>
<tr>
<td>4.</td>
<td>1.4(1.5)</td>
<td>1.83(1.88)</td>
<td>0.010(0.0025)</td>
<td>-10.</td>
<td>2.0(4.5)</td>
<td>2.23(2.07)</td>
<td>0.028(0.014)</td>
<td>-20.</td>
<td>0.4</td>
</tr>
</tbody>
</table>

a. The site models examined here are 1 Mn_{2}(III,IV)_{2}O_{2}(bipy)_{4}(ClO_{4})_{2}; 2 Mn(IV)_{2}O_{2}(picolinate)_{4}; 3 Mn(IV)_{4}O_{6}(tacn)_{4}(ClO_{4})_{4}; and 4 Mn(III)_{4}O_{2}(OAc)_{7}(bipy)_{2}(ClO_{4}).

b. Numbers in parentheses are parameters calculated from the crystal structure of each inorganic complex. Only the static contribution to the Debye-Waller factor has been calculated here. Since the thermal contribution is often as large or larger than the static term, the simulated values are invariably larger than the calculated static values.

c. F is the least squares difference between the Fourier isolated EXAFS and the EXAFS calculated using theoretically derived phase and amplitude functions.
given scattering shell. Applying the scale factor obtained from complex 2 to the number of \( \mu \)-oxo bridges determined for the Mn complex in the OEC, one obtains \( 2.3 \pm 0.6 \) oxygens per Mn. Note that this result is inconsistent with a binuclear di-\( \mu \)-oxo bridged manganese complex and two associated mononuclear manganese centers, which would yield an average number of \( \mu \)-oxo bridging oxygens equal to 1.0 per Mn.

The EXAFS due to the first coordination sphere of complex 4 are nearly superimposable on those of the Mn complex in PSII poised in the \( S_3 \) state. Also the simulation results assuming two oxygen waves for the first coordination sphere of the manganese complex in PSII in the \( S_3 \) state most closely resemble those of 4. Unlike that of complex 4, the simulation of the EXAFS of the first shell for the other inorganic complexes improves substantially upon addition of a third O or N wave, which suggests that the complex envelope of the EXAFS of the first shell of these complexes is inadequately modelled by two light element waves. This does not mean that 4 is the best site model for the manganese cluster in the OEC in the \( S_3 \) state; in fact, simulations of the second shell which indicate two distinct Mn-Mn distances (see section 7.3.7) rule this complex out as a reasonable site model. However, as described above the strikingly similar parameter correlations exhibited in simulations of the first shell permits a more accurate determination of the number of scatterers in the first coordination sphere. For the \( \mu \)-oxo bridging shell, a scale factor of 1.07 is obtained. For the first shell EXAFS of the \( S_3 \) state preparation this yields a value of \( 1.9 \pm 0.4 \) bridging oxygens for all Mn present in PSII. This result taken together with the results for the \( S_2 \) state strongly suggests a di-\( \mu \)-oxo bridged structure. This number of bridging oxygens is inconsistent with a higher nuclearity complex such as an adamantane-like complex (e.g. complex 3) or a symmetric cubane tetranuclear cluster, both of which possess three \( \mu \)-oxo bridges to each manganese atom.

7.3.7 Simulations of the Second Shell

Fits to the second shell, assuming a single manganese neighbor, yield similar numbers of manganese atoms (e.g. 1.7 & 1.8 Mn) for both the \( S_2 \) and \( S_3 \) states although the Debye-Waller values obtained are consistently higher in the \( S_3 \) state simulations (See Table 7-5). The assignment of a manganese scatterer to the second peak in the Fourier transform of the EXAFS of the PSII samples is reasonable from several lines of evidence. The k-weighting behavior of this transform peak is indicative of heavy
scatterer such as manganese, although EXAFS is not capable of discriminating between adjacent elements such as Mn and Fe (Kirby et al., 1981, Yachandra et al., 1986). The similarity of the EPR of the $S_2$ state (the multiline signal) to that of binuclear manganese model complexes strongly suggests the assignment of a manganese neighbor (Cooper et al., 1978). Also, the distance obtained (2.72 Å), if a single manganese neighbor is assumed, is consistent with the distances known in di-μ-oxo bridged binuclear manganese complexes (Plaksin et al, 1972, Stebler et al., 1986).

Fits to the second shell also indicate that a single wave (in this case, a manganese scatterer) is inadequate to describe this disordered shell. Unlike the first shell, the origin of the heterogeneity in the second shell is less clearly defined. Based on an examination of the crystallographic data of a variety of manganese models containing biomimetic ligands (e.g. carboxylates, phenoxy complexes or heterocyclic ring structures) it is clear that the second shell must contain some light elements (probably carbon, see Table 7-6). Considering the generally accepted stoichiometry of four manganese per PSII reaction center, the heterogeneity observed in the second shell could be due to static disorder in the Mn-Mn distance, for example, due to variation in the separation between manganese neighbors within two isolated binuclear complexes.

Table 7-5 contains a summary of the simulation results for the second shell. Generally the quality of the fits improves upon addition of a carbon shell. Based on the analysis of model compounds we have found that inclusion of a shell of light atoms greatly improves the accuracy in the determination of the number of Mn present (See section 4.5). In both the one and two component fits (Table 7-5) a substantially higher Debye-Waller factor is obtained for the $S_3$-state preparations. Figure 7-9 contains the best fits to the second shell assuming a Mn neighbor and a carbon shell.

The dominant consequence of the high disorder of the light atoms (carbons) present in the second shell in all the inorganic complexes is a mutual annihilation of EXAFS waves. One positive effect is that the scattering of transition metal neighbors tends to dominate the scattering characteristics of the outer shell, partially because of a lower disorder and partially due to the larger intrinsic backscattering power. The result is that simulation of the outer shells assuming contributions from only the manganese neighbor can yield reasonable estimates of the distance to these dominant scatterers. For example, simulations of complexes 1 and 2 yield values that are within 0.02 Å
### Table 7-5
Simulation Results for the Second Shell of PSII Preparations Poised in the $S_2$ and $S_3$ States

<table>
<thead>
<tr>
<th>k$^3$-weighted EXAFS Data</th>
<th>Single-Component Simulations</th>
<th>Two-Component Simulations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation</td>
<td>Mn - Mn</td>
<td>Mn - C</td>
</tr>
<tr>
<td>$S_2^a$</td>
<td>R(Å) 2.74</td>
<td>3.11</td>
</tr>
<tr>
<td></td>
<td>N 2.2</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>$\sigma^2(\text{Å}^2)$ 0.012</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>$\Delta E_0$ -3.</td>
<td>-10.</td>
</tr>
<tr>
<td></td>
<td>F 5.8</td>
<td></td>
</tr>
<tr>
<td>$S_3^b$</td>
<td>R(Å) 2.73</td>
<td>3.12</td>
</tr>
<tr>
<td></td>
<td>N 2.0</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>$\sigma^2(\text{Å}^2)$ 0.020</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>$\Delta E_0$ -6.</td>
<td>-12.</td>
</tr>
<tr>
<td></td>
<td>F 9.0</td>
<td></td>
</tr>
</tbody>
</table>

a. Average results obtained from simulation of the Mn EXAFS of $S_2$ State preparations. In general, distances obtained for individual samples differed by less than 0.03 Å. Coordination numbers differed by as much as 50% for the light elements (e.g. carbon) in a disordered shell; however, the number of Mn atoms was generally within 20% of the average number.

b. Difference EXAFS of a PSII preparation poised in the $S_3$ state by the ferricyanide procedure. The difference EXAFS of a PSII preparation poised in the $S_3$ state by the PPBQ procedure was in good agreement with the results presented here.
Figure 7-9. Fourier components (______) of the $k^3$-weighted EXAFS of PSII preparations corresponding to the peak at longer distance in the Fourier transforms shown in Figure 7-7. Best fits to this outer scattering shell are also plotted (______). The curves plotted in (a) are the second shell spectra and fits of a PSII preparation poised in the $S_2$ state. The curves plotted in (b) are the second shell difference EXAFS spectra and fits of an $S_3$ state preparation. The simulations plotted are two component simulations assuming a Mn-Mn wave and a Mn-C EXAFS wave. The results of these simulations are contained in Table 7-6.
Wavevector $k(A^{-1})$
of the crystallographic values (See Table 7-6). Best fit parameters assuming a single manganese neighbor and a manganese neighbor and a shell of carbons are also indicated.

Given that backscattering from the manganese tends to dominate, most probably the increase in disorder shown by the fits to the PSII Mn EXAFS upon advancing to the S₃ state is due to an increase in the spread of Mn-Mn distances. This indicates the presence of two inequivalent binuclear complexes in the S₃ state or distortions in a higher nuclearity complex. Simulations including two inequivalent manganese neighbors for the manganese center of PSII indicate that if a heterogeneity in the Mn-Mn distance exists, then the spread in this distance is approximately 0.15 Å. The same conclusion can be drawn from the value of the Debye-Waller factor when a single Mn neighbor is assumed (e.g. the spread indicated by the Debye-Waller factors is 0.1 Å for the S₂ state and 0.14 Å for the S₃ state). The values of the Debye-Waller factors obtained in simulations of inorganic models which possess a very low degree of static disorder in the Mn-Mn distances (e.g. complexes 1 and 2) are much lower than those obtained in the PSII preparations. Although these two complexes differ substantially in the disorder in the carbon shell, this has little effect on the Debye-Waller factor obtained in simulations assuming only manganese in the outer scattering shell. This indicates that the disorder observed in the second shell of the PSII preparations is due to variation in the Mn - Mn distance.

Complex 4 exhibits two distinct Mn-Mn distances which differ by nearly 0.5 Å. Simulations of the second shell assuming one Mn - Mn distance and a shell of carbon atoms are totally inadequate to describe the complex beat pattern exhibited in the second shell of this model. As is evident from the simulation results, the distances to the neighboring Mn atoms in this complex are accurately determined (again to within ± 0.02 Å).

Of the four inorganic model complexes, 2 matches the simulation properties of the second shell of the manganese complex in PSII most closely. In the simulations of both 2 and the manganese center in PSII, a short Mn-Mn distance (2.7 Å) and a shell of carbon atoms near 3 Å are obtained. Applying the scale factor obtained from 2, 1.2 ± 0.5 Mn neighbors is obtained for the manganese cluster in PSII in the S₂ and S₃ states. A single manganese dimer and two monomeric manganese sites would yield an average number of manganese neighbors equal to 0.5 per Mn which is not consistent with the
Table 7-6
Simulation Results for the Second Shell of Four Site Models

$k^3$-weighted EXAFS Data

<table>
<thead>
<tr>
<th>Model No.</th>
<th>Single Component Simulations</th>
<th>Two Component Simulations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mn - Mn</td>
<td>Mn - C</td>
</tr>
<tr>
<td>R(Å)</td>
<td>2.70(2.72)</td>
<td>3.07(3.02)</td>
</tr>
<tr>
<td>N</td>
<td>0.8(1.0)</td>
<td>1.4(8.0)</td>
</tr>
<tr>
<td>1. $\sigma^2$(Å$^2$)</td>
<td>0.005(0.000)</td>
<td>0.010(0.0092)</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>F</td>
<td>37.7</td>
<td></td>
</tr>
<tr>
<td>R(Å)</td>
<td>2.73(2.75)</td>
<td>2.88(2.89)</td>
</tr>
<tr>
<td>N</td>
<td>1.2(1.0)</td>
<td>1.1(6.0)</td>
</tr>
<tr>
<td>2. $\sigma^2$(Å$^2$)</td>
<td>0.009(0.000)</td>
<td>0.010(0.0015)</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-10.0</td>
<td>-2.0</td>
</tr>
<tr>
<td>F</td>
<td>12.2</td>
<td>8.6</td>
</tr>
<tr>
<td>R(Å)</td>
<td>3.24(3.22)</td>
<td>2.90(2.94)</td>
</tr>
<tr>
<td>N</td>
<td>1.6(3.0)</td>
<td>3.2(6.0)</td>
</tr>
<tr>
<td>3. $\sigma^2$(Å$^2$)</td>
<td>0.005(&lt;0.001)</td>
<td>0.010(0.0026)</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-3.0</td>
<td>-1.0</td>
</tr>
<tr>
<td>F</td>
<td>133.</td>
<td>70.3</td>
</tr>
<tr>
<td>R(Å)</td>
<td>2.91</td>
<td>2.87(2.85)</td>
</tr>
<tr>
<td>N</td>
<td>1.7</td>
<td>0.6(0.5)</td>
</tr>
<tr>
<td>4. $\sigma^2$(Å$^2$)</td>
<td>0.023</td>
<td>0.005(0.000)</td>
</tr>
<tr>
<td>$\Delta E_0$</td>
<td>-10.0</td>
<td>-4.0</td>
</tr>
<tr>
<td>F</td>
<td>7.4</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Numbers in parentheses are calculated EXAFS parameters based on the crystal structure data for each inorganic complex. Note only the static contribution to the Debye Waller factor has been calculated.

a. Complex 4 has two Mn - Mn distances which differ by more than 0.5 Å. Simulations of this complex assuming a single Mn neighbor are inadequate to describe the complex beat structure exhibited by the EXAFS of this complex.

b. The low number of manganese neighbors predicted by simulation of the EXAFS of this model is principally a consequence of the fact that two waves (i.e. a Mn-Mn and a Mn-C wave) are inadequate to describe the second shell EXAFS of this inorganic complex. A well defined shell of oxygens at 3.44 Å also exists in this complex. Inclusion of a third oxygen wave improves the fit quality substantially (e.g. F=46.) and yields a much better estimate of the number of manganese neighbors (e.g. 2.4).
lower limit of this range. Taken together with the results from the first coordination
sphere and given the stoichiometry of four Mn per PSII reaction center, this implies
the presence of two binuclear manganese complexes. The absence of a Mn shell in the
region around 3.2 Å indicates that discrete tetranuclear complexes such as complexes
3 or 4 are also not reasonable structural models for the Mn center in PSII. Symmetric
tetranuclear cubane-like complexes would be expected to yield an average number of
Mn neighbors equal to three, which is also inconsistent with the number determined for
the Mn complex in PSII.

It is important to note that while Fourier isolation of individual scattering shells
can greatly facilitate convergence of the non-linear least squares minimization used in
fitting the EXAFS, Fourier isolation also introduces distortions. By truncating side lobes
(apparent in the Fourier transform), Fourier components of the EXAFS waves potentially
important in defining the envelope shape and phase behavior are lost. Simulating a
Fourier isolation of a broader range of frequency components (e.g. including at a
minimum both peaks in the Fourier transform shown in Figure 7-7) substantially reduces
these distortions. Simulations of the EXAFS of the Mn complex in PSII poised in the
$S_2$ and $S_3$ states performed on a Fourier isolate of both scattering shells largely confirm
the results obtained by simulation of individual shells. Figure 7-10 contains the Fourier
isolate of the first two shells together with a three-component fit including one Mn-Mn
wave and two Mn-O waves. Simulation results are indicated in the figure caption.

7.4 Discussion

7.4.1 Factors Limiting Complete $S_3$ State Conversion

Of the two procedures used to prepare PSII membrane preparations poised in the
$S_3$ state, the procedure involving ferricyanide oxidation yielded a significantly higher $S_3$
state composition. This difference is principally due to the elimination of the competitive
photo-oxidation of cyt $b_{559}$ that occurred in the PPBQ experiments. Previous EPR
studies of PSII membranes (dePaula et al, 1985) have shown that cytochrome $b_{559}$ is the
principal electron donor at 77K. These authors showed that only $0.9 \pm 0.1$ cytochrome of
the 2 cytochromes present in PSII is photo-oxidized at 77 K. However, they found that
$2.1 \pm 0.2$ cytochromes could be oxidized in the dark using ferricyanide. Thus, the 77K
illumination used in the oxidation of $Q_{400}$, using PPBQ, resulted in the oxidation of only
Figure 7-10. Fourier components (——) of the k³-weighted EXAFS of PSII preparations corresponding to both peaks at in the Fourier transforms shown in Figure 7-7. Best fits to these scattering shells are also plotted (…). The curves plotted in (a) are the spectra and fits of a PSII preparation poised in the S₂ state. The curves plotted in (b) are the difference EXAFS spectra and fits of an S₃ state preparation. The simulations plotted are three component simulations assuming a Mn-Mn wave and two Mn-O EXAFS waves. Results obtained for the two fits are as follows. For the S₂ state preparation EXAFS shown, simulations yielded 2.0 Mn neighbors at 2.75 Å, 1.8 oxygen ligands at 1.79 Å, and 3.0 oxygens ligands at 2.23 Å. Debye-Waller factors for these three shells were 0.011, 0.010 and 0.013 Å² respectively. For the S₃ state EXAFS, simulations yielded 1.8 Mn at 2.76 Å, 2.0 oxygens at 1.82 Å, and 1.8 oxygens at 2.18 Å. Debye-Waller factors for these three shell are 0.018, 0.012 and 0.010 Å² respectively. These results are in good agreement with results obtained from the simulation of individual scattering shells.
about half of the available high potential cyt b₅₅₉. The remaining reduced cyt b₅₅₉ was found to be a significant competitive donor in the second low temperature illumination used to generate the $S_3$ state. However, the PPBQ procedure used to generate the $S_3$ state did have one significant advantage over the ferricyanide procedure; the charge separated state attained following the double turnover illumination procedure was found to be significantly more stable than that achieved in the ferricyanide experiments.

The incomplete double turnover of PSII observed in the experiments using ferricyanide to generate $Q_{400}$ is probably due to the presence of reaction centers in which $Q_{400}$ is not functional. Ikegami and Katoh (1973) observed fluorescence induction behavior corresponding to as much as a full additional acceptor equivalent in chloroplasts following ferricyanide oxidation and DCMU treatment. However, Petrouleas and Diner (1987) have found through an analysis of the decay kinetics of the C-550 band associated with pheophytin that the reoxidation of $Q_AFe^{2+}$ exhibits biphasic kinetics. A fast 25 μsec component was attributed to reoxidation by $Q_{400}$ while a slower (100 μsec) component was attributed to electron transfer from $Q_A$ to $Q_B$. Quantitation of the amplitude of each of these components led Petrouleas and Diner (1987) to suggest that as little as 50% of the reaction centers contained oxidized acceptor iron complexes. Thus, incomplete oxidation of $Q_{400}$ may have been a limitation in observing a complete double turnover in all reaction centers.

### 7.4.2 X-ray Absorption Edges

Manganese is not oxidized during the $S_2 \rightarrow S_3$ transition. The average inflections of the Mn X-ray absorption edges of the three different $S_3$ state preparations were found to be within 0.2 eV of the edge energy of the $S_2$ state preparations. The invariance in the energy of the edge and the shape of the edge indicate that the manganese cluster remains at the same oxidation state during the $S_2 \rightarrow S_3$ state transition. Significant changes in the structure of the 1s → 3d transition of the Mn K-edge of PSII preparations are observed during the $S_1 \rightarrow S_2$ state transition (Sauer et al., 1987). These changes indicate a valence change from Mn(III) to Mn(IV). The similarity of the pre-edge structure of the $S_3$ state (See inset of Figure 7-5) to the structure that we have previously reported for the $S_2$ state implies that the oxidation state and site symmetry are unchanged during the $S_2 \rightarrow S_3$ state transition.
This absence of change in the K-edge indicates that the oxidative equivalent stabilized on the donor side of PSII during the $S_2$ to $S_3$ transition is stored on a redox active center other than Mn. This result is surprising in light of the loss of the multiline EPR signal associated with the manganese complex in the $S_2$ state and suggests that the oxidative equivalent stabilized on the donor side of PSII is located on another intermediate donor very close to the manganese complex. Presumably, strong exchange coupling between this oxidized intermediate and the manganese complex results in a spin system with zero net spin or in an EPR silent non-Kramers state. The rapid decrease in the magnitude of exchange coupling as a function of the distance between paramagnetic centers (Coffman & Buettner, 1979) indicates that this unknown, presumably paramagnetic intermediate is directly ligated to the manganese complex or very near (< 7 Å away assuming, a strong exchange coupling in the range of $J \sim 100 \text{ cm}^{-1}$). The small change in the manganese structure between the $S_2$ and $S_3$ states indicated by the EXAFS analyses is suggestive of a change in a species coordinated to the manganese complex. Aromatic species that are likely candidates for this center are quinones (Goodin et al., 1984), tyrosines which have recently been associated with the intermediate Z (Debus et al., 1987) or histidines. While partial oxidation of the substrate water might also explain the lack of change in the manganese oxidation state, a recent thermodynamic analysis of water oxidation chemistry indicates that this is unlikely (Krishtalik, 1986). It is of note that the current estimate of the number of quinones in PSII preparations is two per reaction center (Takahashi & Satoh, 1987). Since one of these is presumably $Q_A$, the remaining one is a possible candidate for the intermediate suggested here.

The absence of changes in the oxidation state of the Mn in PSII during the $S_2 \rightarrow S_3$ transition is inconsistent with assertions made by Dekker et al. (1984) based on UV difference optical spectroscopy studies. However, an alternative interpretation of UV difference spectra put forth by Lavergne (1985) suggests that there may not be a change in the oxidation state of manganese during this transition. More recently these interpretations have been revised (Lavergne, 1987). The assignment of specific oxidation state changes to the difference spectra observed in PSII has been questioned by Vincent and Christou (1986) based on optical difference spectra of structurally characterized multinuclear manganese complexes that could be prepared in more than one oxidation state.
Based on studies of proton relaxation rates as a function of flash number, Srinivasan and Sharp (1986) have asserted that there is no oxidation of manganese occurring during the \( S_2 \) to \( S_3 \) transition. More recently, Rutherford & Styring (1987) measured the microwave power saturation of signal II_{slow} (D+) in different S-states and found a change in \( P_{\frac{1}{2}} \) in the \( S_1 \) to \( S_2 \) transition, which they attributed to relaxation enhancement due to oxidation of Mn(III) to Mn(IV) in the \( O_2 \)-evolving complex. No change was detected upon going from \( S_2 \) to \( S_3 \), which they suggest indicates that Mn is not oxidized during this transition. While one would expect that the spin state change during the \( S_2 \rightarrow S_3 \) transition, which is indicated by the loss of the multiline signal, would alter the spin relaxation properties of D+ or nearby protons, their conclusions are consistent with our interpretation of the Mn K-edge data.

7.4.3 EXAFS Analysis

Consistent with the lack of changes in the edge, relatively subtle structural changes are observed through analyses of the EXAFS of the manganese cluster in the \( S_2 \) and \( S_3 \) states. This is true for PSII preparations poised in the \( S_3 \) state by illumination at low temperature, as well as for samples that were "thermally relaxed" by warming to room temperature in the dark. The small increase in disorder observed is inconsistent with a major structural rearrangement from a cubane-like tetranuclear complex to an adamantane-like complex (complex 3) as has been proposed to occur during this transition (Brudvig & Crabtree, 1986).

This small increase in disorder in the second shell observed for \( S_3 \) state preparation is perhaps consistent with the apparent absence of enhancement of the second shell Fourier peak with increasing k-weighting. Enhancement with k-weighting for a scattering shell containing a heavy atom is not always observed. For example, Woolery et al. (1984) did not always observe enhancement of the second shell Fourier peaks in the EXAFS of hemocyanin (which contains a binuclear Cu complex with a Cu-Cu separation of 3.4 - 3.6 Å) depending on the state of the preparation (e.g. oxidized, reduced or inhibitor bound). Similarly, the Fourier peak due to the second Mn shell at a mean distance of 3.34 Å in the open butterfly-like tetranuclear complex (complex 4) is not enhanced with increasing k-weighting (see Figure 4-3 c). Note that the spread of Mn-Mn distances in the second Mn shell of this complex is 0.07 Å. The lack of enhancement is presumably due to beat effects between two Mn waves with similar frequencies which alter the
shape of the envelope of the EXAFS wave.

Fine adjustments based on correction factors obtained from the inorganic complexes (complexes 2 and 4 which mimic the EXAFS behavior of the manganese in PSII suggest that the four manganese present are in the form of two di-\(\mu\)-oxo bridged manganese binuclear complexes. This is indicated by the remarkably similar parameter correlations of 2 with those of the Mn complex in PSII in both the first and second shells (see sections 4.3.2.3 and 4.3.2.4). However, the parameters obtained from the simulation of the EXAFS of the first coordination sphere of manganese in PSII in the \(S_3\) state strongly resemble those of 4. This open tetranuclear model (Complex 4) is a more disordered structure than any of the other three models. All four manganese present are inequivalent (e.g. there are no crystallographically imposed symmetry constraints). The 18 different terminal bond distances in the terminal ligation of this tetranuclear structure more closely approximate a continuous Gaussian distribution of distances, which apparently is adequately described by a single Gaussian Debye-Waller term. This may explain the significantly better fit obtained for the first coordination sphere of the manganese in the \(S_3\) state using only two oxygen waves. Note however, that the general agreement with the crystallographic distances for the terminal shell of complexes 4 and 2 is very poor. This is presumably due to the large disorder present in the mixed O, N terminal coordination spheres present in these complexes. Large absolute errors in the determination of distances to highly disordered shells have been described in other systems (Brown & Eisenberg, 1980). The striking similarity of the fit parameters for the first shell indicates that a \(\mu\)-oxo bridged structure with four inequivalent manganese centers is present in the OEC in the \(S_3\) state. It is interesting to note that those inorganic complexes that exhibit EXAFS most similar to that of the Mn complex in PSII have heterogeneous ligation spheres containing both carboxylates and heterocyclic rings. Based on this observation, one may speculate that the manganese complex in PSII is ligated by a combination of carboxylates derived from acidic side chains and imidazole rings derived from histidine residues.

### 7.5 Conclusions

1. The use of the high potential acceptor, \(Q_{400}\), in a double turnover of PSII produced by a single low temperature illumination yields a PSII preparation with a substantial \(S_3\) state composition.
2. The energy of the Mn K-edge is the same for PSII preparations poised in the $S_2$ and the $S_3$ states. This implies that manganese is not oxidized during this S-state transition. The similarity of the pre-edge transition ($1s \rightarrow 3d$), in amplitude and shape, suggests that the site symmetry and oxidation state are essentially unchanged during the $S_2 \rightarrow S_3$ transition. This evidence and the disappearance of the multiline signal associated with the manganese complex strongly suggests that the oxidative equivalent stored on the donor side of PSII during the $S_2 \rightarrow S_3$ state transition is not stored within the manganese complex, but is stored on another intermediate magnetically coupled to the manganese complex.

3. EXAFS analyses indicate that a small structural rearrangement occurs during the $S_2 \rightarrow S_3$ state transition and that an adamantane-like tetranuclear structure is not a reasonable site model for the $S_3$ state.

4. Fine adjustments to the EXAFS simulation parameters based on inorganic models imply the presence of two di-$\mu$-oxo bridged binuclear complexes in the oxygen evolving complex of PSII. The absence of a change in the Mn K-edge, the disappearance of the multiline signal associated with manganese and the increase in the disorder in the Mn scattering shell of the EXAFS of PSII together suggest a mechanism of action in which the oxidation of an intermediate donor directly bonded to one of the two binuclear complexes results in a small change in the Mn-Mn distance in that complex during the $S_2 \rightarrow S_3$ state transition. Likely aromatic species found in PSII which could serve this function are quinones, tyrosines or histidine residues.
A generalized model for the structure of photosynthetic reaction centers is emerging. This structural model consists of a heterodimeric intrinsic protein complex. The core structure contains both the primary pigments and the electron donors and acceptors which stabilize the initial charge separation. This model is based largely on the remarkable accomplishments of Deisenhofer et al. (1984) in obtaining an X-ray crystal structure of the non-oxygen evolving photosynthetic reaction center isolated from the purple non-sulfur bacterium, *Rhodopseudomonas viridis*. The structure obtained by Deisenhofer et al. contains two intrinsic membrane proteins, each possessing five transmembrane helices. The generalization of this structural model to that of the reaction centers of oxygenic photosynthetic systems is based on several lines of evidence. For a recent review of the evidence supporting this generalization, see Michel and Deisenhofer (1986, 1988) and Hearst (1986).

There is an abundance of physical evidence which establishes similarities in the organization and structure of the functional components of PSII reaction centers to those of the reaction centers of *Rhodopseudomonas* species. Although the exact chemical composition of the primary pigments and secondary electron carriers is not identical, the structural homology between the primary pigments and the acceptor side electron transfer components is remarkable. For example, Klimov et al. (1977) reported the reduction of a pheophytin following excitation of the chlorophyll a complex, P680. An analogous reaction was observed in purple non-sulfur bacteria (Shuvalov & Klimov, 1976). In this case, an excited state bacteriochlorophyll dimer transferred an electron to a bacteriopheophytin. In both of these photosynthetic systems, the next electron carrier is known to be a quinone (a plastoquinone in PSII and an ubiquinone or a menaquinone in purple bacteria). The semiquinone anion of Q₄ in PSII was first detected optically more than ten years ago (Van Gorkom, 1974). Shortly thereafter, semiquinone anions were observed in purple bacteria (Pulles et al., 1976). It is also known that the quinones
in PSII (\(Q_A\) and \(Q_B\)) interact magnetically with a high spin iron (II) atom (Klimov et al., 1980). Similarly, an analogous magnetically interacting Fe(II) had been extensively characterized previously in purple bacteria (Okamura et al., 1979).

Supportive evidence of this heterodimeric intrinsic protein model also comes from studies of sequence homology between the \textit{Rps. viridis} L and M subunits and intrinsic proteins found in oxygenic photosynthetic organisms. Substantial sequence homology exists between the protein residues in the region involved in binding the iron-quinone acceptor complex on the L and M subunits in the \textit{Rps. viridis} structure and the putative binding sites on the D1 and D2 proteins of PSII (Hearst, 1986). Convincing evidence that the D1 and D2 polypeptides in PSII play a similar role to the L and M subunits in \textit{Rps. viridis} comes from a recent isolation of a PSII reaction center which contains only D1, D2 and cyt b\(_{569}\) (Nanba & Satoh, 1987). Although this core preparation was non-oxygen evolving, reversible absorbance changes associated with photochemical accumulation of reduced pheophytin indicate that the primary charge separation takes place within this preparation. These results are strongly suggestive that the primary pigment and the pheophytin acceptors are bound within the D1 - D2 heterodimer. Based on hydropathy plots of the known sequence of D1 from \textit{Chlamydomonas reinhardii}, Trebst (1986) suggested a folding pattern involving five transmembrane helices. Thus, it seems likely that D1 and D2 are functionally and structurally similar to the analogous L and M proteins.

Evidence of the role of D1 and D2 in binding manganese is much less direct; nonetheless, binding of Mn to D1 and D2 on the lumenal side of the membrane has been proposed (Metz et al., 1986; Michel and Deisenhofer (1988). The highly conserved regions of the C-terminal sequences of D1 and D2 in higher plants and cyanobacteria which are not found in the L and M subunits of the purple non-sulfur bacteria (Hearst, 1986) are likely binding sites. Some indirect evidence supporting this hypothesis comes from the observation that a mutant of the green alga \textit{Scenedesmus obliquus}, containing an additional 2 kDa tail on the D1 C-terminus, is incapable of oxygen evolution (Metz et al., 1986). Presumably, this unprocessed psbA gene product is not able to fold in the native conformation and, thus, is unable to bind Mn.

Pursuing this line of speculation, it is reasonable to examine the highly conserved regions of the C-terminal sequences of D1 and D2 (shown in Figure 8-1) to determine
Figure 8-1. C-terminal amino acid sequences of D1 and D2 from higher plants, green algae and cyanobacteria. Acidic residues are shaded, basic residues are in bold face and the unique lysine on the lumen side of the D2 protein of spinach and Chlamydomonas is underlined. See text for further details.
D-1 C-terminal Amino Acid Sequences

1. FNLMGFNQSVDSQDRVLNTWAD II NRANLGMEVMHERNAHFFPLDLASTNSSSN

2. G I AIEAP TNG

3. L K V AGEATPVALTAPS IHG

1. Chlamydomonas reinhardii
2. Spinach
3. Synechococcus 6301

D2 C-terminal Amino Acid Sequences

4. LNLRAWDFVSQEQ RAAEDPEPETFYTKNI LLNEGRAWMAAQDQPHERLVFPEEVLPRGNAL

5. M N I

6. L P NF

4. Chlamydomonas reinhardii
5. Spinach
6. Synechocystis 6803
probable protein derived ligands to manganese. Acidic residues (e.g. glutamates and aspartates) are by far the most abundant of the charged residues in these hydrophilic chains, comprising nearly 16% of the residues present. Positively charged residues are substantially less abundant, constituting about 9% of the total residues in these two hydrophilic sequences. Among the positively charged residues, arginines are the most abundant. Histidines and notably lysines are far less abundant. In fact, there is only one lysine in the sequence of D2 on the lumen side (based on Trebst’s model of protein folding) of the membrane, a curiosity which will be considered in greater detail in Section 8.4. Tyrosines, another possible ligand to manganese, are also relatively low in abundance (e.g. only two are found in the C-terminal sequences of both D1 and D2, although more are found in the hydrophilic lumenal loops).

Considering the ligands to manganese in manganoproteins of known structure (e.g. Mn superoxide dismutase, concanvalin A, phosphoglycerate kinase), it is almost certain that carboxylates derived from acidic side chains play a major role in the binding of the highly cationic manganese centers in PSII. For a review of Mn containing proteins see Reed and Markham (1984). If one considers the four manganese present as a pair of di-μ-oxo bridged binuclear centers and assuming octahedral coordination of each ion, then as many as 16 of the 19 carboxy side chains of the C-terminal residues may be involved in the binding of manganese (or as few as 8, if bidentate coordination of carboxylates occurs). Histidine is also a common ligand to manganese in manganoproteins and potentially could play a functional role in the storage of oxidative equivalents. Side chains, such as lysine, could also serve as ligands to manganese and could serve to facilitate the oxidation of water in a coupled proton-electron transfer scheme depicted in Section 8.3.

8.2 A Closer Look at Possible Redox Active Ligands

As was suggested in the conclusions of Chapter 7, the absence of change in the Mn X-ray absorption edge in advancing from the S2 to the S3 state indicates that manganese is not oxidized during this transition. Further, the loss of the multilne signal and the small conformational change of the manganese complex indicate the generation of a radical species very close, if not directly ligated, to the manganese atoms yielding the multilne signal. Two possible explanations were presented: partial oxidation of the substrate water or oxidation of another redox active component. Histidines, tyrosines
and plastoquinones could play the role of intermediate carriers of oxidative equivalents. A recent thermodynamic analysis of the stability of one-electron oxidation products of water suggests that the first explanation is highly unlikely (Krishtalik, 1986).

Of the three possible redox active ligands, tyrosines are particularly attractive candidates. There are now two well documented cases where tyrosines have been shown to be redox active components of oxidoreductases. The recent tyrosine-specific deuterium labelling studies performed by Barry and Babcock (1987) provide compelling evidence that, contrary to a body of indirect evidence, D and probably Z are not plastoquinones, but tyrosine residues. Similar deuterium labelling studies have demonstrated that a tyrosine is involved in the electron transfer involved in the enzymatic activity of ribonucleotide reductase (Sjöberg et al., 1983). There are two tyrosines in the C-terminal sequences of D1 and D2 (e.g. D2 residues 297 and 316). There are also several tyrosines on the loops connecting the transmembrane helices on the luminal side of the thylakoid membrane. It should be noted that tyrosyl residues are relatively unusual ligands to metal centers in metalloenzymes. However, several proteins have been characterized in which tyrosine is thought to be involved in the binding of iron. For example transferrin (Garber et al., 1974), catechol dioxygenase (Keyes et al., 1978) and purple acid phosphatases (Garber et al., 1978) are all iron-tyrosinate proteins with distinctive visible absorptions arising from tyrosine-to-iron charge transfer.

Recent attempts to quantitate the number of plastoquinones present in PSII particle preparations indicate that there are about 2 per reaction center (Takahashi & Satoh, 1987). Given that one of these two quinones is involved on the acceptor side, there is only one left which may potentially serve as a redox active ligand to manganese. The idea that quinones could serve this role is given support, at least in concept, by the observation of reversible redox equilibria in inorganic complexes between quinones and the high valence manganese complexes to which they are ligated (Pierpont et al., 1982). It should be noted that quinone redox chemistry generally involves two-electron reactions; however, the one-electron chemistry of the primary electron acceptor in PSII (Q_A) demonstrates this is not an absolute rule in biological systems.

Histidines are common ligands to metal centers in metalloenzymes and thus, are also likely candidates as redox active aromatic ligands to manganese. The presence of three highly conserved histidines in the C-terminal sequences of D1 and D2 (His 337
of D2 and His 332 and 337 of D1) supports this hypothesis. In fact, considering the heterodimeric protein ligation of other functional components of the reaction center in *Rps. viridis*, it is intriguing to speculate that this symmetry (actually a pseudo two-fold axis exists in the crystal structure) also exists in PSII reaction centers and extends to the oxygen evolving complex. Symmetric ligation of His 337 from both D1 and D2 to each of the two di-μ-oxo bridged dimers would seem to fit this model nicely.

8.3 A Model for the Accumulation of Oxidizing Equivalents Within the Oxygen Evolving Complex of PSII

In this section a model of the mechanism of photosynthetic oxidation of water is presented. This mechanism is based principally on conclusions drawn from the analysis of X-ray absorption spectra of PSII preparations poised in the $S_0$, $S_1$, $S_2$ and $S_3$ states as described below.

A comparison of the absolute energy of the inflection of the Mn K-edges of PSII preparations poised in the $S_2$ state with those of twenty five inorganic complexes indicates that the manganese present is principally Mn(IV) (see section 5.3.1). Taken together with the mixed valence nature of the $S_2$ state indicated by the multiline EPR signal, the most probable formal valence of the four manganese present is (III,IV,IV,IV). The light-induced change in the 1s → 3d absorption profile of the Mn K-edge of PSII preparations occurring during the $S_1$ → $S_2$ state transition indicates a formal valence change from Mn(III) to Mn(IV) (see section 5.3.3). Although the magnitude of the light-induced Mn K-edge shift occurring during the $S_1$ → $S_2$ state transition could be explained by one- or two-electron oxidations of the manganese complex (see section 5.3.2), for simplicity we will assume that the one-electron photochemistry of the PSII reaction center results in single-electron oxidations of the Mn complex during any single S-state transition in which the manganese complex is observed to be oxidized. Based on this assumption, the most probable formal valence of the four manganese present in PSII preparations poised in the $S_1$ state is (III,III,IV,IV).

The light-induced Mn K-edge shift to lower energy observed for PSII preparations containing low concentrations of hydroxylamine indicates that the hydroxylamine-induced $S_0$ state contains a Mn complex with a lower formal valence than that in the $S_1$ state (see section 6.4.1). A dramatic decrease is observed in the amplitude of peaks in
the Fourier transform of the EXAFS of the Mn complex in samples poised in the $S_2$ (the hydroxylamine-induced $S_0$ state) relative to those of preparations poised in the $S_1$ or $S_2$ states (see section 6.3.3). This decrease in amplitude observed in the EXAFS is similar to differences observed between isostructural tetranuclear manganese complexes which differ in formal valence by the presence of a single Mn(II) ion (see section 4.3.2.2). We speculate that the hydroxylamine-induced $S_2$ state exhibits properties similar to those of the native $S_0$ state and that the formal valence of the four manganese present is (II,III,IV,IV).

As described in section 7.3.3 advancement to the $S_3$ state does not involve any changes in the shape or energy of the Mn K-edge and hence strongly indicates that manganese is not oxidized during this transition. As described in the previous section, histidines and plastoquinones are possible redox active ligands which may store the oxidative equivalent accumulated in the OEC during the $S_2 \rightarrow S_3$ transition.

An analysis of the Mn EXAFS of ten multinuclear $\mu$-oxo bridged Mn complexes performed in parallel with the analysis of the EXAFS of the Mn complex in PSII preparations poised in the $S_1$, $S_2$ and $S_3$ states indicates that a pair of binuclear di-$\mu$-oxo bridged manganese complexes is the most probable structure for the four manganese present in these states.

Using the valence assignments and the most probable structure for the Mn complex in PSII described above, a model of the mechanism of water oxidation has been constructed (see Figure 8-2). The choice of histidine as a redox active ligand in the scheme shown in Figure 8.2 is somewhat arbitrary, although the basicity of this amino acid side chain may facilitate the oxidation of water and the formation of the peroxy bridge as described below. The charge stabilizing deprotonation of the $\pi$-cation radical of the histidine ligands depicted is also consistent with the proton release observed during the $S_2 \rightarrow S_3$ state transition. Also included in the mechanism depicted in Figure 8-2 are speculations regarding the origin of the protons known to be released (Saphon & Crofts, 1977) during the $S_0 \rightarrow S_1$ and $S_3 \rightarrow (S_4) \rightarrow S_0$ state transition as well as speculations regarding the valence of the Mn in the $S_4$ state and possible water redox chemistry occurring during the $S_3 \rightarrow (S_4) \rightarrow S_0$ state transition.

If the PSII reaction center is unable to oxidize the Mn complex beyond the formal
Figure 8-2. A proposed mechanism for photosynthetic water oxidation. For details see text.
valence present in the $S_2$ state, as is indicated by the lack of change in the formal valence upon advancement to the $S_3$ state, then, at least transiently, the oxidative equivalent stored during the $S_3 \rightarrow S_4$ state transition may also reside on a redox active ligand of manganese. It is appealing to consider symmetric coordination of the two His-337 side chains of D1 and D2 in this regard. Note that there are not enough plastoquinone molecules in PSII preparations to serve this proposed function for both the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$ state transitions.

Based on a thermodynamic analysis of various possible oxidation intermediates, Krishtalik (1986) suggested that a concerted four-electron process is by far the most favorable of conceivable oxidation schemes. Krishtalik also suggested that the presence of basic residues in the immediate environment of the manganese complex could facilitate oxidation of water and release of oxygen by strongly binding protons released during this process. Following reduction of the histidines, these side chains could serve this function. Subsequent binding and hydrolysis of two molecules of water could then result in the release of two additional protons, as proposed by Andréasson et al. (1985). To account for the observed proton release pattern during the $S_0 \rightarrow S_1$ state transition, one could envision an additional basic amino acid side chain whose pKa is strongly influenced by the oxidation state of the manganese to which it is ligated. It has long been known (Basolo & Pearson, 1967) that an increase in the oxidation state of a metal to which a basic residue is ligated can decrease its Ka by as much as two orders of magnitude. Also upon reduction of the manganese complex, this basic residue could strongly bind an additional proton and thus account for the observed proton release pattern during the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition.

Considering the Lewis acidity of the higher valences of Mn, it seems likely that water will be bound as hydroxide ions during the course of the catalytic cycle. Using a mass spectrometer Radmer and Ollinger (1986) examined the isotopic composition of the oxygen evolved from chloroplasts subjected to short saturating flashes of light following addition of $H_2^{18}O$ labelled water. Based on these experiments they concluded that water is exchangeable until the $S_3 \rightarrow S_4$ state transition. Thus, the mechanism in Figure 8-2 depicts hydroxide ions bound to one of the two binuclear manganese complexes in all of the $S$-states. The oxidation of water is presumed to occur in the final step, consistent with the experiments of Radmer and Ollinger and with Krishtalik's
thermodynamic analysis. Subsequent binding of two water molecules to manganese and release of two protons by hydrolysis is also indicated, as proposed by Andréasson et al. (1985).

8.4 Implications of the Proposed Structural Model

As indicated in the conclusions of chapters 4, 6 and 7, the most probable structure for the four manganese present in PSII is a pair of di-μ-oxo bridged binuclear complexes. The absence of peaks in the Fourier transform of the EXAFS of PSII preparations in the region around 3 Å rules out numerous discrete tetranuclear models proposed for the active site (See section 4.5) and indicates that these two dimers are greater than 4 Å apart. Although a small change in the structure of the manganese complex is observed during the $S_2 \rightarrow S_3$ transition, the magnitude of this change and the structure of the Mn complex in the $S_3$ state are inconsistent with the structural rearrangement proposed by Brudvig and Crabtree (1986). The concept of two binuclear complexes is also inconsistent with the recent speculation (Hansson et al., 1987) that the species giving rise to the g=4.1 signal is due to a Mn(IV) monomer. Like the multiline EPR signal, the g=4.1 signal has been assigned to the $S_2$ state (Zimmermann & Rutherford, 1986) and associated with the manganese complex (Cole et al., 1987b).

Although the EXAFS analyses indicate that tetranuclear structures, such as those described by Brudvig and Crabtree (1986) are unlikely, the primary physical basis of their proposed model is not necessarily inconsistent with the concept of two binuclear complexes, if the two complexes are less than 8 Å apart. The theoretical modelling of the multiline EPR signal by dePaula et al. (1985,1986), which is the primary basis of the model proposed by Brudvig and Crabtree, reduces to a set of three superexchange couplings between the four manganese atoms present in PSII. Antiferromagnetic couplings were assumed between manganese atoms within two binuclear units, and ferromagnetic coupling was assumed between these two centers. The magnitude of the ferromagnetic couplings were estimated to be on the order of 50 cm$^{-1}$. Couplings of this magnitude have been observed for paramagnetic centers more distantly coordinated than dePaula et al. suggested. Estimates of the distance between paramagnetic centers which could yield a coupling of this magnitude indicate that the maximum distance between the binuclear units is 8 Å (Coffman & Buehler, 1979). As described above, the EXAFS analyses indicate that the two binuclear centers are > 4 Å apart. Many exchange cou-
pled, bridged binuclear metalloenzyme systems are known which contain metal centers separated by 4 - 6 Å. For example, the Cu and Zn sites in mammalian superoxide dismutase are separated by 6 Å and are bridged by a histidine (Beam et al., 1977). Similarly the Ca and Mn sites in concanavalin A are 4.25 Å apart, bridged by an aspartate carboxyl group (Becker et al., 1975). The inconsistencies between laboratories in the observation of the non-Curie like behavior (which is the experimental basis of the theoretical modelling of dePaula et al. (1985) might be more easily reconciled in terms of subtle differences between preparative techniques if the binuclear manganese centers were more loosely associated in the manner described above. The coupling scheme described by dePaula et al is appealing in that it accounts for both the multiline EPR signal and the g=4.1 EPR signal.

The concept of two di-μ-oxo bridged binuclear complexes separated by less than 8 Å is also appealing from a mechanistic standpoint. The process of water oxidation may occur (as depicted in Figure 8-2) between the two binuclear centers. One of these two binuclear centers may function to bind the substrate water, effectively serving as a template for the formation of a peroxy bridge, while the role of the other binuclear complex might be to accumulate the necessary oxidative equivalents required for the four-electron oxidation. This physical symmetry but functional asymmetry is perhaps consistent with the generalized structure of the reaction centers described by Michel and Deisenhofer.

8.5 Future Experiments

8.5.1 Cryogenic Stabilization of Other S States

A major portion of the work described within this thesis was directed at obtaining structural information about the manganese complex cryogenically stabilized in various S states. A logical extension of this work would be the cryogenic stabilization of the native S0 state and the S4 state. A slight modification of the procedure used to generate the PSII preparations poised in the S3 state (See chapter 7) could be used to cryogenically trap PSII preparations in the S0 state and potentially in the S4 state. The S4 state is normally considered a transient intermediate at room temperature; however, there is doubtless a substantial activation energy barrier to this final step in the oxidation of water. By employing a double turnover low temperature illumination of samples poised
initially in the $S_2$ state, cryogenic trapping of samples poised in the $S_4$ state might be achieved. The following procedure could achieve this result. Illumination at 195 K of long-term dark-adapted PSII preparations containing 1mM PPBQ will trap the OEC in the $S_2$ state. Warming preparations illuminated in this manner to 20 °C for 30 sec will result in the oxidation of FeQ$_2^-$ and the generation of oxidized Q$_{400}$ without substantial loss of $S_2$ state composition. PSII preparations treated in this manner are now poised for advancement to the $S_4$ state by a single low-temperature double-turnover illumination. As mentioned in section 7.4.1, competitive photodonation of cyt b$_{559}$ limited complete $S_3$ state conversion in the double-turnover illumination procedures of PSII preparations poised in the $S_1$ state. By selectively oxidizing cyt b$_{559}$ in the PSII preparation prior to the 195 K illumination described above, this competitive process could be eliminated. Selective oxidation of cyt b$_{559}$ could be achieved by poising the potential of the PSII preparation at 350 mV at pH 6.0.

By warming PSII preparations cryogenically trapped in the $S_4$ state prepared in this manner, presumably the dominant effect would be the oxidation of water and the generation of a sample poised predominantly in the $S_0$ state. Thus this procedure could yield samples cryogenically trapped predominantly in either the $S_0$ or the $S_4$ states.

Stabilization of PSII preparations in the $S_0$ state at concentrations suitable for X-ray absorption analysis might alternatively be accomplished using a series of three short saturating flashes of light. Single turnover, saturating flashes might be achieved by using high power (e.g. 100 - 200 mJ), frequency doubled, YAG laser pulses and more dilute PSII suspensions (e.g. 2 - 4 mg/mL). By centrifuging dark-adapted PSII preparations illuminated in this manner, a sample poised in the $S_0$ state and concentrated enough for X-ray absorption spectroscopy might be obtained. The relative stability of PSII preparations poised in the $S_0$ state (e.g. a half time of decay of 50 min, Styring & Rutherford, 1987) makes this centrifugation step feasible.

8.5.2 Structural Characterization of the Oxygen Evolving Complex

Ultimately a complete structural characterization of the oxygen evolving complex and the PSII reaction center will be obtained by X-ray crystallography, once crystals of PSII core preparations of sufficient purity are obtained. However, considering the current state of PSII core preparation technology and the lability of the oxygen evolv-
ing complex, it would appear that this goal will not be accomplished for quite some time. Nonetheless, the pursuit of this goal is clearly worth the effort, for at the present time no other physical technique is capable of yielding such detailed three dimensional structural information about a macromolecular complexes of this size. It should be emphasized at this point, however, that once a complete three dimensional structure of the OEC is obtained, the structural details revealed by the X-ray absorption experiments are not rendered useless. X-ray absorption spectroscopy should be thought of as a complementary tool to X-ray crystallography, if the objective of the study of a metalloenzyme is the elucidation of the mechanism of action. Although the quantity of structural information obtainable by X-ray crystallography is unsurpassed, the accuracy of the determination of individual atomic positions in a macromolecular structure is limited to 0.1 Å under ideal conditions (Marquart et al., 1983). In contrast, EXAFS yields only a radial distribution of neighboring atoms in a very limited domain around the active site in a metalloenzyme. However, the accuracy of the distance information obtained and the sensitivity to small changes in the structure of the active site is far superior to that obtainable by X-ray crystallography. Thus, the structure of the active site revealed by X-ray crystallography could be refined by the results obtained by EXAFS and conversely, the interpretation of the changes in the structure observed by EXAFS analyses of different functional states would be greatly facilitated by the knowledge of the complete structure revealed by X-ray crystallography.

Alternative methods of obtaining information concerning the arrangement of functional components within the oxygen evolving complex and probable conformations of the putative Mn binding sites on the C-terminal sequences of D1 and D2 may already exist. By using a combination of site directed mutagenesis and specific spin labelling of amino acid side chains, studies of spin-spin interactions of these labelled residues with paramagnetic centers in the OEC could yield an estimate of distances between the functional components and specific amino acid sites. Ideally a single amino acid site would be labelled and the effect of each paramagnetic center (e.g. the manganese complex, cyt b_{669}, Z, D and Q_{A}) on each specifically labelled amino acid could be examined independently.

While it seems improbable that a macromolecular complex of the size of the PSII reaction center could be specifically labelled at a single site, the presence of a unique
lysine residue on the lumenal side of D1 and D2 (according to the folding model of Trebst) is encouraging in this respect. Amino specific spin labelling reagents are in fact known, e.g. succinimidyl-1-oxy-2,2,5,5-tetramethyl-3-pyrroline-3-carboxylate has been used in this manner, and specific labelling conditions have been established (Rousselet et al., 1984) which are suitable for use with active O₂-evolving PSII preparations. It should be noted that, unlike the sequences of D1 and D2, the sequence of the extrinsic 33 KDa protein from spinach (Oh-oka et al., 1986) is rich in lysine residues (e.g. 23 lysine residues are present). The fact that the 33 KDa extrinsic protein is so rich in lysines may be consistent with its proposed role in stabilizing the Mn complex (Miyao & Murata, 1984). The extrinsic 33 KDa protein may form numerous salt bridges between its positively charged lysine side chains and the numerous acidic residues of the C-terminal sequences of D1 and D2, stabilizing the protein conformation which forms the binding site for the Mn complex.

The presence of these lysines in the extrinsic 33 KDa protein does not prevent specific labelling of the D2 lysine residue, because procedures for removing the 33 KDa protein using CaCl₂ washes (Ono & Inoue, 1984) or urea plus NaCl (Miyao & Murata, 1984) are known. Restoration of the oxygen evolving activity of PSII preparations depleted of the extrinsic polypeptides can be accomplished by addition of sufficient Cl⁻ (e.g. 100 mM Cl⁻). Thus the 33 KDa polypeptide is not essential to oxygen evolution. Our recent X-ray absorption study of PSII particles from spinach depleted of the 33 KDa polypeptide also indicates that the structure of the manganese complex is essentially unaltered upon removal of this extrinsic polypeptide (Cole et al., 1987).

An analysis of the broadening of the nitroxide radical EPR signal due to exchange coupling between paramagnetic neighbors can yield estimates of the distance of separation up to 14 Å (Calvin et al., 1969). Dipolar broadening of nitroxide spin labels has also been used to determine the distance between two immobilized unlike spins. For example, the separation between Mn(II)ADP bound to creatine kinase and a nitroxide spin label specifically attached to a cysteine was determined from the line broadening of the nitroxide EPR signal. A calculation of the lineshape of the immobilized free radical EPR signal interacting with a paramagnetic neighbor yielded an expression which depended only on the magnetic moments of the paramagnetic centers, the electron spin relaxation times of these species and the distance of separation (Taylor et al., 1969).
Separation between the nitroxide spin label and the Mn(II) ion was estimated to be between 7 - 10 Å. Similarly a thiol at the active site of phosphofructokinase has been specifically labelled with a nitroxide spin label. Through an analysis of the line broadening of the nitroxide EPR signal, the separation between the nitroxide radical and a Mn$^{2+}$ bound to ATP which is bound to the enzyme was estimated to be 12 Å (Jones et al., 1973).

Presumably, magnetic interactions with a spin label specifically attached to a hydrophilic residue on the luminal side of the membrane would not be observed for all of the paramagnetic centers (e.g. the manganese complex, cyt b$_{559}$, Z, D and Q$_{A}$) which can be generated in a PSII preparation. Considering the range limitations of the technique (e.g. ~ 14 Å) and the width of a typical lipid bilayer (e.g. 40 Å), it is unlikely that broadening of a nitroxide spin label on the luminal side of the membrane would occur due to a paramagnetic center like Q$_{A}$ near the cytosol side of the membrane. Similarly, if one assumes the folding model proposed by Trebst et al. (1986), then the tyrosine associated with D$^{+}$ (Debus et al., 1988) is probably too far from the lumen side of the membrane to cause significant line broadening effects. Line broadening effects due to the manganese complex are more likely. The radius of a hypothetical globular protein consisting of the C-termini of D1 and D2 and the luminal loops from these two proteins (a crude estimate of the molecular weight based on the Trebst folding model is 28 kDa) is approximately 20 Å. Thus, unless the manganese complex is located precisely in the center of this putative globular protein, it seems likely that one or more surface labelled sites will magnetically interact with a manganese complex bound within a protein of this size. If a set of such specifically labelled side-chain paramagnetic center distances could be assembled for the putative binding sites of the Mn complex in PSII, a distance geometry calculation could yield a set of probable protein conformations. The genes containing the sequences coding for D1 and D2 from Synechococcus 7002 have recently been cloned, and site specific changes to these sequences are in progress (Gingrich, personal communication). The ability to specifically change residues potentially allows specific labelling of other sites on the C-terminal sequences and thus may permit the determination of numerous side-chain functional component separations. Changes in the sequences which cause a minimal disruption of the native folding are obviously advantageous. A change from a positive side chain which is not labelled to a lysine
side chain which can be labelled, probably will not significantly alter the normal protein conformation. The seven arginines in the C-terminal sequences of D1 and D2 provide ideal sites for such site directed-mutagenesis.
References


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