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Direct Detection of Oxygen Ligation to the Mn₄Ca Cluster of Photosystem II by X-ray Emission Spectroscopy**

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Ligands play critical roles during the catalytic reactions in inorganic systems and in metalloproteins through bond formation/breaking, protonation/deprotonation, and electron/spin delocalization. There are well-defined element-specific spectroscopic handles, such as X-ray spectroscopy and EPR, to follow the chemistry of metal catalytic sites. However, directly probing particular ligand atoms like C, N, and O, especially in a large protein matrix, is challenging due to their abundance in the protein. FTIR/Raman and ligand-sensitive EPR techniques such as ENDOR and ESEEM have been applied to study metal-ligand interactions. X-ray absorption spectroscopy (XAS) can also probe the ligand environment; its element-specificity allows us to focus only on the catalytic metal site, and EXAFS and XANES provide metal-ligand distances, coordination numbers, and symmetry of ligand environments. However, the information is limited, because one cannot distinguish among ligand elements with similar atomic number (i.e. C, N, and O). As an alternative and

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a more direct method to probe the specific metal-ligand chemistry in the protein matrix, we investigated the application of X-ray emission spectroscopy (XES). Using this technique we have identified the oxo-bridging ligands of the Mn_4Ca complex of photosystem II (PS II), a multisubunit membrane protein, that catalyzes the water oxidizing reaction in photosynthesis.^[1] The catalytic mechanism has been studied intensively by Mn XAS.^[2] The fundamental challenge, however, is to learn how the water molecules are ligated to the Mn_4Ca cluster and how O-O bond formation occurs before the evolution of O_2 .^[3-5] This implies that it is necessary to follow the chemistry of the oxygen ligands to understand the mechanism.

XES, which is a complementary method to XAS, has the potential to directly probe ligation modes.^[6] Among the several emission lines, $\text{K}\beta_{1,3}$ and $\text{K}\beta'$ lines originate from the metal 3p to 1s transition, and they have been used as an indicator of the charge and spin states on Mn in the oxygen-evolving complex (OEC) (Figure 1).^[7, 8] The higher energy region corresponds to valence-to-core transitions just below the Fermi level and can be divided into the $\text{K}\beta''$ and the $\text{K}\beta_{2,5}$ emission (Fig.1 left scheme). $\text{K}\beta_{2,5}$ emission is predominantly from ligand 2p (metal 4p) to metal 1s, and the $\text{K}\beta''$ emission is assigned to a ligand 2s to metal 1s; both are referred to as cross-over transitions.^[9-11] Therefore, only direct ligands to the metal of interest are probed with $\text{K}\beta_{2,5}/\text{K}\beta''$ emission; i.e. other C, N, and O atoms in the protein media do not contribute to the spectra. In this report, we focus on the $\text{K}\beta''$ spectral region to characterize metal-ligand interactions, in particular contributions from ligated oxygens. The energy of the $\text{K}\beta''$ transition depends on the difference between the metal 1s and ligand 2s binding energies, which reflects the environment of the ligand owing to orbital hybridization. Therefore the $\text{K}\beta''$ energy is affected by the charge density on the metal, the ligand protonation state, and changes in the coordination environment. The $\text{K}\beta''$ intensity is influenced by the spatial overlap between the wavefunction that describes the Mn 1s orbital and the molecular orbitals on the ligands. The $\text{K}\beta''$ intensity is affected by the metal-to-ligand distance, and the number of ligands per metal ion. Shorter distances (e.g. from higher bond order or deprotonation) result in increased $\text{K}\beta''$ intensity with an approximate exponential dependence on distance.^[9] A spread of the molecular wavefunction over next-nearest neighbor atoms will decrease the $\text{K}\beta''$ spectral intensity. Therefore single-atom ligands such as oxo-bridges, or terminal oxo ligands bonded to Mn have predominant contributions to the spectra (see below). This combination of factors makes the $\text{K}\beta''$ spectrum a powerful tool for detection and characterization of oxo-bridges in the Mn_4Ca cluster of PS II.

However, because of the weak intensity of the $\text{K}\beta''$ spectrum, obtaining such spectra from biological samples as dilute as PS II (800 μM Mn) have been difficult. For O ligation in a typical model compound the signal is $\sim 10^3$ times weaker than that of $\text{K}\alpha$, and there is an additional large background from both the $\text{K}\beta_{1,3}$ and the $\text{K}\beta_{2,5}$ spectral features (Fig. 1). Furthermore the work is challenging because of the high sensitivity of the Mn_4Ca cluster to radiation damage.^[12] This study of PS II became possible by using a new high resolution spectrometer equipped with 8-14 analyzer crystals collecting over a large solid angle (see experimental section).

Fig. 2 shows the $\text{K}\beta''$ spectrum of a sample of PS II in the S_1 state compared with a series of Mn oxide spectra. Each spectrum is normalized by the $\text{K}\beta_{1,3}$ peak intensity which is proportional to the number of Mn atoms in the system. The peak position of the PS II S_1 state falls between those of the Mn^{III} and Mn^{IV} oxides. The energy of the $\text{K}\beta''$ feature may be influenced by charge screening effects that depend on the charge density on the Mn ion, i.e. the Mn oxidation state. However, the formal oxidation state, is only an approximation to the actual charge density.^[13]

Fig. 3 shows a comparison of the PS II S_1 state spectrum with those of a series of Mn coordination compounds: Mn^V -oxo (**a**), di- μ -oxo bridged $Mn_2^{III,IV}$ and Mn_2^{IV} (**b**, **c**), cubane-type Mn_2^{III} Mn_2^{IV} and $Mn^{III}Mn_3^{IV}$ (**d**, **e**), and a μ -alkoxide bridged Mn_2^{II} (**f**). These compounds have oxo-bridged Mn (except for Mn^V -oxo) with O or N/O terminal ligands. In Fig. 3, there is no detectable $K\beta''$ peaks from the μ -alkoxide/carboxylate bridged Mn_2^{II} complex (**f**). This is likely due to peak broadening originating from the delocalization of the oxygen 2s electron into a molecular orbital that is spread over the whole methyl/carboxylate group, i.e. atoms that are next-nearest to Mn, and to a lesser degree due to the longer Mn-ligand bridging interactions (~ 2.0 - 2.1 Å).^[14, 15] Similarly, contributions from O and N terminal ligands from carboxylates or histidine/amines are weak, because the molecular orbitals that contain O and N 2s are strongly delocalized. Moreover, terminal ligands are generally at longer distances to Mn than the bridging ligands, and their contributions are smaller.^[9] A similar effect has been observed in Fe cyanides.^[6] Theoretical studies regarding X-ray emission spectra from transition metal complexes are emerging,^[14, 15] yet at present the interpretation of the $K\beta''$ spectra has remained largely empirical. There is a strong peak in the Mn^V -oxo compound, indicating that the $K\beta''$ peak intensity is predominantly sensitive to single atom ligands and short metal ligand atom distances, namely, bridging O ligands and double/triple bonds which all have localized 2s orbitals. The sensitivity of the spectra to even one-electron changes is illustrated in Fig. 3. The spectra are different for the two binuclear and the two cubane molecules in which one of two or four Mn is oxidized from (III) to (IV). The $K\beta''$ peak of the PS II S_1 state is relatively intense compared to other $Mn_2^{III,IV}$ di- μ -oxo bridged compounds, which is suggestive that there are several μ -oxo bridged Mn-O bonds in the S_1 state.^[2]

The involvement of bridging oxo groups^[4] or the high valent $Mn^{IV}=O\bullet$ or $Mn^V\equiv O$ species^[3, 5] have all been implicated in the mechanism for the formation of the critical O-O bond in the water-oxidation reaction of PS II. As we have demonstrated here, it is now feasible to obtain such spectra for the Mn_4Ca cluster in PS II, and the outlook for $K\beta''$ spectroscopy as a tool for studying the nature of the O ligand binding modes and therefore the mechanism of the water-splitting reaction seems promising. Finally, new powerful X-ray lasers such as the LINAC Coherent Light Source, Stanford, can be used for future real-time XES studies of the S-state cycle. In contrast to XAS, XES does not require scanning of the incident X-ray beam, and with a dispersive XES spectrometer, a full spectrum can be collected at once, and the time-evolution of the spectrum can be monitored.

Experimental Section

Mn Model compounds

The Mn model compounds used were the following: a bridging μ -alkoxide Mn_2 compound ($[Mn_2^{II}(\mu-RO)(\mu-CH_3CO_2)](ClO_4)_2$ (**f**),^[16] two di- μ -oxo bridged Mn_2 compounds ($[Mn_2^{III,IV}O_2bipy_4](ClO_4)_3$ ^[17] (**b**), and $[Mn_2^{IV,IV}O_2terpy_2(SO_4)_2]\cdot 6H_2O$ (**c**),^[18] two Mn_4 cubane compounds (hexakis(μ_2 -Diphenylphosphinato)-tetrakis(μ_3 -oxo) $Mn_4^{III,III,IV,IV}$ (**d**), and [hexakis(μ_2 -Diphenylphosphinato)-tetrakis(μ_3 -oxo) $Mn_4^{III,IV,IV,IV}$] CF_3SO_3 (**e**),^[19] and a macrocyclic Mn^V -oxo complex (**a**).^[20] The compounds were prepared following published procedures.

PS II sample preparation

PS II membranes were prepared from fresh spinach.^[21] The PS II sample holders (40 μ L each, about 100) were designed to fit into both EPR and X-ray cryostats. After dark-adaptation for one hour at room temperature, the samples were predominantly in the S_1 state.

K β XES measurement

K β emission spectra were recorded on beamline 6-2 at SSRL using a crystal array spectrometer. The samples were positioned at an angle of 45° between the surface of the sample and the incident X-ray beam. They were kept in an Oxford CF1208 cryostat at a temperature of ~10 K under an ambient pressure He atmosphere. The incident X-ray beam (beam size 1×2 mm²) had a flux of ~4×10¹² photons/sec and an energy of 10.4 keV. The X-ray exposure time on the PS II samples was based on the published value for a radiation damage of less than 1%.^[9] This was also confirmed by the XANES study carried out at BL 9-3 (SSRL) by irradiating PS II samples under conditions used for the K β XES measurements (X-ray energy of 10.4 keV, samples at ~10 K). A fast shutter which opened only during data collection protected the samples during all spectrometer and sample movements. This guaranteed a precise assessment of the X-ray dose to the samples. The model compound spectra shown in Fig. 3 were recorded with an 8-crystal device^[10] using a single-element liq. N₂ cooled Ge detector. The PS II S₁ state spectra were recorded with a 14-crystal analyzer with spherically curved Si(440) crystals (10 cm diameter, 1 m radius of curvature) aligned on intersecting Rowland circles (Fig. 4). An energy-resolving Si drift detector (VortexTM) was used to reduce background mainly from elastic scattering.^[22] The emission spectrum is recorded with ~1 eV resolution.

All the spectra were normalized to the incident flux I₀ measured with a gas filled ion chamber and calibrated to the published value of 6490.40 eV for the 1st moment (integrated from 6485 – 6495 eV) of Mn₂O₃.^[11] In order to evaluate the relative intensities of the cross-over peaks, the spectra of all samples were normalized to the intensity of their respective K β _{1,3} peaks. Only a negligible difference was found when using the integrated versus peak intensity. The K β cross-over spectrum of PS II was collected between 6511 eV to 6529 eV at a step size of 0.32 eV. 1035 sweeps, each with 1 sec/step exposure (56 sec total/sweep/position), were added. Second order polynomial functions were used to fit the background of the K β cross-over region.

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Figure 1.

(A) Energy diagram of Mn Kβ transitions in MnO. The Kβ'' and Kβ_{2,5} transitions are from valence molecular orbitals; Kβ' is the O 2s to Mn 1s 'cross-over' transition. (B) Logarithmic plot of the MnO Kβ spectrum. The O-Mn cross-over Kβ'' transition is highlighted.

Figure 2. Mn $K\beta''$ emission spectra from Mn oxides and the PS II S_1 state. These spectra referred to as cross-over peaks are assigned to emission from ligand 2s2p levels to the Mn 1s core level.

Figure 3. The $K\beta''$ emission spectra from a series of multinuclear Mn complexes with oxo-bridging groups, a Mn^V-oxo complex and PS II in the S₁ state. The cross-over peak from the O ligand is prominent when short bridging Mn-O distances are present.

Figure 4.

Schematic drawing of the setup for X-ray emission spectroscopy (right) and the 14-crystal analyzer (left). Only 7 analyzers are shown in the schematic. 14 spherically curved Si(440) crystals, the detector and the sample are in an approximate Rowland circle geometry. The simultaneous vertical movement of the analyzer (d mm) and the detector (2d mm) changes the Bragg angle of the Si crystals with respect to the emission from the sample and thus the wavelength of detection.