X-ray Spectroscopy

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Direct Detection of Oxygen Ligation to the Mn_4Ca 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 and breaking, protonation and deprotonation, and electron and spin delocalization. There are well-defined element-specific spectroscopic handles, such as X-ray spectroscopy and EPR spectroscopy, to follow the chemistry of metal catalytic sites. However, directly probing particular ligand atoms such as C, N, and O, especially in a large protein matrix, is challenging owing to their abundance in the protein. FTIR and Raman spectroscopy and ligand-sensitive EPR spectroscopy 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 it is impossible to distinguish among ligand elements with similar atomic number (i.e. C, N, and O). As an alternative and 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 manganese 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 monitor 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, $K\beta_{1,3}$ and $K\beta'$ 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 $K\beta''$ and the $K\beta_{2,5}$ emission (Figure 1a, left). The $K\beta_{2,5}$ emission is predominantly from ligand 2p (metal 4p) to metal 1s, and the $K\beta''$ emission is assigned to a ligand 2s to metal 1s transition; both are referred to as crossover transitions.[9–11] Therefore, only direct ligands to the metal of interest are probed with $K\beta_{2,5}$/$K\beta''$ emission; that is, other C, N, and O atoms in the protein media do not contribute to the spectra. Herein, we focus on the $K\beta''$ spectral region to characterize metal–ligand interactions, in particular contributions from ligated oxygen atoms. The energy of the $K\beta''$ transition depends on the difference between the metal 1s and ligand 2s binding energies, which reflects the environment of the ligand owing to orbital

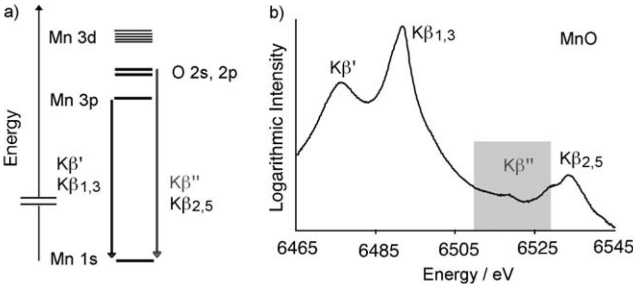

Figure 1. a) Energy diagram of Mn Kβ transitions in MnO. The $K\beta''$ and $K\beta_{2,5}$ transitions are from valence molecular orbitals; $K\beta''$ is the O 2s to Mn 1s "crossover" transition. b) Logarithmic plot of the MnO Kβ spectrum. The O–Mn crossover $K\beta''$ transition is highlighted.

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hybridization. Therefore the Kβ″ energy is affected by the charge density on the metal, the ligand protonation state, and changes in the coordination environment. The Kβ″ intensity is influenced by the spatial overlap between the wavefunction that describes the Mn 1s orbital and the molecular orbitals on the ligands. The Kβ″ intensity is affected by the metal-to-ligand distance and by the number of ligands per metal ion. Shorter distances (e.g. from higher bond order or deprotonation) result in increased Kβ″ intensity, with an approximate exponential dependence on distance.[9] A spread of the molecular wavefunction over next-nearest-neighbor atoms will decrease the Kβ″ 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 Kβ″ 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 Kβ″ spectrum, obtaining such spectra from biological samples as dilute as PS II (800 μM Mn) has been difficult. For O ligation in a typical model compound, the signal is approximately 10^3 times weaker than that of Kα, and there is an additional significant background from both the $K\beta_{1,3}$ and the $K\beta_{2,5}$ spectral features (Figure 1 b). 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 the Experimental Section).

Figure 2 shows the Kβ″ spectrum of a sample of PS II in the S_1 state compared with a series of Mn oxide spectra. Each spectrum is normalized to the $K\beta_{1,3}$ 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 Kβ″ feature may be influenced by charge screening effects that depend on the charge density on the Mn ion, that is, the Mn oxidation state. However, the formal oxidation state is only an approximation of the actual charge density.[13]

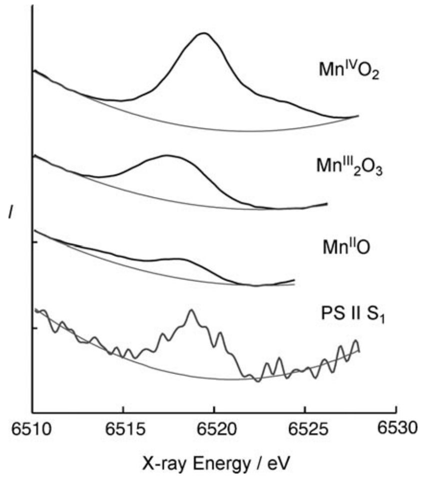

Figure 2. Mn Kβ″ emission spectra from Mn oxides and the PS II S_1 state. These spectra, referred to as crossover peaks, are assigned to emission from ligand 2s2p levels to the Mn 1s core level.

Figure 3 shows a comparison of the PS II S_1 state spectrum with the spectra 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 centers (except for Mn^V oxo and $Mn_2{}^{II}$ alkoxo) with O or N/O terminal ligands. In Figure 3, there are no detectable Kβ″ peaks from the μ-alkoxide/carboxylate-bridged $Mn_2{}^{II}$ complex (**f**). This result 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 (atoms that are next-nearest to Mn), and to a lesser degree due to the longer Mn–ligand bridging interactions (ca. 2.0–2.1 Å).[14,15] Similarly, contributions from O and N terminal ligands from carboxylates or histidines and amines are weak, because the molecular orbitals that contain O and N 2s are strongly delocalized. Moreover, terminal ligands are generally found at longer distances to Mn than the bridging ligands, and their contributions are smaller.[9] A similar effect has been observed in iron cyanides.[6] Theoretical studies regarding X-ray emission spectra from transition-metal complexes are emerging,[14,15] yet at present the interpretation of the Kβ″ spectra has

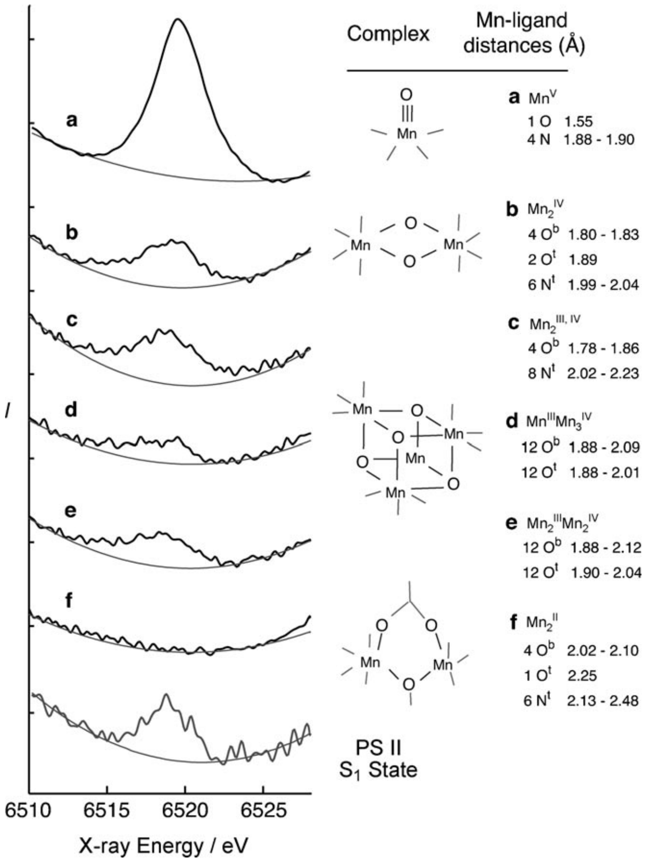

Figure 3. The Kβ″ emission spectra from a series of multinuclear Mn complexes with oxo bridging groups, a Mn^V oxo complex, and PS II in the S_1 state. The crossover peak from the O ligand is prominent when short bridging Mn–O distances are present.

remained largely empirical. There is a strong peak in the Mn^{V} oxo compound, indicating that the Kβ'' peak intensity is predominantly sensitive to single-atom ligands and short metal–ligand atom distances, namely, bridging O ligands and double or triple bonds, which all have localized 2s orbitals. The sensitivity of the spectra to even one-electron changes is illustrated in Figure 3. In both the binuclear (**c** to **b**) and tetranuclear (**e** to **d**) cases, a one-electron change results in an observable spectral difference. The Kβ'' peak of the PS II S$_1$ state is relatively intense compared to other $\mathrm{Mn_2^{III,IV}}$ di-μ-oxo bridged compounds, which suggests 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· or Mn^{V}≡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 herein, it is now feasible to obtain such spectra for the Mn$_4$Ca cluster in PS II, and the outlook for Kβ'' 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; 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

Manganese model compounds were the following: a bridging μ-alkoxide Mn$_2$ compound ($[\mathrm{Mn_2^{II}}(\mu\text{-RO})(\mu\text{-CH_3CO_2})](\mathrm{ClO_4})_2$ (**f**),[16] two di-μ-oxo bridged Mn$_2$ compounds ($[\mathrm{Mn_2^{III,IV}O_2bipy_4}](\mathrm{ClO_4})_3$[17] (**c**, bipy = 2,2′-bipyridine), and $[\mathrm{Mn_2^{IV,IV}O_2terpy_2(SO_4)_2}]\cdot 6\,\mathrm{H_2O}$ (**b**, terpy = 2,2′;6′,2“-terpyridine),[18] two Mn$_4$ cubane compounds (hexakis(μ_2-diphenylphosphinato)tetrakis(μ_3-oxo)$\mathrm{Mn_4^{III,III,IV,IV}}$ (**e**), and [hexakis(μ_2-diphenylphosphinato)tetrakis(μ_3-oxo)$\mathrm{Mn_4^{III,IV,IV,IV}}$ trifluoromethane sulfonate (**d**),[19] and a macrocyclic Mn^{V} oxo complex (**a**).[20] The compounds were prepared according to published procedures.

PS II membranes were prepared from fresh spinach.[21] The PS II sample holders (40 μL 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.

Kβ X-ray 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 approximately 10 K under an ambient-pressure He atmosphere. The incident X-ray beam (beam size 1×2 mm^2) had a flux of approximately 4×10^{12} photons per second 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 level of radiation damage was also confirmed by the XANES study carried out at BL 9–3 (SSRL) by irradiating PS II samples under conditions used for the Kβ XES measurements (X-ray energy of 10.4 keV, samples at ca. 10 K). A fast shutter which opened only during data collection protected the samples during all spectrometer and sample movements. This procedure guaranteed a precise assessment of the X-ray dose to the samples. The model-compound spectra shown in Figure 3 were recorded with an eight-crystal device[10] using a single-element N$_2$(l) cooled germanium detector. The PS II S$_1$ 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 (Figure 4). An energy-resolving Si drift detector (Vortex) was used to reduce background mainly from elastic scattering.[22] The emission spectrum is recorded with approximately 1 eV resolution.

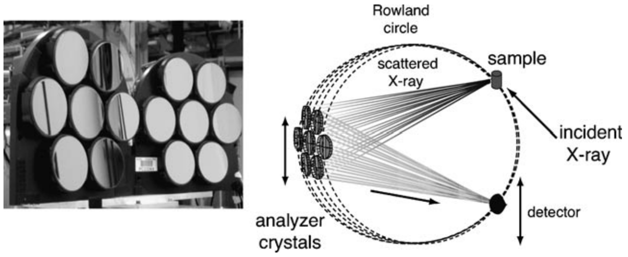

Figure 4. Schematic drawing of the setup for X-ray emission spectroscopy (right) and the 14-crystal analyzer (left). Only seven analyzers are shown in the schematic. The 14 spherically curved Si(440) crystals, the detector, and the sample are in an approximate Rowland circle geometry. The simultaneous vertical movement of the analyzer (*d* mm) and the detector (2*d* mm) changes the Bragg angle of the silicon crystals with respect to the emission from the sample and thus the wavelength of detection.

All the spectra were normalized to the incident flux I_0 measured with a gas-filled ion chamber and calibrated to the published value of 6490.40 eV for the first moment (integrated from 6485–6495 eV) of Mn$_2$O$_3$.[11] To evaluate the relative intensities of the crossover peaks, the spectra of all samples were normalized to the intensity of their respective K$\beta_{1,3}$ peaks. Only a negligible difference was found when using the integrated versus peak intensity. The Kβ crossover spectrum of PS II was collected between 6511 and 6529 eV at a step size of 0.32 eV. A total of 1035 sweeps, each with 1 s per step exposure (56 s total per sweep per position), were added. Second-order polynomial functions were used to fit the background of the Kβ crossover region.

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