

Table VII. Proposed Ligand Structure of the Photosynthetically Active, "Loosely Bound" Pool of Manganese in Spinach Chloroplasts

ligand ^a	average distance ^b	no.
CNO ^c	1.81 (2)	2-3
CNO	2.15 (5)	2-4
MnFe ^c	2.72 (2)	1

^a CNO = carbon, nitrogen, or oxygen; MnFe = manganese or iron or copper. ^b Average results for various fits. ^c Bridged structure with CNO ligands and MnFe ligands in approximately square or trigonal bipyrimid arrangement.

the second peak may not be due to bridged Mn atoms but are due to other Mn atoms that are also released during inactivation. If this is the case, then it will be necessary to study the photosynthetically active Mn in its native environment free of contamination of other Mn atoms that may be present and serve other functions. The possibility of multiple sites would also explain the low absolute number of atoms predicted by the fitting results, but at the present time this should be treated as conjecture until some new evidence indicates otherwise.

The change in the ligand ratio is most likely due to the poorer quality of the difference data but should not be totally discounted.

Conclusion

Three di- μ -oxo Mn dimers were examined by using the EXAFS technique. The crystal structure of one of the dimers has been published.¹¹ Using this published crystal structure, we made determinations concerning certain of the structural parameters for the other two compounds. These results were presented in part a of the Discussion section above.

Part of the study of the Mn dimers was concerned with determining why the nitrogen atoms in the first coordination sphere of the dimers did not appear in the EXAFS spectra. A simulation study showed that large disorder in the Mn-N distances are responsible. This simulation study then led to identification of the first two peaks of the active chloroplast Fourier transforms (see Figure 1).

EXAFS studies on the two chloroplast samples and comparison of the chloroplast results with the bridged Mn dimer models has resulted in a prediction for the local structure of the "loosely bound pool" of Mn in chloroplasts, which is implicitly related to pho-

tosynthetic oxygen evolution. The analysis is compatible with a CNO-bridged transition-metal dimer (or multimer) similar to the core of the Mn dimer models studied. The chemistry of Mn suggests that oxygen is the most likely bridging ligand but carbon and nitrogen cannot be excluded. The partner transition metal is most likely another Mn, but Fe and Cu cannot be excluded due to their relative abundance in chloroplasts. The remainder of the first coordination ligands is most likely CNO. The distances for the bridging ligands and the other transition-metal ligands are quite accurately predicted, but the distance for the other first coordination sphere ligands is poorly defined primarily due to a large spread in the individual ligand distances (see Table VII).

Future Work

Obvious extensions now in progress are the effects of actinic light, the effects of various redox reagents and known cofactors such as Cl⁻. Most important will be the results obtained from a study of the Mn-containing protein, whose isolation has been only recently reported by Spector and Winget.²⁴

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State of Manganese in the Photosynthetic Apparatus. 2. X-ray Absorption Edge Studies on Manganese in Photosynthetic Membranes

J. A. Kirby, D. B. Goodin, T. Wydrzynski, A. S. Robertson, and M. P. Klein*

Contribution from the Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. Received December 1, 1980

Abstract: X-ray absorption spectra at the manganese K edge are presented for spinach chloroplasts and chloroplasts that were Tris treated and hence unable to evolve oxygen. A significant change in the electronic environment of manganese is observed and is attributed to the release of manganese from the thylakoid membranes with a concomitant change in oxidation state. A correlation of the K-edge energy, defined as the energy at the first inflection point, with coordination charge has been established for a number of manganese compounds of known structure and oxidation state. Comparison of the manganese K-edge energies of the chloroplast samples with the reference compounds places the average oxidation state of the chloroplasts between 2+ and 3+. With use of the edge spectra for Tris-treated membranes which were osmotically shocked to remove the released manganese, difference edge spectra were synthesized to approximate the active pool of manganese. Coordination charge predictions for this fraction are consistent with an average resting oxidation state higher than 2+. The shape at the edge is also indicative of heterogeneity of the manganese site, of low symmetry, or both.

The production of oxygen in photosynthesis by green plants and blue-green algae has been the subject of innumerable studies in

the last 50 years. One of the results of these studies has been the demonstration of a manganese requirement for oxygen evolution.¹⁻³

A membrane-bound form of manganese as a necessary component of the electron-transport chain has been the subject of many other studies.⁴⁻⁷ In addition, experiments by Kessler⁸ and more recently by Cheniae and Martin⁹ have placed the site of manganese sensitivity on the donor side of photosystem II near the site of water oxidation.

A currently popular scheme employing the cycling of five states, S_0 through S_4 , was proposed by Kok¹⁰ in 1970 to explain the observed oscillations in oxygen evolution¹¹ after each of a series of brief (10 μ s) flashes of light. The first evidence that manganese may be directly involved in the proposed "S" intermediates was provided by its effect on proton NMR relaxation rates¹² and an EPR study of Mn^{2+} released from thylakoid membranes by heat.¹³

It thus appears likely that a protein-bound manganese site is involved in accumulating oxidizing equivalents and/or acting as the catalytic site for photosynthetic water oxidation. Unfortunately, the crucial questions concerning the oxidation states of the membrane-bound fraction of manganese and its ligand environment have been unavailable to scrutiny by conventional spectroscopic techniques.

We have undertaken a comprehensive study of manganese in spinach chloroplasts, utilizing X-ray absorption spectroscopy, and we report the first direct observation of manganese bound to photosynthetic membranes. Reported here are the results of the X-ray absorption edge spectroscopy (XAES) study of chloroplasts, which revealed information about the oxidation state and ligand site symmetry of this previously shrouded component. Results and analysis of extended X-ray absorption fine structure are presented in a companion paper.¹⁴

Materials and Methods

Model Compounds. Samples of di- μ -oxo-tetrakis(1,10-phenanthroline)dimanganese(IV,IV) perchlorate [$Mn(4,4)phen$], di- μ -oxo-tetrakis(2,2'-bipyridyl)dimanganese(III,IV) perchlorate [$Mn(3,4-bpy)$], di- μ -oxo-tetrakis(1,10-phenanthroline)dimanganese(III,IV) perchlorate [$Mn(3,4)phen$], and μ -oxo-bis(phthalocyaninato)pyridine)manganese(III) dipyrindinate [$Mn^{III}(pcpy)_2O$] were provided by S. R. Cooper of the Department of Chemistry, Harvard University, and were synthesized according to literature methods.¹⁵ Samples of $[(C_6H_5)_4P]_2Mn^{II}(SC_6H_5)_4$ were provided by D. Coucouvanis, Department of Chemistry, University of Iowa. " Mn_2LCl_2 " and " Mn_2LBr_2 " were obtained from J. F. Dodge, Department of Chemistry, California Institute of Technology. All other manganese compounds were obtained from commercial sources.

Chloroplast Preparation. Broken spinach chloroplasts and broken chloroplasts treated with tris(hydroxymethyl)aminomethane (Tris) were prepared as described by Blankenship and Sauer,¹⁶ except that the initial sucrose isolation medium contained 1 mM EDTA to remove free and loosely bound manganese. The total chlorophyll content was determined by the method of MacKinney.¹⁷

O_2 Evolution. O_2 evolution was measured with a Beckman 39065 Clark-type electrode polarized at -0.8 V vs. $Ag|AgCl$. The media and measuring procedure was that of Blankenship and Sauer.¹⁶

X-ray Absorption Samples. Active chloroplast samples were made from preparations evolving no less than 150 μ mol of O_2 (mg of chloro-

phyll)⁻¹ h⁻¹. Complete inhibition of oxygen evolution was verified for samples of Tris-treated chloroplasts, and release of Mn^{2+} from the vesicles was monitored with a Varian E-3 EPR spectrometer. Tris-treated, osmotically shocked samples were prepared by incubating Tris-treated chloroplasts in distilled water on ice at approximately 0.1 mg of chlorophyll/mL for 0.5 h. All samples were centrifuged at 5000g for 5 min, and the pellets were layered, to a thickness of about 2 mm, on sample holders consisting of millipore air pollution filter disks. A drop of sucrose isolation buffer¹⁶ was applied to the samples periodically to retain moisture.

Samples of model compounds were prepared by pressing pellets of the appropriate material in a powdered cellulose matrix. The quantity used was that required to produce an absorbance change of 90% across the K absorption edge.¹⁸ Samples of $[(C_6H_5)_4P]_2Mn(II)(SC_6H_5)_4$ were prepared under an argon atmosphere in a gas-tight sample cell with Kapton windows. The sample cell was stored under argon until use.

X-ray Absorption Measurements. The XAES spectra were collected at the Stanford Synchrotron Radiation Laboratory, Stanford, CA (SSRL). The XAES spectra were collected by using the standard absorption method described elsewhere.¹⁸ This consisted of a tunable monochromatic X-ray source, two ion chambers, and the absorbing sample. The sample was placed between the two ion chambers, which were used to obtain a measure of the incident and transmitted photon fluxes. The ratio of the incident to transmitted flux was collected as a function of incident photon energy, and the natural logarithm of this ratio was used as a measure of the total X-ray absorption cross section.

Data for the chloroplast samples were collected, utilizing detection of the Mn $K\alpha$ fluorescence excitation spectrum with a three-channel lithium drifted silicon detector.¹⁹

Data on samples of a reference material ($KMnO_4$ or $MnCl_2 \cdot 4H_2O$) were collected for each fill of the storage ring to allow reliable energy registration between samples and from day to day. Energy calibration of each sample studied was made by requiring a feature of the reference material to have a predetermined value ($KMnO_4$ preedge transition at 6543.3 eV, $MnCl_2 \cdot 4H_2O$ edge at 6546.7 eV).

The X-ray absorption edge energy was defined as the energy of the first major point of inflection on the edge. The derivative at a point was obtained by fitting a polynomial to a fixed width energy region of the spectrum, evenly spaced on both sides of the point of interest. The polynomial was then differentiated to obtain a good approximation to the true derivative at that point. In the analysis presented here, the polynomial used was a quadratic and the energy widths were 2.5 and 5.0 eV for the model compounds and the chloroplast samples, respectively. The chloroplast samples required a larger width due to a significantly higher noise level.

Theory

It has been known for many years that the binding energies of the inner-core electrons and the nuclei of atoms are affected by chemical bonding. Photoelectron²⁰ and Mössbauer spectroscopy²¹ are good examples of this effect. As with these methods, XAES can be used to gain information related to the oxidation state, ligand-site symmetry, and environment of metal atom.²²

The features of an X-ray absorption edge are due to transitions from a core level to "bound" states in complexes or band structure states in crystalline solids. For this study the 1s or K-shell absorption edge (K edge) of Mn was measured.

In 1967, S. S. Batsonov and co-workers²³ reported a linear correlation between the X-ray edge position and a quantity called the "coordination charge", defined as

$$\eta = Z - \sum_{i=1}^N C_j \quad (1)$$

where η is the coordination charge, Z is the formal oxidation state of the absorbing atom, C_j is the degree of covalence for the j th first coordination sphere ligand, and N is the total number of first

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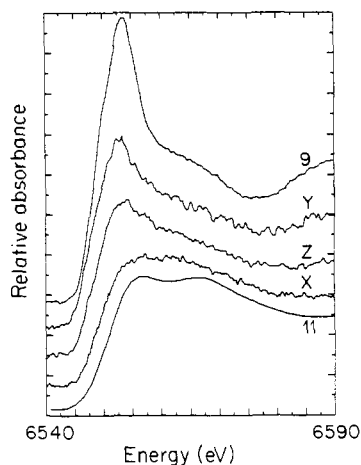


Figure 1. X-ray absorption K edges for manganese in three different chloroplast samples and two model complexes. Spectrum 9 is 100 mM solution of MnCl_2 , 11 is manganese(III) tris(acetylacetonate), X is the active chloroplasts, Y is the Tris-treated chloroplasts, and Z is the Tris-treated, osmotically shocked chloroplasts (see text and Table I).

sphere ligands. The degree of covalence is defined as $C_j = 1 - i_j$, where i_j is the ionicity of the bond between the absorbing atom and the j th ligand. The ionicity is calculated from Pauling's formula,²⁴ giving

$$i = 1 - \exp[-(\chi_A - \chi_L)^2/4] \quad (2)$$

where χ_A and χ_L are the electronegativities of the absorber and ligand, respectively. Cramer et al.²⁵ have recently used this technique with some success in a study of the coordination environment of molybdenum in nitrogenase.

Results

The X-ray absorption edges were obtained, utilizing fluorescence detection,¹⁹ for chloroplasts prepared in three conditions. The results are presented in Figure 1. Normal, active chloroplasts (labeled X) are those possessing full oxygen-evolving capabilities. These preparations are reported to have four–six manganese atoms per photochemical reaction center.⁹ This manganese does not give rise to an aqueous Mn^{2+} EPR signal⁹ and it is assumed to be membrane bound. Second are chloroplasts which have been treated with 0.8 M Tris buffer at pH 8.0 (labeled Y) and lack O_2 -evolution capacity.⁹ Samples prepared under these conditions have released roughly two-thirds of their manganese to the inside of the thylakoid vesicles where it appears as hexa-aquaquated Mn^{2+} by EPR.²⁶ The third sample consists of Tris-treated thylakoid vesicles which have been osmotically ruptured and concentrated by centrifugation (labeled Z). This treatment allows removal of the aqueous manganese trapped on the inside of the vesicles.¹⁶ This was verified by observing the loss of hexa-aquaquated Mn^{2+} EPR signal for this sample.

For comparison, the edge spectra of manganese(III) tris(acetylacetonate) (labeled 11) and $\text{Mn}(\text{H}_2\text{O})_6^{2+}$ (labeled 9) are also shown. A number of characteristics of these spectra are particularly striking. Active chloroplasts exhibit an edge which is quite similar in position and shape to manganese(III) tris(acetylacetonate), yet the edge of Tris-treated chloroplasts is markedly different. The edge position and shape for the latter are much more like that of $\text{Mn}(\text{H}_2\text{O})_6^{2+}$, which exhibits a strong absorbance peak at the edge. It can also be seen that the osmotically shocked chloroplast sample exhibits an edge intermediate between these two extremes.

Absorption edges (not shown) for a variety of manganese compounds were collected for the purpose of the edge correlation analysis. Coordination charges were calculated for these com-

Table I. Manganese Coordination Charges and X-ray K-Edge Energies for Model Complexes

label	compound	coordination charge, e^s ^a	K edge, eV ^e
1	$\alpha\text{-MnS}$	-3.04	6542.15
2	$\text{Mn}^{\text{II}}(\text{SPh})_4 \text{P}(\text{Ph})_4$	-1.36	6545.11
3	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	-1.02	6546.97
4	$\text{Mn}_2\text{LCl}_2 \cdot 2\text{H}_2\text{O}^b$	-0.67	6546.81
5	$\text{Mn}_2\text{LBr}_2 \cdot 2\text{H}_2\text{O}^b$	-0.70	6546.63
6	$\text{Mn}^{\text{II}}(\text{C}_4\text{O}_4) \cdot 2\text{H}_2\text{O}^b$	-0.46	6546.82
7	$\text{Mn}^{\text{II}}(\text{C}_5\text{H}_7\text{CO}_2)_2 \cdot 4\text{H}_2\text{O}$	-0.46	6547.91
8	$\text{Mn}^{\text{II}}(\text{acac})_2 \cdot 2\text{H}_2\text{O}^c$	-0.46	6547.41
9	Mn^{2+} (aqueous)	-0.46	6548.54
10	$\text{Mn}^{\text{II}}\text{P}_2\text{O}_7$	-0.46	6547.18
11	$\text{Mn}^{\text{III}}(\text{acac})_3$ ^c	0.54	6550.70
12	Mn_2O_3	0.54	6549.55
13	$\text{Mn}^{\text{IV,IV}}\text{phen}^d$	0.86	6551.04
14	$\alpha\text{-MnO}_2$	1.54	6552.06

^a Units are electrons. ^b L is the dianion ϵ^f 11,23-dimethyl-3,7,15,19-tetraazatricyclo[19.3.1.1^{9,13}]hexacos-1(25),2,7,9,11,13(26),14,19,21,21-decaene-25,26-diol. ^c Acetylacetonate = acac. ^d Di- μ -oxo-tetrakis(1,10-phenanthroline)dimanganese-(IV,IV) perchlorate. ^e Error estimated to be ± 0.2 eV.

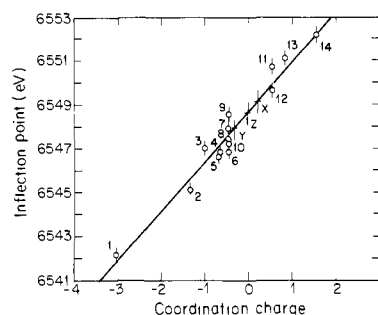


Figure 2. Manganese K-edge inflection point energy vs. coordination charge for 14 compounds and complexes (labeled 1–14). The three chloroplast samples (X, Y, and Z) were placed on a linear fit to points 1–14 (see Tables I and III for label description).

pounds, using the electronegativities created by Allred and Rochow²⁷ and extended by Little and Jones.²⁸ These coordination charge values are tabulated with the experimental edge inflection point energies in Table I.

The values presented in Table I, which includes both simple crystalline Mn compounds and complexes which are primarily σ bonded, are plotted in Figure 2. It is seen that a reasonably good linear relationship is observed. Figure 2 demonstrates that, at least for simply bonded complexes, there exists a linear relationship between coordination charge and X-ray K-edge energies. A least-squares linear fit applied to the values in Table I gives:

$$\text{K edge (eV)} = (2.27 \pm 0.15)\eta + 6548.69 \pm 0.16$$

This fit was subsequently used to estimate the coordination charges corresponding to the K edges of the three chloroplast samples. The results are tabulated in Table III and presented graphically in Figure 2 as the points X, Y, and Z.

In order to test limits to which this approach can be applied, we included as model samples a number of mixed valence compounds and a number of compounds which are known to have metal–ligand π -bonding interactions. The coordination charge values for these compounds are presented in Table II and in Figure 3. It is evident from Figure 3 that many of these complexes exhibit behavior decidedly different in edge inflection point positions than those which involve σ -type bonding.

Discussion

Estimation of the effective quantity of charge on a metal by using correlations of K-edge inflection points with coordination charge is possible within certain limitations. Examination of Table

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Table II. Coordination Charges and X-ray K-Edge Energies for Model Compounds of Unusual Chemical Environments

label	compound	coordination charge, $e's^a$	K edge, eV^g
A	Mn_3O_4	0.48 [0.54] ^b	6547.13 [6550.43] ^b
B	$Mn^{III,IV}bpy^c$	0.36	6549.67
C	$Mn^{III,IV}phen^d$	0.36	6549.66
D	$KMnO_4$	5.36	6557.60
E	$[Mn^{III}(pcpy)]O_2^e$	-0.31	6551.44
F	$Mn\ TPP\ Cl^f$	-0.43	6549.74
G	$K_3Mn(CN)_6$	-1.92	6555.12
H	$Mn_2(CO)_{10}$	-4.10	6553.52

^a Average of individual Mn coordination charges; units are electrons. ^b Second edge, using only Mn(III) sites. ^c Di- μ -oxo-tetrakis(2,2'-bipyridine)dimanganese(III,IV) perchlorate. ^d Di- μ -oxo-tetrakis(1,10-phenanthroline)dimanganese(III,IV) perchlorate. ^e μ -Oxo-bis(phthalocyaninato)pyridine)manganese(III) dipyrindinate. ^f Chloro(5,10,15,20-tetraphenylporphyrinato)aquomanganese(III). ^g Error estimated to be 0.2 eV.

Table III. Experimentally Determined Coordination Charges and K-Edge Energies for Chloroplast Samples

figure label	sample (see text)	coordination charge, $e's^a$	K edge, eV^b
X	active	0.18 (18)	6549.1
Y	Tris-treated	-0.35 (18)	6547.9
Z	Tris-treated, osmotically shocked	-0.04 (18)	6548.6
W	active - [(Tris-treated, osmotically shocked)/3]	0.36 (18)	6549.6

^a Calculated from fit to values in Table I (see text); units are electrons. ^b Error estimated to be 0.3 eV. ^c Synthesized from curves X and Z.

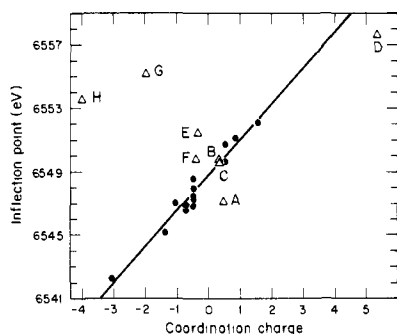


Figure 3. Manganese K-edge inflection point energy vs. coordination charge for complexes containing manganese with mixed valence, high oxidation state, or ligands providing π overlap (points A-H) (see Table II for label description).

I and Figure 2 for compounds in which manganese is simply ligated by σ interactions shows a significant dependence of edge position with charge. Also evident is a general grouping of the Mn(II) compounds and a gap before encountering higher oxidation states. Closer examination shows five compounds with the same coordination charge but different edge energies (labeled 6-10). This spread in points is not due to uncertainties in edge determination but to the simplistic coordination charge calculation which treats all oxygen ligands the same, when in reality they have different properties. This inherent spread must be considered a very real limitation to the technique and, in practice, tends to reduce the reliability of using the method to predict oxidation states.

This effect is even more evident when examining compounds which have unusual coordination environments (see Table II and Figure 3). Points A, B, and C represent complexes which have manganese in more than one oxidation state. It has been observed that edges of this type exist as composites.²² However, the coordination charges for these three complexes were calculated from the average coordination environment of the manganese sites.

Thus, if the resolution of edge features is sufficient to give separate inflection points for both oxidation states, selection of the first inflection point can be expected to give erroneous results. For Mn_3O_4 (labeled A in Table II and Figure 3), which exhibits two edge inflections, this seems to be the case. Mn_3O_4 contains one Mn(II) and two Mn(III) sites. The second inflection point of the edge is included in brackets in Table II opposite the coordination charge calculated for the Mn(III) site. This value is in much better agreement with the fit from Figure 1 (not shown). For Mn-(3,4)bpy and Mn(3,4)phen (labeled B and C), the separate edges are apparently not resolved, and an average edge position is obtained in good agreement with the σ -bonded complexes.

A second condition under which this approach may break down is the occurrence of manganese in high oxidation states. However, we observe that $KMnO_4$ (labeled D) is in fair agreement with the extrapolated line of Figure 3. The electronegativity value for Mn(II) was used in the calculation due to the unavailability of values for Mn(VII) and is the likely cause for an erroneously large coordination charge. In addition, coordination charge values for complexes which involve metal-ligand interactions can be expected to underestimate the amount of charge donated by the ligands. The four observed compounds of this type (labeled E, F, G, and H) show a significant deviation from the predicted line and in the direction expected.

Thus, the following limitations for estimation of coordination charge values of manganese in chloroplasts must be kept in mind. The presence of multiple oxidation states will provide an average coordination charge value unless individual edge inflection points can be discerned. For the chloroplast edges reported here, in which no indication of multiple edges is evident, average coordination charge values will be expected. In addition, if the manganese complex in chloroplasts contains ligands which are π donors, artificially large estimates of coordination charge will be made. The most likely biological complexes in which π interaction may be expected are the manganese porphyrins. However, complexes of this type have not been considered likely candidates as the moderately strong charge-transfer bands have not been observed in chloroplasts and would not be expected to reversibly release manganese upon Tris treatment.

With these limitations in mind, a number of inferences concerning the oxidation state and ligand symmetry can be drawn for chloroplast manganese. It is evident that the difference in edge shape observed for active and Tris-treated chloroplasts shown in Figure 1 reflects a significant change in the electronic environment of the metal. The appearance of the relatively narrow transition at the edge for the Tris-treated sample, which is characteristic of Mn^{2+} ion, is in marked contrast to the broad, featureless edge of active chloroplasts. The latter is much more reminiscent of the edge of manganese(III) tris(acetylacetonate).

We have observed that with the exception of α -MnS, all complexes of Mn(II) measured in this study exhibit a large absorption maximum at the K edge such as observed for $Mn(H_2O)_6^{2+}$ and that none of the observed higher oxidation state complexes of Mn have such a feature. These trends have been seen for complexes of various metals²² in which a pronounced broadening of the K absorption edge appears to be correlated to a loss of symmetry at the metal site. In this instance, the onset of Jahn-Teller distortion with higher oxidation states may be sufficient to cause a significant broadening of "p" states responsible for the absorption maximum and produce the difference observed between the $Mn^{III}(acac)_3$ and the $Mn(H_2O)_6^{2+}$ edges.²⁹ The anomaly seen with α -MnS has been reported with other metal-sulfur complexes,²² and although the effect is not fully understood, a pronounced broadening of states may be caused by the high degree of covalency of the metal-sulfur bond.

Consideration of the above trends in K-edge shape affords the following statements concerning nature of manganese in the chloroplast samples. The characteristically broad edge of untreated

(29) See, for example, Yeh, H. C.; Azaroff, L. V. *J. Appl. Phys.* **1967**, *38*, 4034, and the manganese(III) tris(acetylacetonate) spectrum in Figure 1a, which is distorted by the Jahn-Teller effect.

Table IV. Coordination Charge Calculations for Manganese Dimers with Different Combinations of Oxygen and Nitrogen Ligands

Mn oxidation state	ligands	coordination charge
II,II	6 oxy	-0.46
	5 oxy	-0.05
	2 oxy, 2 N	0.02
	3 oxy, 1 N	0.19
II,III	4 oxy	0.36
	4 oxy, 2 N	-0.30
	5 oxy, 1 N	-0.13
	6 oxy	0.04
	3 oxy, 2 N	0.11
	4 oxy, 1 N	0.28
III,III	5 oxy	0.45
	4 oxy	0.86
	2 oxy, 4 N	-0.14
	3 oxy, 3 N	0.03
	4 oxy, 2 N	0.20
	5 oxy, 1 N	0.37
III,IV	6 oxy	0.54
	5 oxy	0.95
	6 N	0.02
	1 oxy, 5 N	0.19
	2 oxy, 4 N	0.36
	3 oxy, 3 N	0.53

chloroplasts is indicative of a manganese site of low symmetry. Likely causes of this condition include a significant portion of chloroplast manganese in oxidation states higher than 2+ or a manganese site constrained to an unusual ligand configuration.

Secondly, the observably different edge of Tris-treated chloroplasts is interpreted to reflect a considerable content of manganese in the 2+ oxidation state and a somewhat more symmetric environment. This is to be expected in view of the idea that inhibition of oxygen evolution is coincident with the release of manganese to an EPR detectable aqueous state. We have, in fact, observed that the Tris-treated chloroplast edge can be reconstructed by addition of approximately two-thirds of a normalized $Mn^{10}OAc_2 \cdot 4H_2O$ edge to one-third of the Tris-treated, osmotically shocked chloroplast edge. Since Mn(III) is subject to disproportionation to Mn(II) and Mn(IV) in solution, release of either Mn(II) or Mn(III) from the membrane will result in the observed aqueous Mn(II).

Unfortunately, the intermediate appearance of the Tris-treated, osmotically shocked sample (Z of Figure 1) is not so easy to interpret. As this sample should contain that fraction of manganese not released by Tris extraction, this edge should provide information about the nature of the manganese pool not considered necessary for O_2 evolution. It appears that either this fraction contains some manganese in a site similar to $Mn(H_2O)_6^{2+}$ or that removal of the aqueous fraction by osmotic shock was not complete. Although no Mn^{2+} EPR signal could be detected in samples prepared under these conditions, it is conceivable that a portion remains which is nonspecifically bound to membrane components.

Placement of the chloroplast edge inflection point energies on the coordination charge plot (Figure 2) finds support for these ideas. The active chloroplast sample (labeled X) is in the gap between observed Mn(II) and Mn(III) complexes. This indicates that the manganese is formally of oxidation state 2+ or 3+ or a mixture of the two. The first of these possibilities is considered unlikely. Indeed, if the observed edge position were for a 2+ oxidation state, Figure 2 indicates that it would be the most ionically ligated divalent complex of this study. This is not a desirable property for a site which has been proposed to accumulate oxidizing equivalents. The concept of a mixed oxidation state is feasible for a couple of reasons. Of the five S states proposed to act as charge accumulators, S_0 and S_1 , in the ratio of 1:3, are believed to be stable in dark-adapted chloroplasts.¹⁰ Secondly, results of the extended X-ray absorption fine structure (EXAFS)¹⁴ on these samples indicate a dimeric manganese site. Thus, the presence of Mn(II)-Mn(III) and/or Mn(III)-Mn(III)

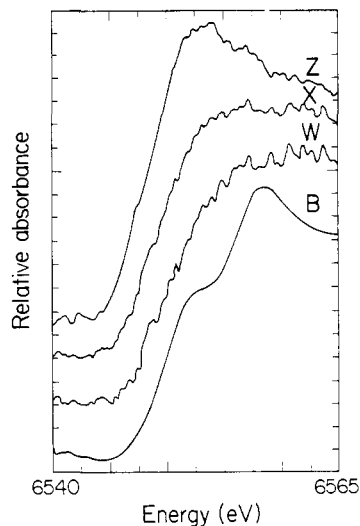


Figure 4. X-ray absorption K edges for manganese in chloroplasts. Z denotes the spectrum for Tris-washed, osmotically shocked chloroplasts, X denotes the edge for active chloroplast, and W denotes an edge constructed by subtraction of one-third of the normalized Tris-shocked chloroplast edge from that of active chloroplasts. Edge B denotes that of di- μ -oxo-tetrakis(2,2'-bipyridine)dimanganese(III,IV) perchlorate [$Mn(3,4)bpy$], which is included for comparison.

site may be indicated (see Table IV).

Points Y (Tris-treated chloroplasts) and Z (Tris-treated, osmotically shocked chloroplasts) on the coordination charge plot (Figure 2) also support the ideas presented on the basis of edge shape. Tris-treated chloroplasts, suspected to contain hexaquated Mn^{2+} , give an average coordination charge value of -0.35, a value quite consistent with the divalent compounds having six oxygen ligands. The Tris-treated, osmotically shocked edge has a coordination charge which is intermediate between that of the other two chloroplast samples. This is indicative of either a mixture of coordination environments or a divalent manganese site with a highly ionic character.

It must be kept in mind that the above interpretations reflect the environment of all manganese retained by chloroplasts after initial isolation in 1 mM EDTA. The release of only a fraction of the manganese from the membranes by alkaline Tris buffer and the resulting total loss of oxygen-evolving capability raise valid concerns about the nature and involvement of the remaining fraction of manganese.

It was indeed our intent to construct the edge for the Tris releasable fraction of manganese by subtraction of one-third of the normalized Tris-treated, osmotically shocked spectrum from that of active chloroplasts. This difference edge is shown in Figure 4. Shown also for comparison are the edges of active and "Tris-shocked" chloroplasts. Like the active chloroplast spectrum, the difference edge is broad and featureless but is shifted to slightly higher energy, giving a predicted coordination charge of 0.36 (Table III). This value is even more in line with that of observed Mn(III) compounds than is the total manganese environment considered earlier.

Also shown in Figure 4 is the edge of di- μ -oxo-tetrakis(2,2'-bipyridyl)dimanganese(III,IV) perchlorate [$Mn(3,4)bpy$]. This compound has special importance in light of the results of EXAFS analysis presented in the companion paper.¹⁴ These results show that the EXAFS of the active manganese site is consistent with a dimeric structure similar to that of the bipyridyl complex. It is seen that the difference chloroplast edge has a coordination charge quite close to that of the $Mn(3,4)bpy$ and $Mn(3,4)phen$ complexes but lacks the characteristic edge structure exhibited by these models. The absence of such edge structure in chloroplasts may reflect a significant difference in ligand environment but may also be due to disorder, as small differences in ligand symmetry will significantly reduce resolution of edge features. Possible sources of this disorder include residual heterogeneity of manganese sites and an expected equilibrium of two environ-

ments representing the S_0 and S_1 states which are stable in the absence of illumination.

To more accurately predict the oxidation state and ligands for the essential Mn, we made several coordination charge calculations, using different combinations of oxygen and nitrogen ligands. These calculations are presented in Table IV. The estimated error in the chloroplast edge determinations results in a predicted range for the coordination charge of +0.1 to +0.5. Several of these hypothetical structures are in agreement with the range of coordination charges under consideration: Mn(II,II) with four oxygen or three oxygen and one nitrogen ligands, Mn(III,III) with six ligands, the majority being oxygen, or Mn(III,IV) with six ligands, the majority being nitrogen.

The previous arguments against a Mn(II,II) complex hold because an unlikely ligand environment must be invoked to explain the observed coordination charge.

Replacement of oxy ligands with complexed water molecules will increase the observed coordination charge because of differences in the ligand donating properties. Indeed, a penta-coordinate Mn(II,III) site both agrees with the observed edge properties and provides a possible site for water substrate binding. Such a configuration is in complete accord with a model of the water splitting site proposed by Wydrzynski and Sauer.¹³ This model contains a dimeric manganese complex and exists in the dark as a 3:1 mixture of Mn(II,III) and Mn(II,II).

Conclusions

The experiments reported here and in the companion paper¹⁴ demonstrate the first direct observations of membrane-bound manganese in chloroplasts. The shape observed and coordination charge deduced for the Mn absorption edge of active chloroplasts suggest an average environment for manganese sites. This proposed environment consists of a portion of the manganese in an oxidation state higher than 2+. Both the proposed dimeric structure derived from the EXAFS analysis¹⁴ and the dark stability of more than one S intermediate of the charge storage observed cycle make a mixture of oxidation states probable. Such a mixture of oxidation states and the ensuing changes in coordination environment may well explain the absence of edge features and a coordination charge which is intermediate between that of observed Mn(II) and Mn(III) complexes. The model of Wydrzynski and Sauer,¹³ involving a manganese dimer in which a mixture of Mn(II,II) and Mn(II,III) in the ratio of 1:3 in the dark, is consistent with the average environment observed in chloroplasts.

We have observed a drastic change in manganese environment upon inactivation of oxygen evolution by Tris treatment. This

change is indicative of the previously reported release of manganese from the putative water oxidation site into the inner thylakoid space where it appears as an aqueous Mn(II).

Finally, we propose that subtraction of one-third of the Tris-treated, osmotically shocked chloroplast edge from that of active chloroplasts may provide a closer estimate of properties for the fraction of manganese sensitive to Tris release and hence to oxygen-evolution capacity. Provided that the difference edge is a valid representation of the active pool of manganese, the observed coordination charge of +0.36 virtually assures that a significant fraction of the manganese present is in oxidation states greater than 2+.

With the sensitivity of the probe of the chemical state of chloroplast manganese established, we are presently investigating effects of illumination and chemical agents known to affect water oxidation. Results of these studies will hopefully provide a view of the mechanistic involvement of manganese in photosynthetic water oxidation. A study of the Mn-containing protein, whose isolation has only recently been reported by Spector and Winget,³⁰ hopefully will provide more detailed information on the oxidation states and ligation of the Mn atoms.

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(30) Spector, M.; Winget, G. D. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 959.