Anion Binding in Biological Systems

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Abstract. We compare aspects of biological X-ray absorption spectroscopy (XAS) studies of cations and anions, and report on some examples of anion binding in biological systems. Brown algae such as Laminaria digitata (oarweed) are effective accumulators of I from seawater, with tissue concentrations exceeding 50 mM, and the vanadate-containing enzyme haloperoxidase is implicated in halide accumulation. We have studied the chemical state of iodine and its biological role in Laminaria at the I K edge, and bromoperoxidase from Ascophyllum nodosum (knotted wrack) at the Br K edge. Mo is essential for many forms of life; W only for certain archaea, such as Archaeoglobus fulgidus and the hyperthermophilic archaeon Pyrococcus furiosus, and some bacteria. The metals are bound and transported as their oxo-anions, molybdate and tungstate, which are similar in size. The transport protein WtpA from P. furiosus binds tungstate more strongly than molybdate, and is related in sequence to A. fulgidus ModA, of which a crystal structure is known. We have measured A. fulgidus ModA with tungstate at the W L3 (2p3/2) edge, and compared the results with the refined crystal structure. XAS studies of anion binding are feasible even if only weak interactions are present, are biologically relevant, and give new insights in the spectroscopy.
1. Introduction

X-ray absorption spectroscopy (XAS) studies in biology, including Extended X-ray Absorption Fine Structure (EXAFS) and X-ray Absorption Near-Edge Structure (XANES), have so far mainly focussed on the chemical environment of transition metal cations. In recent years increasing attention has been paid to metalloids, halogens, and oxyanions [1]. We discuss here some features common to the studies of cations and anions, as well as differences. Transition metal cations interact in a dynamic way with a number of electron-donating ligands in order to achieve a favourable coordination number (or secondary valence); halogens are subject to solvation and H-bonding in their anionic form, and can form single covalent bonds (primary valence) in systems where they can still be H-bond acceptors or themselves be part of a halogen bond.

Figure 1. Experimental (thin red lines) and simulated (solid lines) EXAFS (left panel) and corresponding Fourier transforms (right panel) of Cu K edge of [Cu(pyridine)$_4$(NO$_3$)$_2$] (top), I L$_3$ edge of 3-iodotyrosine (middle), and Br K edge of 4-bromophenylalanine (bottom). Insets, structures on which the simulations are based, including distances of the respective absorbers to the nearest atom. For the Cu spectrum, 2 oxygens of the weakly coordinating nitrate anion were also included in the simulation at 2.5 Å [2]; for the Br spectrum, the R group was not included, whereas for the I spectrum none of the other ring substituents was included [3].

Figure 1 shows that the complex of a transition metal ion like Cu with an aromatic ligand gives the same type of EXAFS spectrum as that of I and Br covalently bound to aromatic groups such as in the amino acids phenylalanine and tyrosine. The geometries of the aromatic systems are such that there are many sets of atoms (absorber-close backscatterer-remote backscatterer) with angles approaching 180°, which means that the photoelectron wave emanating from the absorber is likely to be subjected to multiple rather than single scattering before it returns to the absorber. All systems require the same type of simulation for the EXAFS, viz. multiple scattering with restrained refinement [4]. The Cu EXAFS is stronger because it coordinates 4 such ligands, whereas the I and Br are each bound to only one. The relative weakness of the EXAFS of elements of single valence implies that other factors that determine the XAS become more important, such as the double electron excitation contributions that have been noted for Br [5]. In the cases shown in Figure 1, the amorphous BN in which the compounds were diluted for the measurements does not appear to contribute to the EXAFS. It is
possible to discriminate between halogens bound to sp²- and sp³- hybridized carbons using EXAFS, due to shortening of the carbon-halogen bond with increasing s-character of the bonding orbital [3].

2. Halogens in biological systems

Brown algae such as Laminaria digitata (oarweed) accumulate iodine to concentrations 10⁶ times that of surrounding seawater. XAS as a non-invasive technique is the method of choice for the chemical characterization of this system because any (bio)chemistry involving the haloperoxidases that might occur upon disruption of the algae and lead to erroneous interpretations is avoided. Iodine of Laminaria gives a weak EXAFS that, by comparison to the spectrum of NaI in water, was shown to represent iodide ions with their solvation shell displaced by H-bonding to biomolecules at a comparable distance (3.5-3.6 Å) [6]. On this basis a physiological role for accumulated iodide as an inorganic oxidant is proposed.

Figure 2. Br K edge EXAFS (top panel) and Fourier transform (bottom panel) of native bromoperoxidase from Ascophyllum nodosum [7]. Top traces: experimental (thin red line) and complete simulation (solid line); bottom traces: aromatic ring component (solid blue line), nearest C (dashed line), and bromine (thin green line). Inset: dibromotyrosine structure highlighting the aromatic ring; substituents other than Br were not included in the simulation.
Figure 2 illustrates one of the ways by which haloperoxidases, in this case the vanadium-containing bromoperoxidase of *Ascophyllum nodosum* (knotted wrack), can be involved in the halogen chemistry of the algae. The Br K edge EXAFS of the native enzyme (without added Br) reveals a spectrum that is characteristic of a 1,3-dibrominated aromatic ring, with contributions of the ring at 1.90 (nearest C atom) and a remote bromine at 5.70 Å [7]. It is worth noting that the spectrum is almost entirely accounted for by the ring contribution, except for the Br atom which becomes stronger at high k; in addition, the calculated contribution of the Br is much stronger when it is part of the ring, because of the forward scattering and focusing effects of the ring carbons, than when it is on its own. Mass spectrometric analysis and a reinterpretation of the crystal structure [8] corroborate that the enzyme has (bis)hrominated one of its own surface tyrosine residues. The incorporation of the Br in the non-reactive aromatic ring rules out that it is a reactive intermediate, and the physiological significance of this phenomenon is not clear.

3. Oxyanions in biological systems

Molybdenum (Mo) is an essential metal for many forms of life, tungsten (W) only for certain archaea and some bacteria. The metals are bound and transported as their oxo-anions, molybdate (MoO$_4^{2-}$) and tungstate (WO$_4^{2-}$). The hyperthermophilic archaeon *Pyrococcus furiosus* is strictly dependent on the presence of tungstate for the expression of five W-containing aldehyde oxidoreductases. It will not [9] or only reluctantly use molybdate to incorporate Mo in the active site of these enzymes, even when present in 1,000-fold excess; this is remarkable in view of the similarity in size with tungstate.

The tungsten transport protein A (WtpA) from *P. furiosus* has now been isolated and characterized, and found to bind tungstate more strongly than molybdate by 3 orders of magnitude (K$_D$ 10$^{-11}$ vs. 10$^{-8}$ M) [10] in 1:1 stoichiometry. WtpA is related in sequence to *Archaeoglobus fulgidus* ModA of which the crystal structure with tungstate has been solved to 1.55 Å resolution [11]. Interestingly this structure features octahedral W coordination; this differs from a number of molybdate binding proteins which contain Mo in a tetrahedral geometry with Mo-O distances of 1.76 Å [12]. In crystal structures of octahedral W complexes, such as that of bis(ammonium) D-bis((R)-mandelato-O,O')-dioxo-tungsten(VI) dihydrate [13], typically 3 different (1.7, 2.0, and 2.2 Å) W-O distances are found.

Figure 3 shows the EXAFS of *A. fulgidus* ModA with tungstate till approx. 800 eV above the W L$_3$ (2p$_{3/2}$) edge [14]. The resulting k range of 14 Å$^{-1}$ affords a resolution, calculated as $\Delta R = \pi/2\Delta k$, of 0.1 Å, and allows W-O contributions at the aforementioned distances to be resolved. The major peak in the Fourier transform represents O atoms at 1.79 Å, but a shoulder at the high-R side representing O atoms at 2.24 Å is present. An interesting feature of this spectrum is the high-frequency oscillation at low k in the EXAFS, which leads to the peak at R just below 4 Å in the Fourier transform. Proteins in which the metal is coordinated by aromatic groups (cf. Figure 1) such as the imidazole residues of the amino acid histidine normally give rise to such spectra, but in the present case the imidazole would have to be very close to the W, and moreover the crystal structure gives no indication for it. The contribution might be due to heteroatoms that are weakly linked to the oxyanion by H-bonds, but simulations built on this hypothesis did not give satisfactory agreement with the experimental data.

It turns out that the high-frequency oscillation is due to multiple scattering pathways in which the photoelectron wave goes back and forth through the W absorber atom, being backscattered by O atoms on opposite sides (see inset in Figure 3, where one of the three possibilities is highlighted with arrows). This gives further corroboration that the W environment has octahedral symmetry, with O$_w$-W-O$_a$ angles approaching 180°; this geometry and the corresponding spectroscopic features are absent for tetrahedral oxyanions. Such features had hitherto only been recognized recently for square-planar Ni ions in biological [15] and chemical [16] systems. The exact position of the multiple scattering feature also allows the conclusion that there are combinations of distances W–O$_a$ and W–O$_b$ of 1.79 + 2.24 and 1.79 + 2.06 Å, but not of 1.79 + 1.79 or 2.24 + 2.24 Å. This implies that the O atoms at the shortest (1.79 Å) distance from W have *cis* orientation with respect to each other, as well as the atoms at the longest (2.24 Å) distances, and that there is an atom at the intermediate distance of 2.06 Å.
Figure 3. W L$_3$ EXAFS (top panel) and Fourier transform (bottom panel) of tungstate bound to A.fulgidus Wtpa. Experimental (thin red line), multiple scattering simulation (solid line), single scattering simulation (dotted blue line), and difference between the simulations (dashed green line). Inset, W octahedrally surrounded by O with the 4th order multiple scattering pathway highlighted for the W and the top (O_a) and bottom (O_b) oxygen atoms, and distances (Å) from the refined simulation.

4. Conclusions and outlook
XAS studies of biological anion binding are of high biological and biochemical relevance. They are feasible even if the interactions to be studied are weak or low in number, and lead to new insights in the spectroscopy. For the halogens, H-bonding to the anions as well as the sp-hybridization of covalently bound C atoms can be derived. In addition to the EXAFS highlighted above, the XANES appears to be very sensitive to the exact geometry of the oxyanions and the systems discussed here are
interesting starting points for a coherent EXAFS/XANES interpretation, including 3-dimensional arrangement of the atoms, of the XAS.

In order to complement the present results, it is now of interest to turn to the vanadate (VO$_4^{3-}$) in the haloperoxidases, as the V is expected to feature covalent bonds (to the O in the oxyanion) in addition to coordinative bonds (imidazole residue) and H-bonds (from amino acids lining the active site). The enzyme’s active site and the geometry of the vanadate are expected to undergo structural changes during the process of halide activation by hydrogen peroxide. As discussed above, XAS is perfectly suited to detect these small changes due to its high sensitivity to local structure.

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