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**The Role of Calcium in the Oxygen Evolving
Center of Photosystem II**

M.J. Latimer
(Ph.D. Thesis)

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The Role of Calcium in the Oxygen Evolving Center of Photosystem II

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Abstract

The Role of Calcium in the Oxygen Evolving Center of Photosystem II

by

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The photosynthetic oxygen evolving complex (OEC) contains a cluster of four manganese atoms and requires both Ca and Cl for activity. Ca can be replaced by Sr with retention of activity. The role of Ca in the OEC has been investigated by performing Mn X-ray absorption experiments on Ca-depleted samples of photosystem II (PS II) and on PS II samples depleted of Ca and reconstituted by either Ca or Sr.

Mn X-ray K-edge spectra exhibit no significant differences in oxidation state or symmetry between Ca- and Sr-reactivated preparations, but differences are observed in the extended X-ray absorption fine structure (EXAFS). The amplitude of a Fourier transform peak arising from scatterers at distances greater than 3 Å is larger for samples reactivated with strontium relative to calcium. Curve-fitting analyses of the EXAFS data using FEFF 5-calculated parameters favor a model where both manganese and calcium (or strontium) scatterers contribute to the ~3 Å Fourier peak (Mn-Mn at 3.3 Å and Mn-Ca(Sr) at 3.4-3.5 Å). Possible structural arrangements for a calcium binding site are discussed.

Analysis of Mn K-edge spectra from Ca-depleted samples in the S_1 , S_2 , and S_3 states shows an edge shift on the S_1 - S_2 transition, but no edge shift on the S_2 - S_3 transition, supporting a model where the oxidizing equivalent from the S_2 to S_3 transition is stored on a ligand or nearby protein residue rather than on the Mn cluster. Parallels between Ca-depleted and native samples are discussed.

The design and construction of a simple in-hutch mirror assembly for X-ray harmonic rejection is described. The mirror apparatus reduces the second harmonic to 0.5% of the total flux while retaining >50% of the flux at the fundamental.

Finally, structural parameters derived from EXAFS are compared to multinuclear Mn complexes, and, using structural motifs derived from such comparisons, a working model of the Mn cluster including a Ca-binding site is presented.

*For my parents, Howard and Mary Ann,
and for my wife, Melissa*

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Abbreviations

A ₀ , A ₁	electron acceptors in photosystem I
ATP	adenosine triphosphate
Chl	chlorophyll
Cyt	cytochrome
DCBQ	2,6-dichloro- <i>p</i> -benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
Benz	benzoate
Bipy	bipyridine
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'- tetraacetic acid
EPR	electron paramagnetic resonance
ESE	electron spin echo
ESEEM	electron spin echo envelope modulation
EXAFS	extended X-ray absorption fine structure
KS model	Klein-Sauer model for the Mn cluster of photosystem II
MES	2-[N-morpholino]ethanesulfonic acid
NADP	nicotinamide adenine dinucleotide phosphate
OEC	oxygen evolving complex
P ₆₈₀	primary electron donor of photosystem II
P ₇₀₀	primary electron donor of photosystem I
PC	plastocyanin
Ph	phenyl

Pheo	pheophytin
PS I	photosystem I
PS II	photosystem II
Py	pyridine
Q _A , Q _B	plastoquinone electron acceptors in photosystem II
S ₂ '-annealed	Ca-depleted S ₂ '-state formed by annealing an S ₃ '-state sample at 0°C in darkness.
S ₂ '-DCMU	Ca-depleted S ₂ '-state formed by illumination of an S ₁ '-state sample containing DCMU.
SSRL	Stanford Synchrotron Radiation Laboratory
XAS	X-ray absorption spectroscopy
Y _Z , Y _D	redox-active tyrosine residues in photosystem II

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Chapter 1

Introduction

Importance of Oxygenic Photosynthesis

All life on earth is dependent on the energy from the sun. Plants, algae, and other photosynthetic organisms absorb energy from sunlight, using it to drive chemical reactions. The products of this process, reduced carbon compounds (carbohydrates), are used as an energy source by heterotrophic organisms, which include animals, fungi, most bacteria, and, of course, you the reader. One of the byproducts of photosynthesis, molecular oxygen, is also a key ingredient in aerobic respiration.

These two processes, oxygenic photosynthesis and aerobic respiration form a cycle that took hold as the earth moved from a reducing to an oxidizing atmosphere about 2 billion years ago. Oxygen-evolving photosynthetic organisms (algae, cyanobacteria, and plants) are the source of virtually all the molecular oxygen in the atmosphere and, as they gradually changed the atmosphere from a

reducing to an oxidizing environment, created the conditions for aerobic organisms to evolve. All but a tiny fraction of life on earth is now sustained by photosynthesis and aerobic respiration.

The Reactions of Photosynthesis

The overall photosynthetic process may be described by the simple equation

where water is the source of electrons used to reduce CO_2 . In reality, this process occurs in many steps which can be separated into two broad categories; reactions that require light (light reactions) and reactions involving CO_2 -fixation (carbon reactions).

The light reactions consist of a series of light-driven electron transfer steps whereby electrons extracted from water molecules travel along a chain of electron transfer components, ultimately reducing NADP^+ . The carbon reactions use the NADPH to reduce CO_2 to carbohydrates in an involved process known as the Calvin cycle.

The process of photosynthesis in plants occurs in a specialized organelle called the chloroplast. The chloroplast is enclosed by an outer membrane or envelope, which has a relatively simple structure, and a highly structured inner membrane (the thylakoid). The thylakoid membrane divides the chloroplast into two spaces, the stroma (between the thylakoid and the envelope membrane) and the lumen (enclosed by the thylakoid). The carbon reactions occur in the stroma while the light reactions occur in two pigment-protein

complexes called photosystems which reside in the thylakoid membrane.

The Photosystems

There are two distinct photosystems which are localized in separate regions of the thylakoid membrane. Photosystem II (PS II) reaction centers are located mainly in appressed regions of the thylakoid membrane called grana. Photosystem I (PS I) reaction centers are found in unstacked, stroma-exposed regions of the thylakoid membrane. The three-dimensional structure of the thylakoid membrane is complex; stroma-exposed membranes are arranged in tilted helical planes around tightly stacked grana disks. The grana stacks are connected by bridges through the helical membranes, and the entire thylakoid encloses a single continuous lumen. (For a more complete, illustrated discussion see Ort, 1986).

The electron transport chain through the photosystems, known as the Z-scheme (Hill & Bendall, 1960), is illustrated in Figure 1.1. The flow of electrons proceeds "downhill" energetically, except for photo-excitation events within the reaction centers of PS II and PS I. Excitation of P_{680} (probably a chlorophyll dimer) in PS II produces an excited state (P_{680}^*) which is capable of electron donation to a pheophytin molecule (Pheo) which in turn can transfer the electron to a bound plastoquinone molecule (Q_A). Q_A then transfers the electron to an exchangeable plastoquinone molecule (Q_B) which, when doubly reduced to the quinol form, is released into the membrane plastoquinone pool and is replaced by another plastoquinone.

Figure 1.1. The Z-scheme of photosynthetic electron transfer. Electrons are extracted from water by the OEC and are transferred, through photoexcitations in the reaction centers of PS II and PS I, to Ferredoxin (Fd) and finally provide reducing equivalents to produce NADPH from NADP^+ . Adapted from Miller & Brudvig (1991).

Plastoquinol molecules are then oxidized by a membrane bound cytochrome complex (cyt *b-f* complex) which can, in turn, reduce a small soluble protein, plastocyanin, in the lumenal space. Plastocyanin carries reducing equivalents to the PS I complex where they are used to reduce P₇₀₀ after it has been excited and transferred an electron to acceptor A₀. Electrons are then passed through a series of electron acceptors in PS I, finally resulting in the reduction of a soluble ferredoxin, which supplies the electrons used to reduce NADP⁺ to NADPH for use in the Calvin cycle.

Back at the start of the electron transport chain, oxidized P₆₈₀ is reduced by a tyrosine residue (Y_Z) which in turn is reduced by the oxygen evolving center (OEC) which obtains electrons from water via the reaction:

The entire process efficiently couples light-driven single-electron transfers at P₆₈₀ and P₇₀₀, two-electron reductions of quinone molecules to quinols, and the four-electron chemistry of water oxidation. In addition, water oxidation (and also the quinone/quinol cycle) create a proton gradient across the thylakoid membrane which is used to produce ATP molecules. A more detailed discussion of electron transfer in photosynthesis can be found in Ort (1986).

Photosystem II and Oxygen Evolution.

Measurements of PS II oxygen yield with flashes of light showed a period-four oscillation of maximum oxygen yield, with a

burst of oxygen after every fourth flash of light (Joliot & Joliot, 1968). These experiments were taken as evidence that the reaction centers worked independently and required absorption of four quanta of light to extract four oxidizing equivalents from two water molecules. A model was proposed by Kok et al. (1970) to explain this behavior which invoked an enzyme cycle with five intermediate states designated S_0 - S_4 .

Figure 1.2 The Kok S-state clock model for the oxygen evolving complex of PS II.

Steps which involve the absorption of light and extraction of an electron from the OEC drive the advance from S_0 to S_4 ; the S_4 -state decays spontaneously to S_0 with the release of oxygen. In the absence of light, the S_1 - and S_0 -states are the most stable (Kok, et al., 1970). S_2 and S_3 decay to S_1 in the dark, predominantly through recombination with electron acceptors on the reducing side of PS II. The S_0 -state is relatively stable in the dark, but can be slowly

oxidized on dark adaptation to S_1 by a stable tyrosine radical Y_D^+ (Styring and Rutherford, 1987).

The reaction center in PS II shares considerable structural and functional similarities with the reaction centers crystallized from the purple bacteria *Rhodospseudomonas viridis* and *Rhodobacter sphaeroides* (Diner, 1986; Hearst, 1986; Michel & Deisenhofer, 1988). The first acceptor (pheophytin), and the quinone two-electron gate system are present in both the purple bacteria and PS II. The most obvious differences are on the oxidizing side, where the purple bacteria receive electrons through a bound cytochrome via a cyclic mechanism and PS II extracts electrons from water by way of a redox-active tyrosine molecule (Y_Z) and the OEC.

A model of the polypeptide composition of PS II is presented in Figure 1.2. An extensive discussion of the polypeptides in PS II can be found in Debus (1992); a brief description of the major proteins and their roles in PS II is given here. The two reaction center polypeptides (D1 and D2) are membrane-bound proteins that bind the electron transfer components (P680, Pheo, Q_A , Q_B) and also contain the tyrosine residues Y_Z and Y_D . Y_D forms a stable cation radical in PS II. The CP47 and CP43 polypeptides are also present in the membrane and each binds about 18 chlorophyll molecules. CP47 and CP43 function as light harvesting antennae and also appear to stabilize the structure of PS II. Cytochrome b-559 is present in the membrane and several low molecular weight polypeptides of unknown function are also closely associated with PS II.

Figure 1.3 Polypeptide schematic of the PS II reaction center. Arrows indicate the path of electron transfer from the Mn-complex associated with oxygen evolution to the exchangeable quinone Q_B . Descriptions of the polypeptides and their functions are given in the text. Adapted from Debus (1992).

In plants there are also three extrinsic polypeptides of 33, 24, and 17 kDa associated with the luminal side of PS II; cyanobacteria appear to possess only the 33 kDa protein. All three can be removed by treatments at high ionic strength, with the 17 and 24 kDa proteins more weakly associated than the 33 kDa protein. The 33 kDa protein is associated with stable oxygen evolution and appears to protect a bound complex of Mn atoms. Removal of the 24 kDa protein results in an increased requirement for Ca for oxygen evolution. The removal of the 17 kDa protein creates an increased need for Cl.

Mn in PS II

Mn has long been known to be an essential co-factor in the OEC (reviewed in Radmer & Chennia, 1977), and it is generally agreed that there are four Mn/PS II reaction center (see Debus, 1992). Mn ions, which can exhibit a range of oxidation states, are a likely charge accumulator in the OEC and are also likely participants in the water oxidation chemistry. A considerable body of spectroscopic evidence supports the charge accumulation role, and treatments that have direct effects on the Mn-cluster have been shown to inhibit oxygen evolution.

Electron paramagnetic resonance (EPR) spectroscopy has been a useful probe of the Mn-complex of PS II. The observation of a Mn "multiline" EPR signal was the first spectroscopic evidence that Mn was involved in the charge-accumulation process in the OEC (Dismukes & Siderer, 1981). This signal was found in chloroplasts which had been illuminated and then frozen and is specifically associated with the S₂-state of the OEC. The multiline signal is

centered at $g=2$ and exhibits ~19 lines which arise from Mn nuclear hyperfine structure. The signal is similar to multiline signals seen in Mn(III)Mn(IV) di- μ -oxo bridged binuclear model complexes (Cooper et al., 1978). The multilines from model complexes have only 16 lines however, and the larger number of lines in the PS II signal has been interpreted as arising from a higher nuclearity in the Mn-cluster in PS II, though other interpretations exist.

A second EPR signal centered at $g=4.1$ and also associated with the S_2 -state has also been found in PS II (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984). Experiments which showed Mn hyperfine structure on the $g=4.1$ signal in partially oriented ammonia-treated PS II samples provide support for the view that both the multiline and $g=4.1$ signals arise from a single tetranuclear Mn-cluster (Kim et al., 1990). The $g=4.1$ signal is thought to arise from structural differences in the Mn complex relative to the multiline state (Kim et al., 1990, Liang et al., 1994).

The primary spectroscopic method employed in the work described in this dissertation is X-ray Absorption Spectroscopy (XAS). XAS is an excellent tool for examining the immediate structural environment of metal ions in proteins. XAS has the advantage of being element-specific and does not require a crystalline sample; thus, it has proved to be a useful technique for probing the structure of the Mn complex of PS II where many components (non-heme iron, cytochrome, etc.) normally present in active preparations can interfere with other spectroscopic techniques. X-ray absorption edges can be examined to determine the oxidation states and site symmetry of the Mn, and extended X-

ray absorption fine structure (EXAFS) gives information about the radial distribution of atoms around the Mn atoms of PS II. In addition to distances, EXAFS is sensitive to the numbers and atomic number of atoms around the absorbing atom (for reviews of XAS in biological systems see Scott, 1984; Cramer, 1988; Yachandra, 1995).

Evidence from the Mn K-edge spectra of PS II is indicative that Mn oxidation occurs on the steps S_0 - S_1 (Guiles et al. 1990b) and S_1 - S_2 (Goodin et al., 1984; Yachandra et al., 1987). PS II samples which were advanced to the S_3 -state by a low-temperature illumination protocol showed no edge shift on the S_2 - S_3 transition, indicating that no Mn oxidation has occurred (Guiles et al. 1990a). This view has been challenged by Ono and co-workers who worked on samples advanced through the S-state cycle at physiological temperatures by laser flashes and observed an edge shift of ~ 1 eV on the S_2 - S_3 transition (Ono et al., 1992). However, recent XAS experiments on similarly prepared samples where the S-state composition of the XAS samples was carefully monitored by EPR (which Ono and co-workers did not do) has indicated that there is no Mn oxidation on the S_2 - S_3 transition (Liang, 1994).

Previous results from EXAFS on PS II preparations (reviewed in Sauer et al., 1992) have indicated that the structure contains a Mn-Mn distance of 2.7 Å, which is a characteristic distance in di- μ -oxo bridged Mn model complexes (Wieghardt, 1989; Pecoraro, 1992). Consistent with this result, short bonds to oxygen/nitrogen atoms of 1.8 Å have also been detected which are also characteristic of distances to bridging oxo-groups in model complexes. A longer distance interaction has also been detected, but the interpretations

differ. Among them are: 1) a single Mn-Mn distance of 3.3 Å (George et al., 1989; DeRose et al., 1994); 2) a Mn-Mn or Mn-Ca at 3.3 Å (Penner-Hahn et al., 1990); 3) Mn-Mn and Mn-Ca at ~3.3 Å (Yachandra et al., 1993; DeRose et al., 1994); and 4) a single Mn-Ca at 3.7 Å (MacLachlan et al., 1992).

We have published potential models for the OEC based on a Mn-Mn interaction at 3.3 Å, but at the time we were unable to make a full determination concerning Ca contributions at that distance (DeRose et al., 1994).

Ca and Cl in PS II

Calcium and chloride ions have also been identified as required cofactors for oxygen evolution. Chloride competes with inhibitors of PS II oxygen evolution (anions such as fluoride and acetate, or amines). Depletion of chloride results in an S₂-state with no multiline or g=4.1 EPR signals (Yachandra et al., 1986; Ono et al., 1986). Replacement of chloride by fluoride enhances the amplitude and modifies the shape of the g=4.1 signal and causes changes in the EXAFS, resulting in increased distance heterogeneity in the 2.7 Å Mn-Mn interactions (DeRose et al., 1995). The effects on the Mn EPR signals and the structural perturbations of the Mn complex caused by chloride depletion and fluoride inhibition indicate that it is likely that chloride is bound directly to the Mn cluster, but conclusive evidence has remained elusive.

Ca has been identified as a necessary co-factor for oxygen evolution in plants through experiments where the 17 and 24 kDa polypeptides have been removed by salt washing. In these

preparations, it was found that maximal oxygen evolution rates required addition of Ca (Ghanotakis et al., 1984; Miyao & Murata, 1984; Nakatani, 1984). In later experiments it was found that the 24 kDa protein helps to maintain a high-affinity calcium binding site (reviewed in Yocum, 1991). Differing results using these preparations caused much confusion until it became clear that illumination was necessary to fully inhibit salt washed preparations and that, in fact, calcium binding affinity is affected by the redox-state of the Mn-cluster (Boussac & Rutherford, 1988c).

Ca-depleted PS II preparations do not evolve oxygen and appear to be unable to advance beyond the S₃-state (reviewed in Rutherford et al., 1992). In Ca-depleted preparations the S₃-state exhibits a novel EPR signal centered at g=2 with a width of 130-160 G depending on whether the 17 and 24 kDa polypeptides are present (Boussac et al., 1989; Boussac et al., 1990). Similar signals have been reported in Cl⁻-depleted and F⁻-inhibited preparations (Baumgarten et al., 1990). The identity and significance of this signal has been the subject of much debate, and a relevant question to ask is what is the valence state of the Mn-cluster in the Ca-depleted S-states. This topic is explored further in Chapter 4.

Inactivation of oxygen evolution in Ca-depleted preparations can be reversed by addition of Ca or, to a lesser degree, by Sr (Ghanotakis et al., 1984) or vanadyl (Lockett et al., 1990). Reactivation with Sr affects the rate of the oxygen evolution reaction and results in a modified S₂-state multiline EPR signal indicating that the structure of the Mn-complex has been perturbed (Boussac and Rutherford, 1988a,b).

Thesis Goals

The goal of much of the work presented in this dissertation is to gain information from experiments using X-ray absorption spectroscopy about the binding sites and possible functional roles for calcium in the oxygen evolving center of photosystem II. Mn XAS experiments on Ca-depleted preparations reactivated with Sr were undertaken to determine whether a Ca binding site exists in close proximity to the Mn cluster in PS II (see Chapter 3). Mn K-edge spectra were obtained from Ca-depleted preparations in different oxidation states of the OEC to determine the state of the Mn ions in the modified S-states displayed by these samples (see Chapter 4). From these studies, the possible roles for Ca in PS II and proposals for the structure of a Ca binding site close to the Mn cluster are discussed.

The main spectroscopic tool used in the studies presented here, X-ray absorption spectroscopy, is a rapidly developing technique which continues to benefit from improvements in theory and instrumentation. To that end, the use of improved curve fitting analysis parameters derived from advances in XAS theory is described in Chapter 2. And finally, interesting problems often arise in the practice of XAS, sometimes requiring modifications of equipment or new technical development. One such problem was encountered with harmonic contamination of the X-ray beam and its effects on Mn XAS experiments on samples containing Br. This problem was alleviated through the use of a simple X-ray harmonic

rejection mirror, the design and construction of which is described in Chapter 5.

Chapter 2.

Materials and Methods

In the first section of this chapter the biochemical methods for Ca^{2+} -depletion and reactivation by Ca^{2+} or Sr^{2+} of PS II preparations are described. X-ray absorption spectroscopic methods are described in the second part, with an emphasis on improved curve-fitting of EXAFS data using theoretical parameters calculated from the program FEFF 5.

Biochemical Methods and Sample Characterization

Preparation of Ca^{2+} -depleted and Ca^{2+} - and Sr^{2+} -reactivated PSII

Triton X-100-extracted PS II particles were prepared from market spinach according to the procedure of Berthold et al. (1981) with modifications as described by DeRose (1990). This method employs a detergent to solubilize unstacked regions of broken thylakoid membranes from spinach. Stacked regions (grana stacks), containing predominantly PS II reaction centers, are easily separated from the solubilized portions by centrifugation. Oxygen evolution rates from these preparations were typically 500-600 $\mu\text{mol O}_2/\text{mg Chl/h}$.

Ca-depletion treatments were performed according to the low-pH/citrate procedure of Ono & Inoue (1988). Briefly, PS II particles in 0.4 M sucrose, 25 mM MES, pH 6.5, 15 mM NaCl were pelleted by centrifugation (15 min at 40,000xg), then resuspended to approximately 6 mg Chl/ml in 0.4 M sucrose, 0.25 mM MES, pH 6.5,

15 mM NaCl buffer. The particles were then diluted to 2 mg Chl/ml in 0.4 M sucrose, 20 mM citrate, pH 3.0, 15 mM NaCl buffer. After a 5 min incubation at 0°C, the preparation was brought to pH 6.5 with buffer A (0.4 M sucrose, 50 mM MES, pH 6.5, 15 mM NaCl, 100 μ M EGTA) and pelleted by centrifugation (15 min at 40,000xg). The membranes were then resuspended in buffer A and pelleted again.

At this point in the preparation, Ca-depleted samples for EPR and X-ray spectroscopy were made from the pellet. The treated PS II was packed into lucite sample holders which were designed for both EPR and X-ray spectroscopy cryostats. Problems were encountered with incomplete saturation of samples during illumination; thus, samples which were to be illuminated were spread into a thin layer (<0.5 mm) to ensure saturation. The samples were frozen without delay; dark adaptation was unnecessary as all steps above were performed in darkness or very dim green light. Delays in freezing of the Ca-depleted samples invariably resulted in partial degradation of the Mn-complex, which could be detected by the appearance in the EPR spectra of a characteristic Mn(II) signal. Some Ca-depleted samples were made with a small amount of DCMU (0.5 mM added from a stock solution in DMSO) to limit these samples to a single turnover of the reaction center.

Ca- and Sr-reactivated preparations were made from the Ca-depleted PSII pellets by resuspension in buffer B (25 mM MES, pH 6.5, 15 mM NaCl, 100 μ M EGTA) with either 50 mM CaCl_2 or SrCl_2 (for Ca- and Sr-reactivated PS II samples respectively). The resuspensions with CaCl_2 and SrCl_2 were performed under ambient light and were homogenized slowly by hand to ensure a uniform

suspension and illumination. Ethanol has been shown to minimize the $g=4.1$ EPR signal in PS II preparations from spinach (Zimmermann & Rutherford, 1986), and we have found that addition of ethanol also suppresses the $g=4$ EPR signal in Sr-reactivated preparations; thus, samples were made with 2.5% ethanol (v/v) in the final resuspension step. Finally, the Ca- and Sr-reactivated PS II particles were again centrifuged (30 min at 40,000 $\times g$), and the resulting pellets were transferred to lucite sample holders and allowed to dark-adapt for about 1 h on ice, then were frozen in liquid nitrogen.

All samples were stored and transported in liquid nitrogen, and care was taken to prevent warming when transferring them to the liquid helium cryostats used for EPR and X-ray spectroscopy.

All glassware, centrifuge tubes, etc. were acid washed, and all buffers used in the Ca-depletion procedure were treated with Chelex-100 (Bio-Rad) to remove any Ca contamination in the chemicals used in the buffers.

Oxygen Evolution Measurements

Oxygen evolution activity was measured using a Clark-type oxygen evolution electrode as described by DeRose (1990). The electron acceptor DCBQ was added from a stock solution in DMSO to a final concentration of 2 mM. The oxygen evolution activity of untreated PS II particles was 500-600 $\mu\text{mol O}_2/\text{mg Chl}/\text{hr}$. Oxygen evolution of Ca-depleted preparations was measured in buffer A (see above) with no additions or with 50 mM CaCl_2 or SrCl_2 . Oxygen evolution activity was inhibited in Ca-depleted PS II to ~15% of the

value measured in untreated preparations. Addition of Sr^{2+} resulted in reactivation of oxygen evolution activity to ~40%, while addition of Ca^{2+} reactivated activity to ~80% of that in untreated preparations. Chlorophyll assays were performed on acetone extracts following the method of Arnon (1949).

EPR Spectroscopy

Low-temperature EPR spectra were collected using a Varian E-109 EPR spectrometer equipped with an Air Products Helitran liquid helium cryostat. All samples were characterized by low-temperature EPR prior to exposure to X-rays and were examined afterward to ensure that no damage (Mn release) had occurred from X-ray exposure or in sample handling. Ca- and Sr-reactivated samples were advanced to the S_2 -state by illumination for 10 min at 195K with a 400 W tungsten lamp employing a 5 cm aqueous CuSO_4 filter (5%, w/v) for thermal isolation. Ca-depleted samples were illuminated in the same apparatus, except that the temperature was maintained at 0°C. Ca-depleted samples were illuminated for 2 min to form the Ca-depleted S_3 -state, then quickly (less than 1 sec) frozen in liquid nitrogen. The Ca-depleted S_2 -state was formed in two ways: by illumination for 2 min of a sample containing DCMU then quick freezing, or by illuminating a sample for 2 min followed by incubation in darkness for 15 min at 0°C.

X-ray Absorption Spectroscopy

The data presented in this dissertation were collected at the National Synchrotron Light Source (NSLS) at Brookhaven National

Laboratory and at the Stanford Synchrotron Radiation Laboratory (SSRL). Synchrotrons provide high-intensity broad band radiation (IR to hard X-rays) and are excellent sources for spectroscopy. In the case of XAS on dilute protein samples, no other source can provide the intensity and brightness needed to perform these experiments in a finite/reasonable amount of time. The synchrotron radiation laboratories at NSLS and SSRL have been described (McDermott, 1987; Guiles, 1988; DeRose, 1990). In the following sections, a brief description is given of the XAS experimental setup and data reduction process. In addition, the curve-fitting of EXAFS data using improved theoretical parameters calculated by the program FEFF 5 (Rehr et al., 1992) is described, as well as the use of the program FEFF 5 itself.

XAS Experimental Apparatus

The basic XAS experimental setup is shown in Figure 2.1. The broad band radiation from the synchrotron is passed through a double crystal monochromator which operates by reflection from selected Bragg planes in the crystals (more discussion of X-ray monochromators and beamline optical components can be found in Chapter 5). A signal proportional to the incident intensity (I_0) is measured by a photomultiplier tube equipped with a plastic scintillator which detects photons scattered from a strip of kapton placed in the beam. The intensity of the X-ray beam after passing through the sample (I_1) is measured by an ion chamber, as is the intensity after the energy reference (I_2). Energy calibration (Goodin et al., 1979) is maintained by simultaneous measurement of the

Figure 2.1. The basic XAS experimental setup. The arrangement of the sample, energy reference, and detectors is shown. A top view of the beam path and fluorescence detector is shown in the lower picture. The fluorescence detector is placed at 90° to the incoming X-ray beam to minimize scatter contributions to the signal.

Figure 2.1
XAS experimental setup:

sample spectrum and the spectrum of a reference compound (KMnO₄).

Mn XAS data were recorded as fluorescence excitation spectra (Jaklevic et al., 1977) using either a single element lithium-drifted silicon solid-state detector (described in Guiles et al., 1990) or a 13 element solid-state Ge detector (Cramer et al., 1988); all EXAFS spectra were collected with the Ge detector. All Mn XAS data were collected using a Si(220) double crystal monochromator. For both Mn K-edge and EXAFS experiments, samples were maintained at 10-12 K using an Oxford Instruments CF 1208 liquid helium flow cryostat. The use of the cryostat and Ge-detector is described in DeRose (1990).

XAS Data Collection

X-ray absorption spectra are collected by stepping the double-crystal monochromator through a range of energies. At each point the incident and transmitted flux is measured (for a transmission experiment) and fluorescent photons are counted (for fluorescence detection). The absorption coefficient μ of a sample of thickness x is given by the equation

$$\mu x = -\ln(I_1/I_0) \propto F/I_0$$

Fluorescence detection is the preferred method for dilute proteins and, for Mn in PS II, a large number of individual scans must be averaged to achieve spectra with an acceptable signal-to-noise ratio (S/N).

In earlier work from this group (Guiles, 1988, and others earlier), spectra were signal averaged by weighting each individual scan by the initial I_0 value of each scan. This was an approximation to weighting by the S/N because each data point was collected for a fixed period of time; thus, a high I_0 value results in a larger number of fluorescent photons which in turn leads to better counting statistics. With the advent of the use of an energy resolving 13-element Ge detector (DeRose, 1990), a more accurate weighting was added to the process where the summed scans for each individual detector element were weighted by the S/N for that element. The S/N value can be estimated in an XAS spectrum from $(N_a - N_b)/(N_a)^{1/2}$ where N_a is the measured fluorescence counts above the X-ray edge energy and N_b is the fluorescence counts measured just below the edge.

Proper weighting of the spectra, however, requires that the data from each detector element for each individual scan be weighted individually (Scott, 1984). The weighting factor W_i for each channel i is given by

$$W_i \propto (S_i/N_i)^2/S_i$$

These weighting factors are proportional to the square of the S/N and also normalize the data to unit edge jump so that channels with equal S/N contribute equally to the weighted sum. The weighted fluorescence $\langle F \rangle$ is then given by

$$\langle F \rangle = \frac{\sum_i W_i F_i}{\sum_i W_i}$$

where F_i is the fluorescence signal in each channel. The most recent version of our signal averaging program, EXAVERAGE, employs this weighting scheme.

XAS Data Analysis

Data reduction for EXAFS and edge spectra were performed as reported previously (Guiles et al., 1990; DeRose et al., 1994; Yachandra, 1995). A brief description is given here.

Mn K-edge spectra were subjected to very little processing compared to EXAFS spectra. A straight line was fit to the data before the absorption edge rise and subtracted to remove a slight slope in the background and to set the value at the bottom of the edge to 0.0. The edge spectrum was then normalized to unit edge height as estimated by extrapolation of a polynomial fit to EXAFS data above ~6600 eV; the point at which the extrapolated line crosses the maximum absorption in the edge was set to a value of 1.0. Second derivatives of Mn K-edge spectra, which accentuate the shape of the edges, were derived by analytical differentiation of a third order polynomial fit over a ± 2.5 eV range around each data point.

EXAFS spectra were treated similarly to edge spectra in pre-edge background removal and normalization. After normalization the spectra were divided by the free atom absorption (McMaster et al., 1969) to remove the effect of decreasing absorption cross-section at higher photon energies. The EXAFS was then extracted by subtraction of a line fit through the data above ~6600 eV and extrapolated back through the edge region. EXAFS spectra were then

converted from energy (eV) to photoelectron wave vector (k -space, $\text{\AA}^{-1}$) using the equation:

$$k = (2\pi/h)[2m_e(E-E_0)]^{1/2} \quad (2-1)$$

where h is Planck's constant, m_e is the mass of the electron, E is the X-ray energy, and E_0 is the ionization threshold, which was chosen as 6563.0 eV for all spectra (a point near the peak of the edge). All spectra were weighted by k^3 and Fourier transforms were calculated from k -space spectra truncated to zero crossings in the data (~ 3.6 - $11.2 \text{ \AA}^{-1}$) to minimize apodization distortions. It was found that the removal of a two-domain spline in energy space was most effective in reducing the amplitude of background peaks at $R' < 1 \text{ \AA}$ in these Fourier transforms, and the data presented in Chapter 3 have been processed in this manner with no further background removal in k -space. The relationship between the energy-space spectrum and the extracted k -space spectrum is shown graphically in Figure 2.2-A.

Fourier isolation is commonly used to help simplify curve fitting of EXAFS data, allowing analysis of the contribution of single peaks. The isolation process, however, can introduce distortions in the extracted k -space data, especially at the start and end of the spectrum (Teo, 1986). Closely spaced Fourier peaks can also be distorted if isolated individually. There is a tradeoff in this however for a small peak next to a larger one. If the peaks are isolated together then the spectrum is dominated by the contribution from the larger peak, and the structural information in the smaller peak

Figure 2.2. A graphical display of the data analysis process. The complete, unprocessed, X-ray absorption spectrum is shown in panel A; the extracted k -space spectrum is shown in the inset. Dashed lines indicate the relationship between the two spectra. The Fourier transform of the k -space data in the inset of panel A is shown in panel B. The peaks can be isolated and back-transformed to yield simplified k -space spectra which contain only the frequencies contained in the individual peaks (inset). Dashed arrows indicate the k -space spectra derived from each peak.

can be difficult to extract reliably. If the peaks are isolated individually then the isolation process could greatly distort the smaller peak, as well as the resulting curve-fitting analysis. For analysis of the data presented here, Fourier peaks were isolated individually and in pairs to help simplify the spectra and to minimize the effects of distortions due to Fourier isolation (Zhang et al., 1988). Ranges of the Fourier transforms were isolated for analysis by application of a Hamming window function (applied to the first and last 15% of the range, the middle 70% left unmodified) to the transform. The Fourier filtering process is illustrated in Figure 2.2-B, where the inset figure displays the isolated k -space oscillations for each of the peaks in the transform.

Curve-fitting of EXAFS Data

The Fourier transform shows an approximate radial distribution function of scatterers around the absorbing atom, but the distances at which the peaks appear is lower than the true distance of the scatterers owing to a phase shift caused by the effect of the electron traversing the potentials of the absorbing and backscattering atoms. Curve-fitting of the EXAFS data is required to determine correct distances and coordination numbers.

The following equation was used to calculate the EXAFS ($\chi(k)$):

$$\chi(k) = S_0^2 \sum_i N_i B_i(k) \frac{|f_{eff}(\pi, k, R_i)|}{k R_i^2} e^{-2\sigma_i^2 k^2} e^{-2R_i/\lambda(k)} \sin[2kR_i + 2\delta_i^c(k) + \phi_i(k)]$$

(2-2)

where for each shell i , N is the number of scatterers at distance R , S_0^2 is an overall amplitude reduction factor, $B(k)$ is an amplitude reduction factor caused by inelastic losses in the central atom, $f_{eff}(\pi, k, R)$ is the effective backscattering amplitude of the scattering atom, $\delta^c(k)$ and $\phi(k)$ are phase shifts for the absorber and backscatterer respectively, σ^2 is a Debye-Waller term, and $\lambda(k)$ is the mean-free-path of the photoelectron. Coordination numbers (N) are calculated on a per Mn basis and are interpreted here in the context of a total of four Mn atoms/PS II. This means that a single Mn-scatterer interaction in the complex would result in a coordination number of 0.25. More scatterers would appear in multiples of 0.25. In the case of Mn-Mn interactions though, one Mn-Mn interaction would give a minimum coordination number of 0.5 since both the Mn would "see" each other at the same distance. More Mn-Mn interactions at the same distance would give multiples of 0.5 for the coordination number.

As can be seen in equation 2-2, curve-fitting of EXAFS spectra requires knowledge of the backscattering amplitude of scattering atoms and the phase shifts of the photoelectron caused by the potentials of the absorbing and scattering atoms. These parameters can be obtained either empirically, by extracting them from the EXAFS spectra of compounds of known structure, or they can be derived via *ab initio* calculations. The quality of these EXAFS standards is crucial to obtaining accurate information about coordination numbers, distances, and static and thermal disorder effects from EXAFS spectra.

Empirically derived standards (Cramer et al., 1978) have the inherent advantage that they contain all the effects of chemical environment, allowing calculations which have been shown to give reliably good distance determinations. The phases and amplitudes extracted from EXAFS spectra of "known" compounds also contain the effects of possible distortions of the EXAFS spectrum introduced during the process of data analysis (i.e. background removal, Fourier isolation). If both "known" and unknown spectra are collected and analyzed similarly, these distortions will be present in spectra from both the "known" and "unknown" samples and the effects on determined parameters can thus be minimized.

The empirical procedure requires parameterization of the phase and amplitude functions and then fitting of a compound of known structure, keeping R and N fixed to crystallographically determined values, to determine the parameterization coefficients. Successful extraction of the coefficients requires that the scattering interaction of interest in the EXAFS spectrum of the known compound must be well-separated from other scattering interactions. The structural environment must also be similar between the "known" and "unknown" structures. In practice, standards that meet the above requirements can be extremely difficult to obtain, even for the first coordination shell which is usually the simplest to extract. The problems become much worse for longer distance interactions where there are more closely spaced shells, making Fourier isolation of a single interaction virtually impossible.

The theoretical approach differs from the empirical one in that phase shift and scattering amplitude information is calculated from

electron-atom scattering theory. This method was first described by Teo and Lee (1979). Calculating phase and amplitude information from theory has the advantage that there is no need to depend on a crystallographically characterized standard structure. The information that can be obtained, however, is critically dependent upon the quality of the theoretical calculation.

Some key elements in the theoretical calculations appear to be the construction of the potentials that the photoelectron must traverse in the excited and backscattering atoms and the treatment of the photoelectron as a plane or curved wave. The $1s$ initial state is relatively well understood, but the excited state of the absorbing atom has a core hole, which can either be described simply as a ground state of a $Z+1$ atom, or more exactly as an excited state which can relax. Relaxation of the excited state results in a decrease in the lifetime of the excited state and is considered a "many body" effect leading to a decrease in EXAFS amplitudes. The plane wave approximation is useful for higher energies, but breaks down at low photoelectron energies (closer to the edge). (A discussion of EXAFS theoretical calculations can be found in Stern, 1988).

The calculation strategy employed by Teo and Lee (1979) was based on an electron-atom scattering theory introduced by Lee and Beni (1977). The phase and amplitude calculations they performed took into account many body effects in the central atom, but treated the photoelectron as a plane-wave instead of the more exact curved wave. Inelastic losses outside the central atom were not calculated. The result of their work was a series of tables of scattering

amplitudes and phase shifts for roughly half the atoms in the periodic table.

The fitting procedure using Teo and Lee parameters employed a simplified version of equation 2-2. The mean-free-path term and the central atom amplitude reduction factor were ignored (effectively combining them with the Debye-Waller term). To obtain accurate distance information using the tables of Teo and Lee it was necessary to use the energy reference, E_0 , as a free parameter in each scattering shell. The variable E_0 (ΔE_0) compensated for inaccuracies in the theory and, in effect, had no direct physical interpretation. Also, the accurate determination of coordination numbers required calibration of a multiplicative factor by fitting compounds of known structure.

Improved tables of phase and amplitude information came from the work of McKale et al. (1988) where full curved-wave calculations were performed. The treatment of the photoelectron as a curved, rather than plane, wave improved the ability of the theory to describe the EXAFS at lower photoelectron energies, effectively extending the range of data available for analysis. In addition, the curved wave calculations included a distance dependence; thus phase and amplitude information was given in tables for atoms at two distances (2.5 and 4.0 Å) and the data could be interpolated to allow calculations for any particular distance. Again, use of these tabulated values requires a variable energy reference for each shell, which has no direct physical interpretation. In addition, the calculations of McKale et al. (1988) are based on ground state potentials and ignore the many body effects in the central atom.

Recent improvements in theory, computer calculation strategies and speed have resulted in the development of computer programs which can be used by non-theoreticians to calculate EXAFS phase and amplitude information. These programs, titled FEFF (after the effective backscattering amplitude, f_{eff}) were developed by J. J. Rehr and co-workers (Rehr et al. 1991; Rehr et al., 1992). The most recent version in use in our lab is FEFF 5 (version 5.05).

FEFF 5 represents an important advance over previous tabulated values in a number of areas. Its features include:

- 1) The calculation is based on a proposed structural model (or crystal structure coordinates) and potentials are calculated taking into account the coordination environment of the absorbing atom. This allows the user to calculate fitting parameters based specifically on a proposed structure, or structural element.

- 2) Many body effects are calculated based on the structural model, including the mean-free-path, $\lambda(k)$. The explicit calculation of mean-free-path allows a more reliable estimate of Debye-Waller parameters.

- 3) An exact treatment of curved wave effects is employed, which results in improved reliability of the parameters at lower values of k . ($<3.5 \text{ \AA}^{-1}$).

- 4) A well defined energy reference, E_0 , is calculated. The freely floating E_0 required when using the tables provided by Teo and Lee (1979) and McKale et al. (1988) can be restricted in range, or allowed to vary as a single parameter to describe all shells in a fit (O'Day et al., 1994)

5) Multiple scattering contributions are calculated and the relative contributions of all scattering paths within the structural model are estimated. Multiple scattering refers to scattering pathways where more than one scatterer is involved. Typically this occurs when there are rigid ligands present in the structure, such as histidine rings. The presence of multiple scattering can greatly complicate a spectrum at longer distances ($R \geq 3 \text{ \AA}$) and is typically strongest at low photoelectron energies. With FEFF 5 a proposed structural model can be evaluated for significant multiple scattering pathways, and the calculated parameters can be used in curve-fitting.

The input requirement for FEFF 5 is the structural coordinates of a model, either proposed or derived from crystal structure determinations. The simplest model is a pair of atoms; an absorbing atom and a scattering atom at a set distance. More complicated models can be constructed which more closely approximate real structural environments by including nearest neighbor atoms or even longer distance interactions. The best model is based on complete crystal structure coordinates which is, of course, impossible for unknown structures.

To determine the best model to use for simulations of Mn EXAFS in PS II, the results of calculations based on a simple pair model and a more complicated model (containing first coordination shells and longer distance interactions) were compared to calculations based on a crystal structure of a Mn complex referred to here as the dipicolinate dimer ($\text{Mn}_2\text{O}_2(\text{picolinate})_4 \cdot \text{MeCN}$; Libby et al., 1989). The intermediate model tested (the Klein-Sauer, or KS

Model) was based on the structural model proposed for the Mn cluster in PS II (Yachandra et al., 1993) and is illustrated in Figure 2.3. To facilitate calculations on variations of this model, (different Mn-O or Mn-Mn distances or angles) a VAX-Fortran program was written (KSMODEL) which allows the user to set Mn-Mn distances, Mn-O bridging and terminal ligand distances, and angles between the two 2.7 Å dimer units. KSMODEL outputs two files; an input file in the correct format for input to FEFF 5, and a coordinate file for use with chemical structure display programs (i.e. CSC Chem3D Plus, Cambridge Scientific Computing, Inc.)

The effect of the different coordination environment on the calculation of a FEFF 5-calculated backscattering amplitude (f_{eff}) for a Mn-Mn 2.7 Å interaction is illustrated in Figure 2.4. The amplitude function calculated from a simple pair of atoms is quite different from either the KS Model or dipicolinate dimer-based result, especially at low values of k (0-5 Å⁻¹). Comparison of other model structures and other crystal structure-based calculations indicates that inclusion of the first coordination shell in a model yields a more realistic amplitude function. Similar effects are observed for other parameters ($\lambda(k)$, $\delta^c(k)$, $\phi(k)$, $B(k)$) calculated by FEFF 5.

The KS Model structure provides a convenient framework on which to base FEFF 5 calculations, owing to the presence of all the structural elements thought to be in the Mn cluster in PS II (i.e. Mn di- μ -oxo binuclear units). Additional scattering atoms (C, Ca, Sr, etc.) are easily added to the model, allowing calculation of proposed structural elements in the presence of known elements. While the single-scattering EXAFS is relatively insensitive to the exact

Figure 2.3. KS Model structure used for FEFF 5 calculations. This model contains structural elements which are known/proposed to be present in the Mn cluster in PS II. The inclusion of a first coordination shell was found to be important to calculate more realistic fitting parameters.

Figure 2.4. FEFF 5 Amplitude functions calculated for a Mn-Mn 2.7 Å interactions from several models. The top panel was calculated using simply a pair of Mn atoms separated by 2.7 Å. The middle panel was calculated using the KS Model structure shown in Figure 2.3. The lower panel was calculated from the crystal structure coordinates of a di-μ-oxo bridged binuclear Mn compound (Mn₂O₂(picolinate)₄•MeCN; Libby et al., 1989). The amplitude function calculation based on the simple pair of atoms is quite different from the other models where the first coordination shell is present.

FEFF 5 Amplitude Functions

geometrical arrangement of these structural elements, multiple scattering contributions are strongly dependent. No features in the EXAFS spectra from PS II samples have been identified as arising from multiple scattering, and all the fitting presented in Chapter 3 is based on single scattering; but multiple scattering contributions cannot be ruled out. The evaluation of potential models for the Mn-cluster and data fitting using FEFF 5 calculations is an ongoing endeavor, and the work presented here should be regarded as a starting point for more detailed exploration.

Curve-fitting using FEFF 5 parameters and the complete form of equation 2-2 was implemented in the VAX-Fortran program FEFFIT, and is based on the program EXFIT (Goodin, 1983).

For the curve-fitting presented in Chapter 3, FEFF 5 parameters ($f_{eff}(\pi, k, R_i)$, $B(k)$, $\delta^c(k)$, $\phi(k)$, $\lambda(k)$) were calculated from the KS Model structure. Also included in the model were Mn-Ca, Mn-Sr, or Mn-C interactions (calculated for a distance range of 3.0-3.6 Å) depending on which were needed for curve fitting.

The use of FEFF 5 calculated parameters allowed use of a further simplification in that a single ΔE_0 value was used in multishell fits. It has been shown by O'Day et al. (1994), that in fitting the EXAFS from inorganic complexes with FEFF 5-calculated parameters they were able to use the ΔE_0 value obtained from fits to the first shell in fits to higher shells and obtain correct distance information. In our case, the first peak is not fit well enough to allow a confident estimation of the ΔE_0 parameter for use in fitting longer distance interactions. What we have chosen to do instead is to limit each fit to a single ΔE_0 parameter for all shells.

Curve-fitting Error Statistics

The quality of the fits presented here was evaluated using the normalized error sum, Φ , given by the equation:

$$\Phi = \sum_i^N (1/s_i^2)(\chi^{exp tl}(k_i) - \chi^{theor}(k_i))^2 \quad (2-3)$$

where N is the number of data points and s_i is defined as

$$1/s_i = k_i^3 / \sum_j^N k_j^3 |\chi_j^{exp tl}(k_j)| \quad (2-4)$$

It is important to note that the Φ value is not a true chi square parameter because the standard deviations of the data points are not included. The normalization (division by s_i) does allow comparison of overall fit quality between data sets of varying EXAFS amplitude.

The ε^2 error takes into account the number of variable fit parameters (p) and the number of independent data points (N_{ind}):

$$\varepsilon^2 = [N_{ind} / (N_{ind} - p)] N^{-1} \Phi \quad (2-5)$$

The number of independent data points is estimated to be equal to $2\Delta k \Delta R/p$, where Δk is the k -range of the data used and ΔR is the width of the Fourier-filtered peak (for details see Bunker et al., 1991 and Binsted et al., 1992). A negative value for ε^2 indicates that the fit is underdetermined and the fit solution is generally considered not to be unique. The ε^2 value allows one to distinguish whether the inclusion of additional shells in a fit (thus increasing the number of free parameters p) actually improves the quality of the fit by explicitly accounting for the degrees of freedom. It is important to note that the limits on numbers of parameters is a mathematical limitation on the number of freely floating parameters which can be uniquely determined based on the information content of the EXAFS

data. One way to deal with complicated data, such as those from the Mn-cluster of PS II, is to propose chemically reasonable structural models and evaluate the data within that framework. A model-based approach (where certain parameters (e.g. N , σ^2) were kept fixed) was adopted for several of the multi-shell fits in Chapter 3, resulting in the same (or even fewer) number of free-floating parameters as fits with fewer shells. These fits are specifically model dependent, and this should be kept in mind when reviewing fit error parameters.

Chapter 3

Evidence for the Proximity of Calcium to the Manganese Cluster of Photosystem II: Determination by X-ray Absorption Spectroscopy

Introduction

Calcium has been shown (in addition to manganese and chloride) to be an essential co-factor in oxygen evolution (reviewed in Yocum, 1991; Debus, 1992). Most commonly, treatments with high NaCl concentrations (1.2 M) or with a low pH (3.0)/citrate incubation have been employed to deplete calcium from PS II. These treatments produce preparations in which oxygen evolution is inhibited, but which can be reactivated by the addition of Ca^{2+} . Partial reactivation of oxygen evolution in inhibited preparations can also be achieved by addition of Sr^{2+} (Ghanotakis et al., 1984) or vanadyl ion (VO^{2+}) (Lockett et al., 1990). Although many other metal ions have been shown to compete with Ca^{2+} for binding sites in PS II (Na^+ , K^+ , Cd^{2+} , various lanthanides), none of them results in reactivation of oxygen evolution activity (Yocum, 1991; Debus, 1992).

Addition of Sr^{2+} to calcium-depleted preparations has been shown to reactivate the same number of centers as Ca^{2+} , but with slower turnover in the S-state cycle, producing a lower overall rate of oxygen evolution at saturating light intensities (Boussac & Rutherford, 1988a). Reactivation with Sr^{2+} also produces an altered manganese complex, as evidenced by changes in the S_2 -state multiline EPR signal (Boussac & Rutherford, 1988a,b). The multiline signal produced in Sr^{2+} -reactivated preparations has a narrower

average line spacing (~71 G vs. ~88 G in untreated preparations) and a different pattern of intensities compared to the normal multiline signal from untreated PS II preparations.

The stoichiometry of Ca^{2+} in PS II has been a matter of some confusion, although most results seem to point to there being 2 Ca^{2+} /PS II reaction center: one high-affinity binding site which requires extreme conditions for removal and a second, lower-affinity site from which Ca^{2+} can be removed by the NaCl or low pH treatments discussed above. (For discussions of this point see Debus, 1992; Rutherford, 1992; Yocum, 1991.) Specifically, for the low pH/citrate treatment, it has been reported that one of two Ca^{2+} /PS II is removed (Ono and Inoue, 1988).

The effects on the S_2 -state multiline EPR signal of reactivation with Sr^{2+} and the experiments linking calcium reconstitution with reactivation of activity in Ca-depleted preparations have fueled speculation that there is a Ca-binding site closely associated with the Mn cluster in PS II. A particularly interesting result supporting this view is the observation the Ca binding affinity for PS II varies with the redox state (S-state) of the Mn complex; Ca is most labile in the S_3 -state, less so in S_2 and S_0 , and least in the S_1 -state (Boussac and Rutherford, 1988c).

Previous results from EXAFS on PS II preparations (reviewed in Sauer et al., 1992) have indicated that the structure contains a Mn-Mn distance of 2.7 Å, which is a characteristic distance in di- μ -oxo bridged Mn model complexes (Wieghardt, 1989; Pecoraro, 1992). Consistent with this result, short bonds to oxygen/nitrogen atoms of 1.8 Å have also been detected which are also characteristic of

distances to bridging oxo-groups in model complexes. A longer distance interaction has also been detected, but the interpretations differ. Among them are: 1) a single Mn-Mn distance of 3.3 Å (George et al., 1989; DeRose et al., 1994); 2) a Mn-Mn or Mn-Ca at 3.3 Å (Penner-Hahn et al., 1990); 3) Mn-Mn and Mn-Ca at ~3.3 Å (Yachandra et al., 1993; DeRose et al., 1994); and 4) a single Mn-Ca at 3.7 Å (MacLachlan et al., 1992).

We have published potential models for the OEC based on a Mn-Mn interaction at 3.3 Å, but at the time were unable to make a full determination concerning Ca contributions at that distance (DeRose et al., 1994).

In this chapter, the results of experiments using XAS spectroscopy to determine the effects of replacement of Ca by Sr on the structure of the Mn complex of PS II are described. The dominant effect is an increase in the amplitude of the third Fourier peak in the EXAFS of PS II reactivated with Sr^{2+} relative to that of PS II preparations reactivated with Ca^{2+} . Simulations of the EXAFS and structural constraints favor a model in which there are both Mn-Mn and Mn-Ca/Sr pairs contributing to the third Fourier transform peak in the EXAFS. The Mn-Ca/Sr distance is indicative of an uniquely close association of Ca and Mn. Possible structural arrangements of Ca and Mn are discussed.

Materials and Methods

Ca-depleted and Ca- and Sr-reactivated samples were prepared and characterized by EPR spectroscopy as described in Chapter 2.

Mn X-ray absorption edge spectra were collected at Stanford Synchrotron Radiation Laboratory (SSRL) on beam line IV-2 using a Si(220) double-crystal monochromator. Mn K-edge data were recorded as fluorescence excitation spectra (Jaklevic et al., 1977) using a lithium-drifted silicon solid-state detector (described in Guiles et al., 1990).

EXAFS spectra were collected on beamline X-9A at the National Synchrotron Light Source, Brookhaven National Laboratory, and at SSRL on beamlines VII-3 and IV-2. All EXAFS spectra were collected using a 13 element solid-state Ge detector (Cramer et al., 1988).

Curve-fitting of EXAFS data was done as described in Chapter 2. Both tabulated values (McKale et al., 1988) and calculated values using the program FEFF 5 (version 5.05, Rehr et al., 1992) were employed. Only fit results using FEFF 5-calculated parameters are presented here, although similar information was obtained from fits using the McKale tables.

Results

EPR Spectroscopy

Low temperature EPR spectra from Ca- and Sr-reactivated samples, as well as from a Ca-depleted sample, are presented in Figure 3.1. Each spectrum is the difference between the spectrum after illumination (S_2 -state) and the spectrum from the same dark-adapted (S_1 -state) sample. The spectra displayed in Figure 3.1A show the $g=4$ region. Ca-reactivated samples show no light-induced $g=4$ signal while Sr-reactivated samples do show a small $g=4$ signal. Boussac and Rutherford (1988a,b) have reported similar effects on

Figure 3.1. X-band EPR spectra of Ca- and Sr-reactivated PS II. Also shown is the spectrum of a Ca-depleted sample in a modified S₂-state. Spectra of the g=4 region (A) and the g=2 multiline region (B) are presented. Spectra are the differences between spectra from illuminated samples and the spectra of the same sample prior to illumination (i.e. dark adapted). Samples were illuminated to obtain the S₂ state as described in the text. Spectrometer conditions: microwave frequency 9.21 GHz; microwave power 20mW; modulation amplitude 20 G; temperature 8K. Differences between the samples in the g=4 region are shown in (A), where Sr-reactivation enhances a g=4 signal which is not present in Ca-reactivated or Ca-depleted spectra. Mn multiline spectra are shown with an expanded x-axis in (B) and clearly show the reduced line spacing and different amplitude patterns in the Sr-reactivated sample vs. the Ca-reactivated sample. The Ca-depleted multiline signal has more lines and a smaller line-spacing than do multiline signals from either Ca- or Sr-reactivated samples. The y-scales in both plots are the same allowing direct comparison of the amplitudes of g=4 and g=2 signals.

the $g=4$ region (i.e. an increased $g=4$ signal) in Sr-reactivated samples which had been depleted of Ca using high concentrations of NaCl, chelators, and light. The Ca-depleted sample also shows a signal in the $g=4$ region, but this is due to the disappearance of a signal present in the S_1 -state. This signal has been attributed to an Fe(III)-citrate complex formed in less than 10% of PS II centers during the low-pH Ca-depletion treatment and is associated with the acceptor side of PS II (Tso et al., 1991).

Manganese multiline signals are displayed on an expanded x-scale in Figure 3.1B (both A and B are plotted on the same y-scale). The multiline signal observed in Ca-reactivated PS II is identical to that found in untreated PS II preparations, and in Sr-reactivated PS II a modified multiline signal is observed with changes in overall line spacings (~ 73 G vs. ~ 88 G in Ca-reactivated PS II) and intensities similar to those reported for Sr-reactivated preparations (Boussac & Rutherford, 1988a,b; Ono & Inoue, 1989). For comparison, a spectrum from a Ca-depleted sample made before Ca or Sr reactivation is presented. The Ca depleted multiline has a greater number of resolvable lines, an even smaller line-spacing, and is stable for long periods of time in the dark at 0°C . The conditions for its generation, were the same as reported previously for these preparations (Boussac et al., 1989; Sivaraja et al., 1989). Illumination of the Ca-depleted sample at 200K induced no "normal" multiline signal (data not shown). The absence of a light-induced multiline signal after illumination at 200K is in accord with the reported characteristics of these preparations (Ono & Inoue, 1989) and is evidence of the completeness of inhibition and the lack of

contaminating Ca at a concentration high enough to reactivate a detectable number of the Mn centers to a Ca sufficient/"normal" multiline state.

Mn K-Edge Spectroscopy

Mn X-ray K-edge spectra for Ca- and Sr-reactivated samples in the S_1 -state are presented in Figure 3.2. The two edges are almost superimposable; the edge inflection energies, taken from the second-derivative zero crossing, are within 0.2 eV of each other, with the edge in Sr-reactivated PS II slightly higher than in Ca-reactivated PS II. The second derivative spectra show that the overall edge shapes are also quite similar (Figure 3.2B); furthermore, no differences in shape or edge inflection energy are found between these samples and control samples of untreated PS II in the S_1 -state (also displayed in Figure 3.2). The similarity of the edges provides evidence that there has been no significant change in the oxidation state or ligand environment/symmetry of the Mn cluster in Ca- or Sr-reactivated PS II relative to untreated preparations. The observation that the edges are similar is also significant in that the modifications to the multiline signal and the lower rates of oxygen evolution in Sr-reactivated preparations appear not to arise from a very different environment or oxidation level for the Mn ions in PS II.

Mn EXAFS of Ca- and Sr-reactivated Preparations in the S_1 -state

Background-subtracted EXAFS oscillations in k -space for Ca-reactivated PS II and Sr-reactivated PS II (data multiplied by k^3) are presented in Figure 3.3. Both data sets are of comparable signal-to-

Figure 3.2. Mn K-Edge X-ray absorption spectra of Ca- (solid line) and Sr-reactivated (dashed line) PS II in the S_1 -state. Absorption spectra are presented in (A), second derivatives in (B). The edges from Ca- and Sr-reactivated samples are virtually identical to each other and also to untreated PS II samples. The shapes of the edges are accentuated in the second derivative spectra (B) where, again, all three types of samples are shown to give similar spectra.

Figure 3.3. Background-subtracted k -space EXAFS data from Ca- and Sr-reactivated PS II. These data have been multiplied by k^3 . Differences in the EXAFS between Ca- and Sr-reactivated samples are clear at $k=9.3$ and $11\text{\AA}^{-1}$.

noise and are similar except for small differences in EXAFS position and amplitude at about 9.3 and 11 Å⁻¹. The data are multiplied by k^3 , which offsets the decay of EXAFS amplitudes at higher values of k and emphasizes the contributions of scattering atoms at longer distance and of higher atomic number (Teo, 1986).

All data sets were truncated to $k=11.2$ Å⁻¹ to exclude a glitch which appeared as a spike in the data at about 11.3 Å⁻¹. Glitches are caused by a sudden drop in X-ray intensity and are a property of the particular crystal set (Si(220)) used in the double crystal monochromator.

The Fourier transforms of the k -space data are presented in Figure 3.4. Three peaks appear at $R'=1.3$, 2.3, and 3.0 Å that are above the noise level, and there is possibly a fourth peak at about 3.8 Å. The peaks appear at an apparent distance (R') that is shorter than the actual distances in the complex owing to a phase shift caused by the effect of the potentials of the absorbing and scattering atoms on the photoelectron. Actual distances are determined through curve-fitting of the EXAFS oscillations. The good signal-to-noise of the data is reflected by the absence of major peaks in the Fourier transform in the $R'=5-10$ Å region. The small peak at an apparent distance of 0.8 Å is a result of incomplete background removal.

The three peaks and the apparent distances at which they appear are similar to those reported for low temperature Mn EXAFS from a variety of PS II preparations (Penner-Hahn et al., 1990; George et al., 1989; Sauer et al., 1992; Yachandra et al., 1993; Mukerji et al., 1994; DeRose et al., 1994). It is generally agreed that the first

Figure 3.4. Fourier transform power spectra of *k*-space data presented in Fig. 3.3. The major Fourier peaks are labeled I, II, and III. The spectra are clearly different in the amplitude of peak III, which is enhanced in the spectrum from the Sr-reactivated sample.

peak corresponds to oxygen or nitrogen scatterers at about $R=1.8-2.1$ Å, the second peak is due to Mn at about 2.7 Å, and the third peak is due to scatterers at distances >3 Å. The most obvious difference in these data is a change in amplitude of Peak III in the Sr-reactivated sample vs. the Ca-reactivated sample.

Several different background removal techniques have been employed (splines in energy or k -space), and different ranges of k -space data have been used for Fourier transformation. The same pattern of peaks in the Fourier transform is found, including the change in amplitude of peak III between Ca- and Sr-reactivated PS II. This effect is thus unlikely to arise from truncation artifacts or background removal procedures and corresponds to real differences between the data sets.

The change in the amplitude of the peak III has been observed in three other data sets acquired on the same type of samples (data not shown). Of five Sr-reactivated samples, only one has shown no significant increase in the third peak amplitude. One of the four which displayed an increase in peak III was not used for curve-fitting owing to the presence of crystal glitches which were difficult to remove from the spectrum.

Curve-fitting of Mn EXAFS Data

Curve-fitting results for the EXAFS data from five samples, two Ca-reactivated samples (Ca1, Ca2) and three Sr-reactivated (Sr1, Sr2, Sr3) are presented in Tables 3.1-3.5.

Peaks I and II

The first and second Fourier peaks were isolated together and fit as a combination of two or three shells of scatterers. The results of the curve-fitting procedure for these two peaks are presented in Table 3.1. Overall the results are similar to those presented previously (Sauer et al., 1992; Penner-Hahn et al., 1990; George et al., 1989; MacLachlan et al., 1992; Yachandra et al., 1993; DeRose et al., 1994). Two shells of scatterers were needed to fit the data: a shell of about two O or N atoms at 1.83-1.85 Å and a shell of about 1.0 Mn at about 2.7 Å. Unlike our previous work with native samples (Sauer et al., 1992; Yachandra et al., 1993, DeRose et al., 1994) adding a disordered third shell of O or N at 1.9-2.1 Å does not significantly improve the simulation for these reconstituted samples. The first peak thus appears to be dominated by an O or N 1.83-1.85 Å interaction.

Fitting parameters for Fourier peak II alone are presented in Table 3.2 and agree well with our earlier results on untreated PS II where the peak was fit as a shell of ~1.2 Mn at ~2.7 Å (Sauer et al., 1992; Yachandra et al., 1993, DeRose et al., 1994). There is a small, consistent difference in the distance of the Mn-Mn ~2.7 Å interaction between Ca- and Sr-reactivated PS II. Ca-reactivated samples fit to a slightly longer distance (0.01-0.02 Å longer) than Sr-reactivated samples. This slightly longer distance in Ca-reactivated samples was found in fits to isolates of peak II, peaks I and II together, and peaks II and III together, although the exact distance determined in each fit differs somewhat depending upon the type of fit (peaks I and II, peak II alone, or peaks II and III). This difference, although within

Table 3.1: Two-shell simulations of Fourier Peaks I and II ^a

<u>Mn-O Interaction</u>				<u>Mn-Mn Interaction</u>					
Sample ^b	R(1)(Å)	N(1)	$\sigma^2(1)^c$	R(2)(Å)	N(2)	$\sigma^2(2)$	ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
Ca1	1.83	2.62	0.0051	2.70	1.04	0.0020	-22	0.53	4.22
Ca2	1.83	1.90	0.0037	2.70	0.95	0.0020	-22	0.53	4.16
Sr1	1.83	2.55	0.0056	2.70	0.93	0.0016	-23	0.29	2.28
Sr2	1.83	2.10	0.0036	2.69	0.88	0.0020	-24	0.27	2.14
Sr3	1.85	2.66	0.0051	2.70	0.94	0.0012	-21	0.28	2.23

^a Fit parameters are as defined in the text. $S_0^2 = 0.85$ in all fits. Data were fit from $k=4.5-10.5 \text{ \AA}^{-1}$. The width of the Fourier isolation window employed was $2 \text{ \AA}$. ^bCa1 and Ca2 are Ca-reactivated samples; Sr1, Sr2 and Sr3 are Sr-reactivated samples. ^cUnits are $\text{\AA}^2$. ^dGoodness of fit parameters Φ and ϵ^2 are defined in Chapter 2; Materials and Methods.

Table 3.2 : One-shell simulations of Fourier Peak II^a

Sample ^b	<u>Mn-Mn</u>		σ^2 (Å ²) ^c	ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
	R(Å)	N				
Ca1	2.74	1.00	0.002	-15	0.23	0.71
Ca2	2.74	0.93	0.002	-14	0.13	0.39
Sr1	2.72	1.00	0.002	-17	0.19	0.59
Sr2	2.72	0.84	0.002	-18	0.17	0.53
Sr3	2.72	0.95	0.002	-18	0.20	0.61

^aFit parameters are as defined in the text. $S_0^2 = 0.85$ in all fits. Data were fit from $k=4.5-10.5$ Å⁻¹. The width of the Fourier isolation window employed was 1.0 Å. ^bSamples as defined in Table 3.1. ^cParameter is fixed in the fit. Units are Å². ^dGoodness of fit parameters Φ and ϵ^2 are defined in Chapter 2: Materials and Methods.

the experimental error for each individual fit, is consistently observed and probably corresponds to a real difference in the data.

Peak III

The major difference observed in the EXAFS from Ca and Sr-reactivated samples is the apparent difference in amplitude of peak III in the Fourier transforms (see Figure 3.4). In fitting peak III possible models for the Mn-cluster were evaluated, depending not only on the mathematical quality of the fit, but also on the ability of the models and fit parameters to provide a chemically reasonable physical description of the observed differences between Ca- and Sr-reactivated samples. Fits to peak III are difficult because the third shell contributes a relatively small portion of the total EXAFS and arises from scatterers at a distance (about 3.2-3.5 Å) where there are many possible contributions from low-Z atoms. Glutamate and aspartate residues, which are likely components of the ligand environment for the Mn cluster in PS II, have a number of C and O atoms either two or three bonds away from the liganding atom which would fall in the range 3-3.5 Å. Multiple contributions to a single Fourier peak complicate the fitting and result in greater uncertainties in the fit parameters (Scott and Eidness, 1988). The quality of fits to the third shell is also proportionately more susceptible to the noise level in the data.

It should also be noted that Fourier isolation of such a small feature as peak III can cause distortions owing to the limited range of data used in the isolation (about 0.8 Å) and the presence of a much larger neighboring peak (the Mn-Mn 2.7 Å interaction).

Isolation of a larger range of data can help to reduce distortions, but use of a range including the larger Mn-Mn 2.7 Å interaction results in simulations that are less sensitive to the composition of peak III. The fits presented here are of Fourier isolates of peaks II and III together, although fits have been performed on peak III alone with qualitatively similar results.

Peak III- Single Shell Fits

In Table 3.3 the simplest models are considered for the identity of scatterers comprising peak III: models where a single type of scatterer is present. For each type of fit, Debye-Waller factors (σ^2) were fixed to reasonable values; thus allowing comparisons of coordination numbers between samples. The parameters describing peak II (considered to be a Mn-Mn ~2.7 Å interaction) in each fit are relatively constant for each type of scatterer considered for peak III. In each case, the peak II fit values indicate a slightly shorter distance in Sr-reactivated samples (by 0.01-0.02 Å) which is consistent with the results observed in fits to peak II alone, although the absolute fit distances differ somewhat depending on the scatterer chosen for peak III.

The best single-shell fits to peak III for Ca-reactivated samples are obtained using a Mn shell at about 3.31 Å (Table 3.3, fits [A]), although equivalent fits to the data can also be obtained with a Ca shell at ~3.37 Å (Table 3.3, fits [B]). Samples containing Sr, however, show somewhat better fit quality for a single shell of Sr (Table 3.3, fits [C]), than for fits to Ca or Mn (Φ values, Table 3.3). Finally, all samples were fit to C at about ~3.32 Å; both a well-ordered shell

Table 3.3: Two-shell simulations of Fourier Peaks II and III ^a

[A]

<u>Mn-Mn Interaction</u>				<u>Mn-Mn Interaction</u>				ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
Sample ^b	R(1)(Å)	N(1)	$\sigma^2(1)^c$	R(2)(Å)	N(2)	$\sigma^2(2)^c$				
Ca1	2.75	1.18	0.003	3.31	0.48	0.004	-12	0.18	0.44	
Ca2	2.76	1.09	0.003	3.32	0.58	0.004	-11	0.13	0.31	
Sr1	2.74	1.18	0.003	3.31	0.89	0.004	-11	0.27	0.66	
Sr2	2.74	0.99	0.003	3.30	0.76	0.004	-13	0.30	0.72	
Sr3	2.74	1.06	0.003	3.29	0.73	0.004	-14	0.33	0.80	

[B]

<u>Mn-Mn Interaction</u>				<u>Mn-Ca Interaction</u>				ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
Sample ^b	R(1)(Å)	N(1)	$\sigma^2(1)^c$	R(2)(Å)	N(2)	$\sigma^2(2)^c$				
Ca1	2.75	1.16	0.003	3.37	0.60	0.004	-12	0.18	0.44	
Ca2	2.75	1.07	0.003	3.38	0.72	0.004	-11	0.13	0.32	
Sr1	2.74	1.15	0.003	3.37	1.09	0.004	-11	0.31	0.75	
Sr2	2.74	0.95	0.003	3.35	0.93	0.004	-13	0.33	0.80	
Sr3	2.73	1.08	0.003	3.34	0.90	0.004	-14	0.36	0.87	

[C]

<u>Mn-Mn Interaction</u>				<u>Mn-Sr Interaction</u>				ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
Sample ^b	R(1)(Å)	N(1)	$\sigma^2(1)^c$	R(2)(Å)	N(2)	$\sigma^2(2)^c$				
Sr1	2.71	1.10	0.003	3.51	0.74	0.004	-18	0.22	0.54	
Sr2	2.71	0.91	0.003	3.49	0.60	0.004	-19	0.29	0.70	
Sr3	2.71	1.04	0.003	3.48	0.60	0.004	-20	0.28	0.67	

[D]

<u>Mn-Mn Interaction</u>				<u>Mn-C Interaction</u>				ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
Sample ^b	R(1)(Å)	N(1)	$\sigma^2(1)^c$	R(2)(Å)	N(2)	$\sigma^2(2)^c$				
Ca1	2.75	1.01	0.002	3.32	2.13	0.002	-11	0.20	0.48	
Ca2	2.76	0.94	0.002	3.33	2.63	0.002	-10	0.14	0.34	
Sr1	2.75	1.01	0.002	3.33	3.91	0.002	-10	0.30	0.73	
Sr2	2.74	0.84	0.002	3.31	3.37	0.002	-12	0.33	0.80	
Sr3	2.73	0.96	0.002	3.29	3.29	0.002	-13	0.33	0.80	

^aFit parameters are as defined in the text. $S_0^2 = 0.85$ in all fits. Data were fit from $k=4.5-10.5 \text{ \AA}^{-1}$. The width of the Fourier isolation window employed was $1.8 \text{ \AA}$. ^bSamples as defined in Table 3.1. ^cParameter is fixed in the fit. Units are $\text{\AA}^2$. ^dGoodness of fit parameters Φ and ϵ^2 are defined in Chapter 2: Materials and Methods.

(Table 3.3, fits [D]) and a disordered shell were considered. Within this model, the well-ordered C shell ($\sigma^2 = 0.002$) fit much better than the disordered C-shell ($\sigma^2 = 0.010$, data not shown). When Debye-Waller factors were allowed to vary for the C-shell, all the σ^2 values went to 0, which is a physically unreasonable result.

Previously, peak III has been proposed to be due primarily to Mn at 3.3 Å (George et al., 1989; DeRose et al., 1994), a Mn or Ca at 3.3 Å (Penner-Hahn et al., 1990), a Ca at about 3.7 Å (MacLachlan et al., 1992), or a Mn and a Ca at about 3.3 Å (Yachandra et al., 1993, DeRose et al., 1994). A Mn-Mn interaction was thought by most authors to be the most likely component, owing both to slightly better fits to the EXAFS of native samples and the fact that similar Mn-Mn distances have been observed in synthetic manganese complexes (Wieghardt et al., 1985; Sheats et al., 1987) where the Mn atoms are bridged by a mono- μ -oxo bridge and one or more bidentate carboxylate ligands.

The fits presented in Table 3.3 must be evaluated taking into account all of the information known about the samples and also keeping in mind chemically reasonable structural models. Although satisfactory fits to the peak II and III isolate can be obtained with two shells (Mn-Mn at ~ 2.7 Å and Mn-Mn/Ca/Sr at 3.3-3.5 Å), it is difficult to explain the differences observed in the amplitude of peak III in Ca- vs Sr-reactivated samples in a chemically reasonable way. For example, the fits to a single shell of Mn imply that reactivation with Sr results in an increase in the Mn-Mn coordination number from ~ 0.5 in Ca-reactivated samples to ~ 0.8 in Sr-reactivated samples (Table 3, fits [A]). One could propose that some of the Mn clusters

have lost the Mn-Mn ~ 3.3 Å interactions in the Ca-reactivated sample relative to the Sr-reactivated sample, but Ca reactivates samples more completely (higher O₂ evolution rates, restoration of "native" multiline EPR signal) than does Sr. It is unlikely that a structural feature of the native complex would be diminished in a Ca-reactivated sample, which is closer in activity and chemical composition to a native sample, relative to a Sr-reactivated sample.

Coordination numbers and Debye-Waller factors within a shell are strongly correlated in EXAFS curve-fitting, and it is also possible to fit both Ca- and Sr-reactivated samples using the same Mn coordination number for the ~ 3.3 Å Mn-Mn interaction. In this case the difference between data sets is reflected in an increased Debye-Waller factor in Ca-reactivated samples relative to Sr-reactivated samples, though the fit quality is not so good. Debye-Waller parameters describe both static and thermal disorder and, because the measurements were taken at low temperature and the basic structure appears to remain intact, increased disorder would have to be a result of an increase in the static term. There is no reason however to suppose that a single Mn-Mn interaction in the Mn cluster in Ca-reactivated PS II would be any more disordered than in Sr-reactivated PS II because, if anything, Ca binds better than Sr and should therefore result in a more homogeneous Mn cluster. Thus, although increased static disorder is a possible explanation of the differences in the data sets, it is difficult to reconcile with a physical model.

Decreases in amplitude of peak III have been observed in spectra from native samples exhibiting the $g=4$ S₂-state EPR signal

(Liang et al., 1994) and in ammonia-treated samples in the S₂-state (Dau et al., 1995). In both cases, the decreases could be ascribed to increased distance heterogeneity in peak III, indicating that a single Mn-Mn interaction is unlikely. A more reasonable scenario to consider, as did Liang et al. (1994), is more than one Mn-Mn interaction at ~3.3 Å (see below).

The same situation of increased coordination number for Sr- vs. Ca-reactivated samples also occurs for fits to Ca and to C. In the case of a Mn-Ca interaction (Table 3.3, [B]), an increased coordination number for Ca in the Sr-reactivated sample (N=0.9-1.09 vs 0.6-0.72 in the Ca-reactivated samples) is a virtual impossibility because the sample had been specifically depleted of Ca and reactivated with a relatively high concentration of high-purity SrCl₂. Again, increased disorder can also be considered, but the fit quality is worse and increased disorder seems unlikely for some of the same reasons as given for a Mn-Mn interaction. In the case of a C-shell, a disordered shell provides a very poor fit to the data (fit parameters not shown). A well-ordered C-shell (Table 3.3, [D]), while not fitting the data so well as Mn or Ca, also results in an increased coordination number for Sr (N=3.3-3.9) vs. Ca-reactivated samples (N=2.1-2.6). Again, there is no satisfactory model to account for the change; in addition, the results are suggestive that there would need to be 2-4 C atoms/Mn in a well defined shell at ~3.4 Å. That would be a total of 8-16 Mn-C interactions with very small distance spread, an extremely unlikely situation in a protein where the coordination environment beyond the first ligand sphere would be expected to be relatively disordered. More likely would be a distributed spread of

C/N/O interactions resulting in a disordered shell(s) that could be smeared out beyond the detectable limits of EXAFS.

The Sr-reactivated samples were also fit to a shell of Sr to test the possibility that the change in amplitude of peak III was a result of an exchange of Sr for Ca (Table 3, fits [C]). The fit quality to a single shell of Sr for Sr-reactivated samples is better than fits to Ca or Mn by 10 to 30% (see Φ values, Table 3.3). The best fits were found at a distance of ~ 3.5 Å with a coordination number comparable to that found in Ca-reactivated samples fit with a Ca-shell ($N=0.6-0.74$). This would correspond to $\sim 2-3$ Mn-Ca or Mn-Sr interactions in the Mn complex and is consistent with a model where Ca is replaced by Sr. The increased fit distance (~ 3.5 Å for Sr vs ~ 3.4 Å for Ca) makes sense in that the ionic radius of Sr is 0.1 Å larger than that of Ca. The relatively large coordination number for Ca/Sr (2-3 Mn-Ca/Sr interactions) implies that there is either a single Ca/Sr site with multiple Mn-Ca/Sr interactions, all at about the same distance, or several sites with the same Mn-Ca/Sr distances. The low-pH treatment employed here to deplete Ca from PS II has been reported to remove only one of the two Ca ions in PS II (Ono & Inoue, 1988); thus, a single site is a more reasonable interpretation. In either case several Mn-Ca/Sr vectors of the same distance would need to be present, which is an unlikely, though still possible, model.

Peak III- Two Shell Fits

Because it is difficult to rationalize the results of the previous fits, more complicated three shell fits (two shells for peak III) to the data were undertaken considering two models; one where both Mn-

Mn and Mn-Ca/Sr interactions are present in peak III (Mn + Ca/Sr model) and one where two Mn-Mn interactions (2 Mn model) are present. The small data range available for fitting limits the number of free parameters allowed in a given fit (see Materials and Methods) and precludes adding shells with more free parameters. In the following fits, to address this problem, coordination numbers and σ^2 values were fixed to allow fitting to specific, chemically reasonable models. The only free parameters were distances and a single ΔE_0 for each fit.

The results of fits for the Mn + Ca/Sr model are presented in Table 3.4. All data sets were fit to shells of Mn at ~ 2.7 and 3.3 Å, and a shell of Ca at 3.3 - 3.5 Å (Table 4, fits [A]). The quality for fits to the Ca-reactivated samples is equal to or better than all of the fits in Table 3 (see Φ values, Table 3.3, fits to Ca1 and Ca2 samples). Greater improvement is seen in the ϵ^2 values for Table 3.4 vs. Table 3.3 fits, the reason being that, although there were more shells being fit, there was actually one less free parameter in the fits in Table 4 than in those in Table 3. The Sr-reactivated samples, when fit to Mn and Ca shells (Table 4, fits [A]), fit to a shorter Ca distance (~ 3.34 Å) than the Ca-reactivated samples (~ 3.46 Å); the EXAFS changes between Ca and Sr-reactivated PS II are reflected in the decreased difference in distance (lessened disorder) between the Ca and Mn shells in the Sr-reactivated samples. Sr-reactivated samples were also fit with a Sr shell instead of a Ca shell (Table 3.4, fits [B]). For all the Sr-reactivated samples, the fit quality was improved (20-25% decrease in Φ) by changing the Ca shell to a Sr shell (Table 3.4). The fit quality for two out of three of the Sr samples was better than the

Table 3.4 : Three-shell simulations of Fourier Peaks II and III, Mn and Ca model.^a

[A]

Sample ^b	<u>Mn-Mn</u>		<u>Mn-Mn</u>		<u>Mn-Ca</u>		ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
	R(1)(Å)	N(1) ^c	R(2)(Å)	N(2) ^c	R(3)(Å)	N(3) ^c			
Ca1	2.75	1.0	3.29	0.5	3.47	0.25	-11	0.18	0.29
Ca2	2.75	1.0	3.30	0.5	3.46	0.25	-11	0.12	0.19
Sr1	2.74	1.0	3.31	0.5	3.36	0.25	-11	0.24	0.38
Sr2	2.74	1.0	3.28	0.5	3.36	0.25	-14	0.43	0.69
Sr3	2.73	1.0	3.29	0.5	3.30	0.25	-14	0.26	0.42

[B]

Sample ^b	<u>Mn-Mn</u>		<u>Mn-Mn</u>		<u>Mn-Sr</u>		ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
	R(1)(Å)	N(1) ^c	R(2)(Å)	N(2) ^c	R(3)(Å)	N(3) ^c			
Sr1	2.73	1.0	3.28	0.5	3.55	0.25	-14	0.18	0.28
Sr2	2.72	1.0	3.25	0.5	3.54	0.25	-17	0.36	0.58
Sr3	2.72	1.0	3.24	0.5	3.53	0.25	-17	0.21	0.33

^a Fit parameters are as defined in the text. $S_0^2 = 0.85$ in all fits. Data were fit from $k=4.5-10.5 \text{ \AA}^{-1}$. The width of the Fourier isolation window employed was $1.8 \text{ \AA}$. For all fits $\sigma^2(1) = 0.002 \text{ \AA}^2$, $\sigma^2(2)=\sigma^2(3)=0.003 \text{ \AA}^2$. ^bSamples are as defined in Table 3.1. ^c Coordination numbers were fixed in the fit. ^dGoodness of fit parameters Φ and ϵ^2 are defined in Chapter 2: Materials and Methods.

Figure 3.5. Fits to the EXAFS of Ca- and Sr-reactivated PS II. Both fits are to Fourier isolates of peaks II and III. The parameters used for the fit are found in Table 3.4. The fit to the Ca-reactivated sample (A) was calculated using the fit values for the Ca1 sample in Table 3.4, fits [A]. The fit to the Sr-reactivated sample was calculated using fit values for the Sr1 sample in Table 3.4, fits [B]. The partial waves due to individual scattering contributions are shown as well as the overall fit (top). The individual Ca and Sr partial waves used in these fits are overplotted (C) and demonstrate the clear differences in phase and amplitude for these two contributions.

best fits in Table 3.3 (fits [C]), decreasing Φ by ~20%. As in the case with the single shell Ca/Sr fits (Table 3.3, [B,C]) the Sr distance is longer (3.54 Å) than the Ca distance (3.46 Å) which fits well with a model where Sr (with a larger ionic radius) has substituted for Ca in a binding site closely associated with the Mn-cluster. Fits based on the Mn + Ca/Sr model are displayed in Figure 3.5A (Ca-reactivated sample) and 3.5B (Sr-reactivated sample). The differences in the EXAFS of the two samples, most apparent in the beat pattern at $k=8 \text{ Å}^{-1}$, are well simulated by the change of one Mn-Ca interaction for a Mn-Sr interaction. The individual EXAFS waves for the Ca contribution in Fig. 3.5A and the Sr contribution in Figure 3.5B are overplotted in Figure 3.5C and show clearly that the waves are different in both phase and amplitude.

The fits to the 2 Mn model are presented in Table 3.5. In this case the possibility was considered that there is more than one Mn-Mn interaction giving rise to peak III. To test this possibility the data were fit using a single Mn shell for peak III with a larger coordination number, fixed at 1.0, corresponding to two Mn-Mn interactions (Table 3.5, fits [A]). In these fits the Debye-Waller parameter for the ~3.3 Å Mn shell was allowed to float and indicated a larger disorder for the Ca- reactivated samples relative to the Sr-reactivated samples by a factor of almost two. In the fits labeled [B] (Table 3.5), the Mn shell at ~3.3 Å was split into two equal Mn-Mn interactions representing the same total number of Mn-Mn interactions at ~3.3 Å as in the [A] fits. The spread of Mn-Mn distances in fits to peak III is slightly greater in the Ca-reactivated samples (0.14 Å) than in the Sr-reactivated samples (0.09 Å),

Table 3.5: Three-shell simulations of Fourier Peaks II and III, Two Mn 3.3Å model.^a

[A]

Sample ^b	<u>Mn-Mn</u>		<u>Mn-Mn</u>		$\sigma^2(2)(\text{\AA}^2)$	ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
	R(1)(Å)	N(1) ^c	R(2)(Å)	N(2) ^c				
Ca1	2.74	1.0	3.29	1.0	0.0103	-12	0.16	0.25
Ca2	2.75	1.0	3.32	1.0	0.0081	-11	0.13	0.20
Sr1	2.74	1.0	3.31	1.0	0.0048	-10	0.24	0.38
Sr2	2.74	1.0	3.28	1.0	0.0054	-14	0.46	0.72
Sr3	2.73	1.0	3.28	1.0	0.0058	-14	0.30	0.47

[B]

Sample ^b	<u>Mn-Mn</u>		<u>Mn-Mn</u>		<u>Mn-Mn</u>		ΔE_0	$\Phi \times 10^3$ ^d	$\epsilon^2 \times 10^5$ ^d
	R(1)(Å)	N(1) ^c	R(2)(Å)	N(2) ^c	R(3)(Å)	N(3) ^c			
Ca1	2.74	1.0	3.37	0.5	3.22	0.5	-12	0.15	0.24
Ca2	2.75	1.0	3.38	0.5	3.26	0.5	-11	0.12	0.19
Sr1	2.74	1.0	3.36	0.5	3.28	0.5	-10	0.24	0.38
Sr2	2.74	1.0	3.33	0.5	3.24	0.5	-14	0.46	0.72
Sr3	2.73	1.0	3.33	0.5	3.23	0.5	-14	0.30	0.47

^a Fit parameters are as defined in the text. $S_0^2 = 0.85$ in all fits. Data were fit from $k=4.5-10.5 \text{ \AA}^{-1}$. The width of the Fourier isolation window employed was 1.8 Å. For [A] fits $\sigma^2(1) = 0.002\text{\AA}^2$, for [B] fits $\sigma^2(1) = 0.002\text{\AA}^2, \sigma^2(2)=\sigma^2(3)=0.003\text{\AA}^2$. ^b Sample identification as in Table 3.1. ^c Coordination numbers were fixed in the fits. ^d Goodness of fit parameters Φ and ϵ^2 are defined in Chapter 2: Materials and Methods.

possibly accounting for the difference in amplitude of peak III in the Fourier transforms. Although these fits do describe another reasonable structural model to explain the differences between the Ca- and Sr-reactivated data sets, the fit quality is no better for two Mn shells (Table 3.5, [B]) than for one disordered Mn shell (Table 3.5, [A]); thus, the data neither support nor disprove this hypothesis. This is in contrast to the Mn+Ca/Sr model in which the fit quality was improved by the addition of the Ca/Sr shell.

Discussion

The results presented above indicate that the dominant effect of reactivation of calcium-depleted preparations with strontium rather than calcium is a change in the environment of the manganese cluster at a distance greater than 3 Å. The data presented here are best explained by the replacement of Ca by Sr in a binding site in close proximity to the Mn-cluster in PS II for the following reasons:

- 1) The Mn K-edge spectra indicate that no major changes have occurred in the oxidation states or ligand environments of the manganese ions in Ca- or Sr-reactivated PS II relative to those in untreated preparations. The similarity of the first two peaks in the EXAFS from calcium- and strontium-reactivated preparations to those in untreated preparations is also an indication that the manganese cluster has been restored to an intact state. The fact that high rates of oxygen evolution in these preparations are restored to the same extent, albeit with altered kinetics, for Sr-reactivated preparations is a further indication that substitution of Sr for Ca results in no major changes in the structure of the complex.

2) EPR spectra of Sr-reactivated samples show an altered multiline signal and an enhanced $g=4$ signal relative to Ca-reactivated samples. These changes clearly indicate an effect directly on the Mn-cluster caused by the substitution of Ca by Sr. The Sr-altered multiline is similar to the multiline observed in ammonia-inhibited PS II where ammonia has been shown to bind directly to manganese (Britt et al., 1989). It has been proposed that the modifications in the EPR spectra are caused by replacement of Ca by Sr in a binding site close to the Mn-complex (Boussac & Rutherford, 1988a,b).

3) Finally, the differences in the EXAFS spectra between the Ca- and Sr-reactivated PS II data sets can be ascribed to a change of Ca to Sr at approximately 3.4 Å from a manganese ion. This is a reasonable proposition given the chemical differences between the two preparations (just the presence of Ca or Sr). The enhanced X-ray photoelectron backscattering cross-section of Sr versus Ca is a plausible explanation of the amplitude change in the third Fourier peak in the EXAFS. The data can be fit with Ca/Sr as the sole contributor to Fourier peak III or with Mn + Ca/Sr contributing to peak III. From the structural analyses we present here, the most likely model is one in which both Mn-Ca and Mn-Mn interactions contribute to peak III.

The differences in the EXAFS between the Ca-reactivated and Sr-reactivated data sets can also be simulated as a change in the number of Mn scatterers contributing to the third peak, but this is difficult to reconcile in a chemically reasonable physical model. More Mn-Mn interactions would probably require a relatively major

change in the structure of the complex, possibly resulting in more oxo-bridges. The difference in coordination number is also not sufficient to account for a stoichiometric increase in Mn-Mn interactions. An alternative explanation is that the difference between data sets arises from a lack of reactivation of centers in Ca-reactivated PS II relative to Sr-reactivated PS II, leading to decreased Mn-Mn coordination numbers in Ca-reactivated PS II, but this is also difficult to reconcile with the fact that reactivation of O₂ evolution is greater in Ca- than in Sr-reactivated PS II, and that Ca reactivates with higher binding affinity and produces a "native"-like multiline EPR signal.

Also considered was a model where two Mn-Mn interactions at slightly different distances contributed to peak III. There was no improvement in fit quality vs. other single-shell fits; thus, a two Mn-Mn interaction model is neither supported nor disproved on the basis of the data presented here.

The above interpretations require a note of caution. Even the most complicated models presented here (peak III comprised of two metal-metal interactions) are simplifications in that they do not include C/N/O contributions which, although almost certainly disordered, must be present in the complex. The fits to these data are also rather severely information-limited owing to the small k -range of the data and the relatively small contribution that peak III makes to the total EXAFS. More complicated models including more metal-metal interactions or large numbers of individual C/N/O shells, although certainly possible, cannot be critically evaluated on the basis of the data presented here. The Mn + Ca model for peak III

does however describe the data well and provides a chemically reasonable explanation for the differences observed in the EXAFS of Ca- and Sr-reactivated samples.

The manganese-calcium distance proposed from the EXAFS results presented here is rather short for a calcium-metal interaction. A calcium binding site at this distance from another metal ion (~ 3.4 Å) has not been reported in other biological systems, but an examination of crystallographically characterized protein calcium binding sites and calcium complexes provides clues as to the structural possibilities which might give rise to such an interaction. Some representative structures containing Ca-metal interactions are shown in Figure 3.6 and are discussed below.

The manganese-calcium binding site of concanavalin A, in which two carboxyl groups bridge between Mn and Ca, has a metal-metal distance of 4.2 Å (Edelman et al., 1972; Hardman & Ainsworth, 1972). A close association between two calcium ions is found in thermolysin, which has a binding site in which two calcium ions are bridged by three carboxyl groups, resulting in a metal-metal distance of 3.8 Å (Matthews et al., 1974). Closer associations have been observed in calcium complexes when two metal atoms share the same oxygen atom. In the calcium salt of garcinia acid, a Ca-Ca distance of 3.6 Å is found where the two Ca are bridged by single oxygen atoms from two carboxyls (Glusker et al., 1971). Finally, Bonadies et al. (1989) have reported a manganese complex containing sodium in which the Mn-Na distance is 3.3 Å. The Mn and Na ions are bridged by one acetate molecule and by two oxygen atoms from phenolate groups. Although Mn is bridged to Na and not

Figure 3.6. Ca-to-metal bridging structures and distances for several structurally characterized systems: a) Concanavalin A, Edelman et al. (1972), Hardman & Ainsworth (1972); b) thermolysin, Matthews et al. (1974); c) garcinia acid, Glusker et al. (1971).

Concanavalin A

Thermolysin

Ca, this structure has relevance because Na has an ionic radius equal to that of Ca. The Ca-Ca structures pictured would probably have shorter metal-metal distances if one of the metals was a high-valent Mn ion (i.e. Mn^{3+} or Mn^{4+}) which can exhibit a range of Mn-bridging O distances from 1.75-1.9 Å (Pecoraro, 1992; Weighardt, 1989).

Given the structures above, it seems probable that a calcium-metal interaction at 3.4-3.5 Å would involve one or more bridging atoms, probably oxygen. Bridging oxygen atoms could be derived from the carboxyl groups of aspartate, glutamate, or phenolate residues, protein backbone carbonyls, or possibly from water or hydroxide. Bidentate carboxyl groups, such as in thermolysin or concanavalin A (Figure 3.6), are also a possibility. Water molecules are often found in the coordination sphere of biological calcium binding sites, and a shared water molecule between calcium and manganese is an interesting prospect. Calcium ions range from 6-8 coordinate in biological systems and could provide a storage site with a flexible coordination number for water molecules used in the catalytic cycle.

Based on the structures discussed above, two possibilities are presented for a Ca-binding site in PS II (Figure 3.7). In the first case (Figure 3.7a) the model is considered where the scatterers at >3 Å in the EXAFS arise from Ca (or Sr) alone. In this case a single site with multiple Mn-Ca(Sr) interactions is likely and Ca is shown bridged to two Mn atoms through single O atoms which could be provided by protein residues or water. The second, more likely, possibility is a model where both Mn-Mn and Mn-Ca(Sr) interactions contribute (Figure 7b). Both a Mn-Ca bridged structure with bridging oxygen

Figure 3.7. Possible structural arrangements for the Ca-binding site in PS II. The bridging structure in a) corresponds to a model where Fourier peak III is due to only Ca (or Sr) scatterers. The structure depicted in b) corresponds to a model where both Mn-Ca and Mn-Mn interactions contribute to peak III. In each case, additional bridges may be present, including bidentate and unidentate carboxylates as well as water, hydroxide, phenolate, etc.

a)

b)

atoms as described above and a Mn-Mn bridged structure are shown. The Mn-Mn structure would possess an oxo-bridge and possibly also one or two bidentate carboxylate bridges (not drawn).

Conclusions

We conclude from this study that the structure of the manganese complex in PS II preparations depleted of Ca and reactivated with Ca is largely unchanged relative to that found in untreated PS II preparations. Reactivation with Sr, however, results in changes in the EXAFS spectra which are consistent with a model in which Ca or Sr ions can occupy a binding site 3.4-3.5 Å from one Mn atom of the manganese complex of PS II. The most likely model based on these data is one in which both Mn-Mn and Mn-Ca interactions give rise to Fourier peak III in the EXAFS collected from these samples. A calcium binding site at this distance from a metal cluster represents an unusually close association for a biological system and may reflect an intimate role for calcium in water oxidation.

Chapter 4

Mn X-ray Absorption K-edge Studies of Ca-depleted PS II in States Corresponding to S₁, S₂, and S₃

Introduction

PS II preparations can be depleted of calcium via several methods (low pH/citrate, NaCl-wash) which result in inhibition of oxygen evolution activity. Activity can be restored by addition of Ca²⁺ or to a lesser extent Sr²⁺ or vanadyl ion (see Chapter 3). The exact characteristics of Ca-depleted preparations (extent of inhibition of oxygen evolution, which S-state transition is blocked) have been the subject of much debate, largely owing to different protocols followed in different groups (see Rutherford et al., 1992). The view which appears to have gained wide acceptance is that Ca-depleted samples are blocked at a formal S₃-state (i.e. Ca-depleted samples can undergo two turnovers from an S₁-equivalent state). The oxidized forms of Ca-depleted PS II preparations have yielded interesting EPR signals which are distinct from those present in native PS II samples.

EPR Signals of Ca-depleted PS II Samples

In samples which have been depleted of calcium in the presence of a chelating agent (citrate, EGTA, EDTA), altered stability of the S₂-state is observed. In these samples the S₂-state is formed by illumination at 273 K with DCMU present to limit the samples to one turnover, or they can be dark adapted at 273 K after

illumination. The S₂-state is stable for long periods of time (hours) at room temperature, and an EPR multiline signal is observed which has a smaller average line spacing of ~55 G vs. 88 G in untreated PS II (Boussac et al., 1989; Sivaraja et al., 1989). The stable multiline signal has been shown to be dependent on the presence of chelators during the Ca-depletion treatment (Boussac et al., 1990a; Ono & Inoue, 1990) although the lineshape and stability of the S₂-state multiline signal do not exhibit any variation depending on chelator (citrate, EGTA, EDTA). In the absence of chelators, a normal multiline signal can be observed.

Illumination of Ca-depleted samples at 277 K, with no restriction on the number of turnovers, results in the formation of a broad EPR signal centered at g=2 (Boussac et al., 1989; Sivaraja et al., 1989). This is a gaussian-shaped signal which is quite similar in shape to a simple radical signal, but is much broader (~160 G in the absence of the extrinsic polypeptides, 130 G with the extrinsic polypeptides bound). A width of <90 G is observed in PS II preparations from *Synechocystis* 6803 which have been washed with a Ca-free medium containing EDTA (Kirilovsky et al., 1992). Similar signals have also been observed in a number of different inhibited preparations. The exact width of the signal differs depending upon the type of treatment used; the largest widths (~230 G) are found in acetate-treated PS II (MacLachlan & Nugent, 1993) while smaller splittings (<160 G) in F⁻-treated (Baumgarten et al., 1990), NH₃-treated (Andréasson & Lindberg, 1992, Hallahan et al., 1992) and Cl⁻-depleted samples (Boussac et al., 1992). These signals have been assigned to modified S₃ states in these inhibited preparations.

The relevance of the S-state label in these inhibited preparations is questionable. Illumination of the samples does advance the donor side of PS II through several oxidation steps, but the preparations do not evolve oxygen and thus do not form an S-state cycle. The S-state notation is useful, however, in specifying the oxidation level of the preparation. In the work presented here, the modified S-states in these inhibited PS II samples are referred to as S_1' , S_2' , and S_3' -states to distinguish them from the S-states in native samples.

The identity of the oxidized species giving rise to the S_3' signal has been a subject of debate. Initially it was proposed to be an organic radical, broadened by a magnetic interaction with the Mn-complex (Boussac et al., 1989). Based on comparisons of optical difference spectra this organic radical was proposed to be histidine (Boussac et al., 1990b). This assignment was challenged by Hallahan et al. (1992) who suggested that the radical was Y_Z, the redox active tyrosine between the OEC and P680 in the electron transfer chain in PS II (see Chapter 1). This proposal was rebutted by Boussac & Rutherford (1992) and more recent FTIR results have also been interpreted as favoring the assignment to histidine (Berthomieu & Boussac, 1995). To date, there has been no conclusive proof as to the identity of the species giving rise to the S_3' signal.

The significance of the S_3' EPR signal has also been questioned in a study using pulsed EPR, where it was indicated that the S_3' signal may arise from less than 25% of centers and that the majority of centers in the S_3' -state are EPR silent (Gilchrist et al., 1992). Zimmermann et al. (1993), however, report in another pulsed EPR

study that the modified multiline signal does not disappear in the S_3' -state, but is instead broadened beyond detection by conventional EPR. The first study (Gilchrist et al. 1992) is consistent with Mn oxidation on the S_2' - S_3' transition while the second study indicates that no Mn oxidation has occurred. The question of Mn oxidation is thus central to resolving this issue.

Two X-ray absorption spectroscopy (XAS) studies have been reported on Ca-depleted preparations (Ono et al., 1993; MacLachlan et al., 1994), both of which indicate that the Mn X-ray K-edge inflection point energy shifts to higher energy on both the S_1' - S_2' and S_2' - S_3' transitions. These results were interpreted as indicative of Mn oxidation in both transitions.

In the work presented here, the question of the state of the Mn-cluster in the modified S-states exhibited in Ca-depleted PS II is addressed. XAS edge spectra were obtained for S_1' , S_2' , and S_3' -state samples. The results indicate that there is no evidence for Mn oxidation on the S_2' to S_3' transition, which is consistent with the view that a protein residue (histidine or tyrosine) is oxidized in this step. The relation of the previous XAS studies to the present work is discussed.

Materials and Methods

Ca-depleted PS II samples were prepared and characterized by EPR spectroscopy as described in Chapter 2.

Mn X-ray absorption edge spectra were collected at SSRL on beamlines IV-2, IV-3, and VII-3 using Si(220) double crystal monochromators. All data were recorded as fluorescence excitation

spectra (Jaklevic et al., 1977) using a 13 element solid-state Ge detector (Cramer et al., 1988).

Results

EPR Spectroscopy

The Ca-depleted PS II preparations can be generated by a number of protocols. Samples which have been created by treatment with a high NaCl concentration (1.2 M NaCl treatment in the light and in the presence of chelators) are initially in the S_2' -state owing to the illumination during the protocol and the enhanced stability of the S_2' -state in the presence of chelators. Low pH-treated preparations (pH 3.0 treatment in darkness with citrate) remain in the S_1' -state and must be advanced to S_2' or S_3' by illumination. The low pH treatment was chosen for the experiments reported here because all three Ca-depleted S-states were accessible in these preparations.

EPR spectra from Ca-depleted samples in the S_2' and S_3' -states are shown in Figure 4.1. The S_2' -state multiline signal (4.1A) is different in appearance from the one displayed in Figure 3.1 (see Chapter 3) because of the presence of a signal from Q_A^- . The sample used for S_2' -state experiments contained the herbicide DCMU which displaces the Q_B quinone and prevents electron transfer beyond Q_A . The Q_A^- -signal appears as a sharp downward feature at ~3700 G. The S_2' -state can be generated either by illumination at 273 K where the samples are limited to one turnover (DCMU) or by dark adaptation of a sample illuminated at 273 K where photoaccumulation of the S_3' -state occurs. The results from the DCMU treated sample demonstrate that the multiline signal arises

Figure 4.1. EPR Spectra from Ca-depleted samples in the S_2' (panel A) and S_3' -states (panel B). The S_2' -state sample was a Ca-depleted sample with DCMU added. The S_3' -state sample had no additions. Both were advanced from the S_1' -state by illumination at 273 K. The multiline spectrum in panel A is displayed on an expanded scale (x4.3) to allow comparison with the higher amplitude S_3' -state signal in panel B.

from a single turnover and thus must arise from a formal S_2 -state (denoted S_2' here). The multiline signals observed in each case are identical except for the underlying signal from Q_A^- in the sample containing DCMU.

In the absence of DCMU, illumination of Ca-depleted samples at 273 K results in a broad (~ 160 G) derivative shaped signal centered at $g=2$ (Figure 4.1B). The appearance of this signal coincides with the loss of the S_2' multiline signal and has been shown by others (Boussac et al., 1990b), in Ca-depleted samples which were advanced with single turnover flashes, to result from a state one step oxidized from the S_2' -state; a formal S_3 -state (S_3'). The S_3' -state is relatively unstable and deactivates quickly to the S_2' -state (half life of 300 ms in the presence of reduced Q_A (Boussac et al., 1990)) and can be difficult to trap quantitatively. Initially, samples for XAS experiments were made in the usual manner where the sample was spread in a thick paste into a lucite sample holder (thickness ~ 1.5 mm), but these samples were difficult to saturate during illumination and displayed significant amounts of S_2' -state multiline signal in addition to the S_3' -signal. It was found that spreading a thin layer (< 0.5 mm) onto the mylar backing of the XAS sample holder resulted in more complete advancement to the S_3' -state. Although complete advancement was never achieved, the amplitude of the S_2' -state multiline signal could be reduced to a very low level.

Quantitative determination of the S-state composition of these samples was not possible, although reasonable estimates could be made. The main problem with quantitations using EPR is that it was not possible to unambiguously generate the S_2' -state in 100% yield.

The largest amount of S_2' -state multiline that could be generated in a sample came from dark adaptation of samples illuminated at 273 K containing nothing to limit multiple turnovers. Addition of an electron acceptor (DCBQ) did not appreciably affect the yield of either the S_3' or S_2' EPR signals. Samples which contained DCMU to limit electron transfer to a single turnover generated approximately 70-100% of the S_2' multiline generated by dark adaptation. The lower yield of S_2' multiline is probably caused by recombination with the electron trapped on Q_A . The degree of advancement to S_3' can then be estimated from the disappearance of the multiline signal. Based on the assumption of 100% S_2' -state in dark adapted S_3' -state samples, the S_3' -state samples used for experiments ranged from 55 to >80% S_3' , while DCMU-treated S_2' -state samples were approximately 70-100% in the S_2' -state. There is no guarantee, however, that the amount of multiline generated by dark adaptation of an S_3' -state sample is equivalent to 100% and, in fact, XAS experiments indicate that this is not the case (see below). Thus the S-state compositions reported here represent best estimates.

X-ray Edge Spectroscopy

Mn K-edge spectra are relatively broad and structural features on the edges are quite subtle. The edge spectra in this chapter are presented as second-derivatives which accentuate the shape of the edge and allow clear comparisons of edge structure (see Figure 4.2). The region in the spectrum from 6545-6555 eV corresponds to the rising part of the edge and has been shown to be sensitive to the oxidation state of the absorbing atom (Yachandra et al., 1993).

Figure 4.2. Mn X-ray K-edge spectrum and second derivative of a Ca-depleted sample in the S_3' -state. Features in the absorption spectrum are accentuated in the second derivative spectrum, where negative peaks correspond to positive features in the edge. The inflection point of the rising portion of the edge is indicated by an arrow and is taken as the zero crossing of the second derivative in this region.

Increased oxidation states create a higher positive charge on a metal ion. A higher energy photon is required to extract an electron from a more positively charged atom resulting in an edge shift to higher energy. Edge energies can also be affected by ligands (by the degree of electron donation) and by the symmetry around the atom which can affect the selection rules and relative energy levels of transitions. The inflection point energy can be determined as the zero crossing of the second derivative which in the spectra presented here appears at ~6551-6553 eV. The region above ~6555 eV is more sensitive to ligand environment (Yachandra et al., 1993) and in fact corresponds to transitions to bound states as well as the start of the EXAFS scattering. The relatively low energy of the photoelectron also means that multiple scattering interactions can dominate in this region and the interpretation of the spectra can be quite difficult.

Second derivative spectra of samples in predominantly the S_1' , S_2' , and S_3' -states are presented in Figure 4.3. The noise level in the second derivative spectra can be estimated from the region before the absorption edge (6530-6537 eV). The S_2' -state sample was generated by illumination of a sample containing DCMU (referred to as S_2' -DCMU). The inflection point energies (IPE) for these samples is reported in Table 4.1. The IPE for the S_1' -state sample (6550.7 eV) is lower than that found in native S_1 -state samples (6551.7 eV; Liang, 1994) and indicates that either there has been a reduction of the complex caused by the Ca-depletion procedure, or a structural change has occurred. This lower edge energy has also been observed by Ono et al. (1991) where it was shown that the edge could be restored to the native S_1 -state energy by reconstitution with Ca in

Table 4.1 Inflection point energies (IPE) of Ca-depleted samples in the S_1' , S_2' , and S_3' states.^a

	IPE (eV)
S_1' -state	6550.7 (0.2) ^b
S_2' -state (DCMU)	6552.8 (0.4) ^b
S_3' -state	6553.2 (0.3) ^b
S_2' -state (annealed)	6550.1 (0.3) ^b

^a Values are averages over several samples. ^bStandard deviations are given in parentheses.

Figure 4.3. Second derivatives of Mn X-ray K-edge spectra from Ca-depleted samples in the S_1' , S_2' , and S_3' -states. The S_2' and S_3' spectra are quite similar in the region of the inflection point, while the S_1' sample exhibits a significantly lower inflection point energy.

the absence of illumination. The lack of a requirement for light-induced oxidation of the complex to restore the normal edge supports a structural change where the lower edge energy is caused by the removal of Ca. Removal of Ca from a binding site such as the one described in Chapter 3 could cause such a change.

The S₂'-DCMU and S₃'-state samples have quite similar spectra in the region of the inflection point with only a small shift in IPE (0.4 eV) between them. The IPE of the S₂' and S₃'-state samples (6552.8 and 6553.2 eV, respectively) approach that of the native S₂-state (6553.5 eV; Liang, 1994) and constitute a large shift (2.1-2.5 eV) from the spectrum of the S₁'-state sample. The S₂' and S₃'-state samples are not pure in their S'-state composition; the DCMU-treated S₂' sample shown probably contains 0-30% S₁' and the S₃' sample contains some S₂'-state centers. The estimate of the actual S₃'-state content of the S₃' samples ranges from 55 to >80%; the high number for samples where the multiline was so small it could not be accurately quantitated. There was, however, no systematic trend to higher energies for samples with higher S₃' content: the edge energy seems relatively insensitive to the relative amounts of S₂' or S₃' and the scatter of edge energies was relatively small (standard deviation of 0.2 eV). On the basis of the S₃' spectra, there appears to be no discernable difference in edge energy between samples in the S₂' and S₃'state.

The S₂'-state samples were slightly lower in energy (0.4 eV) than the S₃'-state samples, but the conclusion above, that S₂' and S₃'-states have the approximately the same edge energy, is not appreciably weakened. The S₂'-DCMU samples are a mixture of the

S₁' and S₂'-states and probably contain small amounts of S₁' which could account for the lower edge energy. The structure on the edge after the inflection point (6560-6580 eV) shows reproducible differences among all three types of samples, indicating perhaps subtle structural changes.

Data were also collected on S₂'-state samples made by dark adaptation of S₃'-state samples (S₂'-annealed) as described above. The Mn K-edge spectra from S₂'-DCMU and S₂'-annealed samples are compared in Figure 4.4. Surprisingly, the edge in the S₂'-annealed sample is actually at lower energy (6550.1 eV) than the S₂'-DCMU sample. In fact, it is even lower in energy than the S₁'-state samples (see Table 4.1). The lower edge energy clearly indicates that there is some change occurring in these samples during the dark adaptation period which results in a significant lowering of the edge energy, but which does not result in a major loss of the multiline signal. This is perhaps not surprising, because Ca-depleted samples are more unstable than untreated PS II samples, and delays during the treatment process inevitably lead to samples in which the Mn-cluster has been partially destroyed, releasing Mn(II) ions which can be detected as a six line signal centered at g=2 in the EPR spectrum. The Mn cluster has been shown to be more vulnerable to exogenous reductants in the absence of Ca (Mei & Yocum, 1991; Tso et al, 1991). However, the EPR spectra from the S₂'-annealed samples do not show an increase in a Mn(II) EPR signal, although small increases are difficult to judge in the presence of the S₂'-state multiline.

To test the possibility that Mn(II) release by damaged centers is the cause of the lowered edge, simulations of the edge were

Figure 4.4. Second derivative Mn X-ray K-edge spectra from samples in the S_2' -state prepared by annealing (S_2' -annealed) and by illumination with turnovers limited by DCMU (S_2' -DCMU). The inflection point energy of the edge in the S_2' -annealed sample is much lower in energy than the S_2' -DCMU sample.

attempted by adding various amounts of Mn(II) hexaquo edge spectra to the spectrum from an S₃'-state sample. This technique has been shown to be reasonably accurate for the determination of Mn(II) release by damaged centers (Sauer et al., 1992). The shape of the edge in the region of the inflection point was, however, not well simulated using the hexaquo Mn(II) spectrum.

To a certain extent, general edge shapes can be simulated by addition of spectra from monomeric Mn(II), Mn(III) and Mn(IV) complexes and this technique has been used to determine the probable oxidation state composition of native PS II samples in the S₁ and S₂-states (Yachandra et al., 1993). However, the inflection point regions of mixed valence Mn(II)Mn(III) compounds are not well simulated by the addition of spectra from monomeric complexes, probably owing to the effects of the structural environment, including the proximity Mn ions to each other.

To test the possibility that the lower edge energy in the S₂'-annealed sample was caused by partial reduction of the Mn cluster during the annealing process, but without release of the reduced Mn from the cluster, simulations were attempted where varying amounts of spectra from mixed valence Mn(II)Mn(III) complexes were added to the spectrum from an S₃' sample. Both a Mn₃(II,III)₂ trimer (Mn₃O(O₂CPh)₆(py)₂(H₂O); Vincent et al., 1987a) and a Mn₄(II,III)₃ tetramer (Mn₄O₂(benz)₇(bipy)₂; Vincent et al., 1987b) were tried and gave similar results. The best simulation (using the trimer) is presented in Figure 4.5. In this case, the simulation was created with 35% of the trimer spectrum and 65% S₃'-state spectrum. This would correspond to approximately 12% Mn(II) in the sample.

Figure 4.5. Simulation of the second derivative Mn X-ray K-edge spectrum from a Ca-depleted sample in the S_2' -state formed by dark adaptation of an S_3' -state sample. The simulated spectrum is the sum of the spectrum from a $Mn_3(II,III)_2$ trinuclear model complex (35%) and a spectrum from a sample in the S_3' -state (65%).

The results from the simulation with the tetramer indicated ~8% Mn(II). These simulations should not be taken as a quantitative determination but rather as an indication that the lower edge energy may be caused by a slow reduction of the Mn-complex without release of Mn during the annealing process. A structural change remains another possibility.

Discussion

The Mn X-ray K-edge data presented here on the modified S-states in Ca-depleted samples indicate that there is a large edge shift on the S_1' - S_2' transition, but little or no significant change in the edge position/inflection point for the S_2' - S_3' transition. As discussed above, edge positions are determined by oxidation state and by ligand/structural environment. In the absence of large structural changes, an edge shift can be ascribed to changes in the oxidation state of the absorbing atom. Assuming no major structural changes occurring in the S-state transitions, the results indicate that there is Mn oxidation on the S_1' - S_2' transition, but not on the S_2' - S_3' transition.

These results are consistent with a model where something other than the Mn cluster is oxidized on the S_2' - S_3' transition. These results are in marked contrast with two previous XAS studies that have indicated an edge shift on the S_2' - S_3' transition. These studies are addressed individually below.

In the first study, Ono and co-workers (Ono et al., 1993) performed XAS experiments on Ca-depleted PS II using the low pH/citrate treatment employed here. These samples were advanced

through the S'-states by single laser flashes. They did not perform EPR on the samples used for XAS, but compared the pattern of increasing edge energy on successive flashes with EPR results obtained by other groups on similar samples. The lack of any independent confirmation of S-state composition by EPR calls these results into question. Also, the total S₁'-S₃' edge shift reported by Ono et al. (1.9 eV) is comparable to the shift observed in the spectra presented here for the S₁'-S₂' transition (2.1 eV) where the composition of the samples was confirmed by EPR.

In the second study, MacLachlan et al (1994) report edge spectra from Ca-depleted as well as ammonia and acetate-inhibited preparations which also exhibit a form of the S₃' EPR signal. EPR spectroscopy was performed on parallel samples, but not on XAS samples. Again, the total S₁'-S₃' edge shift observed in their samples (2.2 eV) is comparable to the S₁'-S₂' edge shift observed in the work presented here. A possible reason for this is that MacLachlan et al. poised samples in the S₂'-state by dark adaptation for a short period of time (5-10 min). It is shown in the work presented here that dark adaptation results in a lower edge energy, probably owing to reduction of a small percentage of the Mn clusters. It is possible that the relatively shorter dark adaptation time used by MacLachlan et al. (5-10 min vs. 15 min here) resulted in a small shift to lower energy for their S₂'-state samples. This effect is not readily detectable by EPR.

Recent results from the Klein/Sauer group indicate that in native samples there is also very little edge shift on the S₂-S₃ transition (Liang, 1994). This result has been interpreted as

indicative that there is no Mn oxidation in this transition. There are possible parallels between native and Ca-depleted samples in that perhaps the same species that is oxidized to give rise to the S_3' signal is also oxidized in native PS II, but a different structural environment renders the signal unobservable. Another possibility, however, is that in the native samples, the lack of an edge shift on the S_2 - S_3 transition could be caused by the onset of water oxidation chemistry.

Chapter 5

Design and Construction of a Simple In-hutch Mirror Assembly for X-ray Harmonic Suppression

Introduction

Beamlines used for X-ray absorption spectroscopy in the hard X-ray region (>2 KeV) usually employ double crystal monochromators to provide tunable monochromatic radiation for stepwise scanning through a spectrum. In these monochromators, Bragg reflection from a specified crystal plane is used according to the equation:

$$n\lambda = 2d\sin\theta \quad [5-1]$$

where n is the order of the reflection, λ is the wavelength of the transmitted radiation, d is the spacing of the crystal planes, and θ is the angle of the crystal plane to the incoming beam. For a given angle and d -spacing, harmonics ($n>1$) can be reflected as well as the fundamental ($n=1$).

Harmonic contamination contributes to distortions in X-ray absorption spectra most commonly through a "thickness effect", the origin of which is leakage of radiation through the sample (more extensive discussions of harmonics, thickness effects, and their effects on X-ray absorption spectra may be found in Scott, 1984, and Heald, 1988). The name "thickness" arises because thicker (more absorbing) samples transmit less of the fundamental energy X-rays,

resulting in a larger proportion of harmonic X-rays in the transmitted signal and thus a larger distortion of the signal.

Fluorescence detection (Jaklevic et al., 1977) avoids most of the problems of thickness effects discussed above, but the spectra can still be degraded by harmonics. Monochromator glitches, caused by alignment of additional diffraction planes in the monochromator crystals, result in sharp decreases in X-ray intensity at particular energies (crystal angles) during a scan. Ideally, glitches should disappear in the ratio of fluorescence to incident intensity (F/I_0), but fundamental and harmonic X-rays contribute differently to the response of the fluorescence detectors and the I_0 detector and these differences do not completely disappear in the ratio. In addition, since the absorption cross-section of the element is not zero at the energy of the harmonic, there will be some fluorescent signal arising from absorption of the $n=2$ harmonic throughout the scan of the fundamental energies. This fluorescence signal should exhibit a relatively smooth decay and thus should not appreciably distort the spectrum, but the overall signal-to-noise of the EXAFS spectrum is degraded.

In our experiments at the Mn K-edge (>6.5 KeV) on dilute Mn samples ($<1\text{mM}$) we have encountered a particularly troubling case of harmonic contamination in samples containing considerable amounts of bromine (>10 mM). Midway through a Mn EXAFS scan, we observed a drop in Mn-fluorescence due to absorption of harmonic energy X-rays by bromine (K-edge 13.47 KeV). Bromine in the sample effectively shadows the manganese. Raw data which clearly demonstrates the problem is presented in Figure 5.1. Also

Figure 5.1. Effect of Harmonic Contamination on the XAS Spectrum of a Dilute Mn sample containing Br. The arrows indicate the distortion caused by absorption of the harmonic energy X-rays by Br in the sample. Harmonic energies are in parentheses.

shown is the ratio of incident to transmitted intensity (I_0/I_1) through of the sample, showing that the most significant absorption signal present in the sample arises from the absorption of the harmonic energy X-rays by Br.

Because EXAFS oscillations are only a few percent of the total signal, even a small drop in fluorescence can lead to a serious distortion of the data. In this particular case, we were trying to discern small changes in the EXAFS of samples containing bromine or chlorine and the low signal-to-noise in these dilute samples required that we collect data for long periods of time. To distinguish distortions from real EXAFS features in these experiments we required a rigorously harmonic-free beam.

Harmonic contamination can be removed or lessened in several ways:

- 1) Change the source characteristics to minimize the output at higher energies. This is usually not possible at a synchrotron source, where the accelerator ring energy is kept at a fixed, stable value. The beamline where these experiments were performed also employs a wiggler insertion device which increases flux across the spectrum.

- 2) Use crystals which do not pass even harmonics. The most commonly used crystals in double-crystal monochromators are Si(111), and Si(220). Due to the geometry of the unit cell in Si crystals, some reflections, which would seem to be allowed according to equation 5-1, have no transmitted intensity. Si(111) crystals do not pass the $n=2$ harmonic (2nd order reflection), but they possess an

unacceptable glitch pattern for EXAFS experiments on Mn. We use Si(220) crystals which pass the $n=2$ harmonic.

3) Detune the monochromator crystals. Monochromator detuning is achieved by rocking one crystal away from alignment with the other, thus reducing harmonics due to the narrower rocking curve of the harmonic compared to the fundamental. The greater the detuning the more suppressed the harmonic, however flux at both the harmonic and fundamental energies is reduced.

4) Use a harmonic rejection mirror. X-ray mirrors are used at glancing angles and their reflective properties depend both on angle and on the density of the coating material. Reflectance tends to fall off at higher energies and proper choice of coating material and angle can yield mirrors with excellent harmonic rejection capabilities. The particular beamline used for these experiments, VII-3 at Stanford Synchrotron Radiation Laboratory (SSRL), had no mirror as part of the beamline optics.

In this chapter the construction and use of a tabletop double mirror harmonic suppressor is described. This apparatus can be used in the experimental hutch and need not be part of the permanent beamline optics. The harmonic suppression of the mirror apparatus is also compared to that achieved through monochromator detuning.

Design and construction

Harmonic rejection mirrors take advantage of the critical angle for total reflection to act as low-pass filters. The critical angle (θ_c)

for a given wavelength of radiation (λ) is related to the electronic density (ρ_e) of the mirror material by the equation (Heald, 1988):

$$\theta_c \approx \rho_e^{1/2} \lambda. \quad [5-2]$$

Beyond the critical angle total external reflection does not occur and there is a sharp falloff in reflected X-ray intensity. Denser materials allow for larger angles for a given energy (or λ), but they also have lower reflectivity below the critical angle due to a greater absorption coefficient and the critical angle energy cutoff is not as sharp as it is for less dense materials. Thus there is a tradeoff between larger critical angles (shorter mirror length) and greater reflectivity with sharper cutoffs (but with considerably longer mirrors).

In our case we needed a short, portable mirror apparatus with a reasonably large entrance aperture (>1 mm) that would fit into the X-ray hutch and be relatively easy to adjust. Calculations of the reflectivity of gold at glancing angles over our energy range of interest showed that a gold-coated mirror could give excellent harmonic rejection while passing greater than 50% of the fundamental (Figure 5.2). A further enhancement of the sharpness of the cutoff can be had by using two reflections (the effect is squared). The "periscope" design we chose to employ (illustrated in Figure 5.3) has an advantage over a single mirror in that the beam exits parallel to the entering beam; this allows the use of a range of angles with only small changes in the vertical positioning of the rest of the experimental apparatus. Similar "periscope" designs have been used previously (Goulon et al., 1984; Saintavit et al., 1989).

Figure 5.2. Calculated reflectivity for one and two reflections of a gold mirror at an angle of 0.4 degrees. Data points (boxes) represent experimentally determined performance of the mirror apparatus at two energies.

Figure 5.3. Scale drawing of the double mirror harmonic suppressor and diagram of the ray path through the "periscope" design.

The mirrors were made from two flat strips of float glass (285 x 25.4 x 1.85 mm). Each glass strip had a 500 Å-thick gold coating evaporated over a 20 Å-thick layer of chromium which increases the adhesion of the gold to the glass. The mirrors were mounted on an aluminum plate in a face-to-face arrangement with the upper mirror overlapping the lower mirror for half its length. This mirror plate rests on an aluminum baseplate and angle adjustments are made using a micrometer (Newport) attached to the mirror plate.

The mirrors were kept at the correct spacing by four 1mm-thick lucite shims which extend 3mm in from the sides of the mirrors leaving a 2 cm-wide path for the beam through the apparatus. The lower mirror rests on the mirror plate and is held in place by four plastic screws. The upper mirror is held in place on the spacers by two U-shaped lucite clamps screwed into the mirror plate. The thickness of the shims (1 mm) is optimal for a glancing angle of 0.4° , allowing for a vertical entrance aperture of 2 mm. The angle of the mirror is adjustable over a small range of angles (0.4 - 0.5°) without greatly decreasing the aperture or allowing the beam through without reflection. For sufficiently different angles different lucite shims must be used.

The whole apparatus is protected from dust by a plexiglass cover that attaches to the sides of the baseplate. This cover can easily be modified to allow the mirrors to be kept in a helium gas atmosphere to reduce air-attenuation of the X-ray beam at low energies and also to prevent degradation of the mirror surface over time.

The bottom plate was drilled for attachment to an adjustable optical rail (Oriel) which is standard equipment on beamline VII-3 at SSRL. SSRL adjustable tantalum slits were also employed before and after the mirrors to aid with initial alignment and to define the incident beam. These slits were standard X-ray slit boxes provided by SSRL and were bolted to supports attached to each end of the bottom plate.

The slits and the adjustable rail made initial alignment of the mirror apparatus simple. Initially the slits were adjusted to 0.5mm vertical size at the same height, then the bottom plate with slits was attached to an ion chamber support on the rail and the angle of the rail was adjusted to maximize the beam intensity through the slits. The mirror plate was then put into place on the bottom plate, the angle was set, and slit positions were adjusted to the final positions.

Experimental results

The performance of the double mirror harmonic suppressor was evaluated on beamline VII-3 at SSRL with Si(220) crystals in a fixed separation double-crystal monochromator. The output of one element of a 13 element germanium detector (Canberra) in connection with a pulse height analyzer (Canberra Series 35 Plus MCA) was used to evaluate the performance of the mirror in our standard EXAFS experimental setup (Liang et al., 1994). Relative amounts of fundamental and harmonic X-rays were calculated from MCA trace peaks areas and were corrected for the falloff in scattering cross-section of the sample at the harmonic relative to the fundamental.

The X-ray hutch on beamline VII-3 is 130 cm long and the length of the mirror apparatus (51 cm) and the constraints imposed by the use of an Oxford liquid helium flow cryostat precluded the use of an ion chamber for I_0 (initial intensity) measurements. Typically, however, we measure I_0 using a photomultiplier tube equipped with a plastic scintillator to detect scattered radiation from a piece of Kapton film placed in the X-ray beam. This I_0 setup was easily adapted such that very little space was used (about 7 cm).

The energy spectrum of X-rays incident on the germanium detector at the end of a typical Mn-EXAFS scan (7.1 keV) is presented in Figure 5.4 and illustrates quite well the problem we faced with these samples. The spectrum was collected with the monochromator detuned to 90% of the maximum I_0 flux and consists of both scattered (fundamental and harmonic) and fluorescent X-rays. The amount of Br fluorescence is indicative of the large amount of $n=2$ harmonic present in the incident beam. To evaluate the performance of the mirrors compared to crystal detuning further traces were collected at 6.5 keV where Br fluorescence does not complicate the measurement of peak areas of the scattered $n=2$ harmonic X-rays.

Energy spectra, comparing the effects of monochromator detuning and the double mirror harmonic suppressor, are shown in Figure 5.5. Detuning to 50% of the maximum I_0 flux (trace B) reduced the amounts of $n=2$ harmonic X-rays to a low level (12% of the 90% detuned value in trace A) while maintaining a great deal of fundamental flux (>80%). The proportion of harmonic to fundamental X-rays was still relatively high at 1:9, although greatly improved

Figure 5.4. Energy spectrum of scattered and fluorescent X-rays from a dilute Mn sample also containing Br. The spectrum was collected with the fundamental at 7.1 keV ($n=2$ harmonic at 14.2 keV).

Figure 5.5. Energy spectra collected at 6.5 keV from the sample in Fig. 3. Spectra are presented with the monochromator tuned to 90% (A), 50% (B) and 10% (C) of the maximum flux. Trace (D) was collected with the monochromator tuned to 90% and with the mirror apparatus in the beam. To compare relative harmonic content of the beam, the spectra are normalized to the height of the fundamental peak (individual multiplicative factors are indicated on the horizontal axes.)

from an initial value of 1:1.3. Further detuning reduced the fundamental as well as the $n=2$ harmonic, though not much improvement was observed in the ratio of harmonic to fundamental. At 10% of the maximum I_0 flux (Figure 5, trace C), the flux at the fundamental energy was reduced to 14.5% of the maximum value but the proportion of harmonic to fundamental counts was only improved to 1:18.

The use of the double mirror at an angle of 0.4° (figure 5, trace D) decreased the fundamental to 53% of the maximum value without the mirror, but the harmonic was almost undetectable and the proportion of harmonic to fundamental was 1:200. This behavior was very close to that predicted by reflectance calculations (Figure 5.2) and obviously far exceeds the results obtained by monochromator detuning.

No problems were observed with beam stability or position through the mirrors, though a small spreading of the beam after the mirrors was observed. The beam spreading caused no difficulties with data collection and was probably caused by a slight curvature of the tail end of the top mirror which is unsupported. This problem can be alleviated by affixing an aluminum plate to the top mirror for additional stiffness. Another possibility is to control the bend on the top mirror to focus the beam into a sharp line at the sample.

Conclusions

We have built and tested a double mirror harmonic suppressor for use in an experimental hutch on X-ray beamlines. This apparatus is much more effective in harmonic rejection than monochromator

detuning using Si(220) crystals and maintains better than 50% of the maximum flux of X-rays at the fundamental energy. Moreover, this simple device is easily transported to other beamlines, and can be adjusted to different angles for use at a variety of energies.

Chapter 6

Conclusions and Future Directions

The Evolution of the Klein-Sauer Model of the Mn Cluster

Based on a body of EXAFS results on PS II preparations collected over a number of years by the Klein-Sauer group, a structural model has been proposed for the Mn complex of PS II (Sauer et al., 1992; Yachandra et al., 1993). A brief description of the derivation of the model, including the addition of a Ca-binding site based on the results in Chapter 3, is given here.

EXAFS data collected from native PS II samples can be fit to very similar parameters as the Ca- and Sr-reactivated samples in Chapter 3. There are three peaks in the Fourier transforms of these data. The first Fourier peak corresponds to atoms directly ligated to the Mn atoms of the complex and is dominated in both native and Ca/Sr-reactivated samples by O or N atoms at about 1.8 Å with a possible contribution from O/N atoms from 1.95-2.1 Å. The second Fourier peak is well simulated as 1-1.5 Mn atoms at a distance of about 2.7 Å. The 2.7 Å Mn-Mn distance, as well as the 1.8 Å Mn-O distances, is characteristic of many di- μ -oxo bridged Mn model complexes (Figure 6.1) and indicates that the Mn complex contains 2-3 such units.

The third Fourier peak has been interpreted by most workers (George et al., 1989; Penner-Hahn et al., 1990; Sauer et al., 1992; DeRose, et al., 1994) to be a Mn-Mn 3.3 Å interaction and in the initial model (Sauer et al., 1992) was interpreted as such. Similar

Figure 6.1. Structural elements of the Mn complex. These core bridging structures are based on common Mn model compound motifs.

Mn-Mn distances have been observed in Mn model complexes possessing a single μ -oxo bridge and 0, 1, or 2 bidentate carboxylate bridges. The core structure of a single μ -oxo bridged binuclear Mn compound is shown in Figure 6.1. The two structural units in Figure 6.1 form the basis for a structural model of the Mn complex.

Partially oriented PS II preparations have also been studied by EXAFS and have yielded information about the relative orientations of the scattering vectors in the complex (Mukerji et al., 1994). The ~ 2.7 Å vectors are oriented at an average angle of 60° to the membrane normal, and the 3.3 Å vector makes an angle of 43° . These results place constraints on the arrangement of the Mn atoms in the complex, i.e. that the 2.7 Å vectors must lie closer to the plane of the membrane while the 3.3 Å vector lies closer to the membrane normal. Based on these results, a simple tetranuclear model can be constructed and is shown in Figure 6.2. This model is not presented as the only possible arrangement of the Mn atoms, but the basic structural features of oxo bridged Mn units will be present in any structural model consistent with the EXAFS results. Figure 6.2 was created using the basic structural units in 6.1 and combining them in a single model using the program Chem3D Plus (Cambridge Scientific Computing). The overall model was energy minimized on the basis of optimal values for structural parameters in Chem3D Plus (bond angles, bond distances) which were taken from model-compound crystal structures.

EXAFS studies on the Mn complex in the S_2 -state exhibiting the $g=4.1$ signal (Liang et al., 1993), NH_3 -treated preparations in the S_2 -state (Dau et al., 1995), and F⁻-treated preparations (DeRose et al.,

Figure 6.2. Basic structural model of the Mn complex in PS II. This model is based on the structural units in Figure 6.1 and represents a reasonable assembly based on EXAFS results from partially oriented PS II preparations.

1995) have all shown a decreased amplitude of the 2.7 Å peak relative to native samples, which can be fit as an increased heterogeneity caused by a slight lengthening of a Mn-Mn distance. These studies all support the presence of two Mn-Mn ~2.7 Å units, one of which can be affected by the procedures mentioned above.

This core model has formed a basic structure upon which other results can build. Further additions/improvements to the model have arisen from work on chloride binding sites, nitrogen ligation, and a calcium binding site. The results of these studies are described below and the extended structural model is shown in Figure 6.3.

A large body of indirect biochemical and spectroscopic evidence (see DeRose 1990, DeRose et al., 1995) indicates that there is a chloride (halide) binding site on the Mn cluster. Specifically, the effect of F⁻ treatment (which displaces Cl) is to increase one Mn-Mn ~2.7 Å distance in the complex (DeRose et al., 1995). A Cl⁻ ligand displaced by F⁻ could give rise to such a change, and a Cl⁻ ligand to Mn is included in the structural model.

Nitrogen ligation to Mn has been demonstrated by time-domain EPR studies on PS II preparations from a thermophilic cyanobacterium grown on ¹⁴N and ¹⁵N-media (DeRose et al., 1991). Further work has shown that this nitrogen signal comes from a histidine ligated to the Mn complex (Tang et al., 1994), thus, a histidine ligand has become part of the model.

Evidence for a Ca binding site in PS II comes from the experiments presented here (Chapter 3) on Sr-reactivated samples where an increase was observed (relative to Ca-reactivated samples) in the amplitude of the third Fourier peak in the EXAFS spectrum.

Figure 6.3. An extended model of the Mn complex and associated co-factors. The exact arrangement of co-factors relative to each other is not known at this time.

Figure 6.4. An extended model of the Mn complex including a more detailed model for a Ca-binding site in PS II.

The most reasonable structural interpretation of these data supports a model where both Mn-Mn and Mn-Ca/Sr scattering contributes to the third Fourier peak. This result was initially shown in the structural model as a Ca bridged to Mn by a bidentate carboxylate ligand. Based on a review of Ca-metal bridging structures and distances (see Chapter 3) a more likely bridging structure for the Ca binding site is depicted in Figure 6.4.

The proximity of this binding site to Mn has implications for the role of Ca in the Mn complex. With such a short Mn-Ca distance and probable shared ligands, Ca undoubtedly has an effect on the redox properties of the Mn cluster. This may be important in achieving a high enough potential in the Mn complex to drive water oxidation. Such a closely bound calcium could also be a site where water molecules bind until they participate in water oxidation. Some interesting speculative models where Cl and H₂O molecules are exchanged between Ca and Mn have been proposed (Rutherford, et al., 1992), but there is little evidence to support or deny these propositions.

Future Directions

The Ca-binding site proposed above and in Chapter 3 is a testable hypothesis. Several experiments to further confirm/define the site are described here, as well as experiments to further define the oxidation state changes in the S-state cycles of Ca-depleted and native PS II samples.

The Mn EXAFS of PS II samples is complicated by the fact that there are four Mn atoms/ PS II. The relative contribution of a single

Ca/Sr scatterer is very small in the Mn EXAFS spectrum because only one of the four Mn atoms "sees" a Ca/Sr atom. One way to approach this problem is to perform EXAFS experiments where an atom other than Mn is the central atom.

In native PS II preparations there are 2 Ca/4 Mn. Commonly used treatments to deplete Ca from PS II (high NaCl concentrations and low pH/citrate treatments) remove one of these Ca atoms; the remaining Ca is very difficult to remove (Kalosaka et al., 1989). Reactivation with Sr replaces the more loosely bound Ca. One method to examine the loosely bound Ca (the site which appears to be associated with the Mn complex and reactivation of oxygen evolution) is to perform EXAFS experiments at the Sr K-edge. If the Sr atom is situated 3.5 Å from the Mn complex then the effective Mn coordination number seen by the Sr would be 1 and would lead to a much greater contribution to the EXAFS than for Mn EXAFS where the Sr coordination number seen by Mn is 0.25. Experimentally this is difficult to carry out because the samples must be free of any adventitious Sr while still retaining Sr at the binding site of interest, but recent results in the Klein/Sauer group on samples of this type are encouraging (Roehl Cinco, personal communication).

EXAFS experiments can also be performed at the Ca K-edge. These experiments can be difficult owing to a lower fluorescence yield for Ca vs. Mn, greater attenuation of the Ca fluorescence signal by cryostat and detector window materials, and very low concentration in the sample. (Mn K-edge EXAFS experiments are already difficult because of the low concentration of Mn in PS II samples; Ca would be present at 1/2 the concentration of Mn.) These

experiments are, however, feasible, and they offer the possibility to examine specifically the tight binding Ca site (about which very little is known) as well as the more loosely bound Ca. In addition, Ca-EXAFS experiments could be performed on untreated samples, which have had adventitiously bound Ca removed. The use of native (untreated) samples would remove possible questions about changes in the Mn complex caused by the treatment process.

Information about the oxidation states of the Mn ions in the various S-states is crucial to understanding the charge accumulation process and the mechanism of oxygen evolution. The results presented in Chapter 4 show that there is no significant shift in inflection point energy of the Mn K-edge spectra on the S₂'-S₃' transition in Ca-depleted samples. This result supports a model where there is no oxidation of the Mn cluster on this transition and that another species, possibly a protein residue like histidine or tyrosine is oxidized instead. These samples have interesting parallels with native samples, which also show a very small edge shift on the S₂-S₃ transition. It has been proposed that a redox-active protein residue might be oxidized in this transition (Guiles et al., 1990).

EXAFS is a complementary experiment that can help determine whether edge shifts are the result of oxidation or structural changes. In the low pH-treated Ca-depleted samples, there is EPR evidence that the structure of the Mn-complex has been significantly altered from the native state (i.e. the dark-stable multiline). Mn EXAFS data have been collected from samples in these states (S₁', S₂', and S₃') and are in the process of being analyzed.

Electron spin echo envelope modulation (ESEEM) spectroscopy has been used to examine the oxygen evolving complex and has been instrumental in showing ammonia binding to Mn (Britt et al., 1989) and the presence of nitrogen ligation to Mn (DeRose et al., 1991), specifically a histidine residue (Tang et al., 1994). Zimmermann et al. (1993) have examined Ca-depleted preparations using electron spin echo (ESE) techniques and have found that, using spin echo amplitude to record the absorption spectrum, they are able to observe the absorption from the modified multiline even in the presence of the S_3' -signal. They interpret this result as supporting a model where the S_3' -signal arises from a radical species interacting magnetically with the Mn cluster, resulting in a greatly broadened multiline signal which is no longer visible by conventional EPR, although the absorption amplitude remains. Conventional EPR spectrometers use magnetic field modulation to detect absorption signals producing a first derivative spectrum; broad featureless signals are largely undetected using these instruments.

An interesting experiment would be to look at the direct absorption using the ESE technique in the S_2 and S_3 -states of the native enzyme. Unlike Ca-depleted samples, there has been no signal detected via conventional EPR associated with the S_3 -state in native PS II samples, but the similar behavior of the Mn X-ray K-edge shifts in the two preparations may mean that oxidation of a protein residue occurs in both preparations. In the native samples, the signal from such a species may be too broadened to be observed by conventional EPR, but may be observable via direct absorption measurements.

Even more interesting would be to see if the Mn multiline signal in native samples is just broadened or is actually gone in the S₃-state.

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