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**Title:** Protonation of the oxygen axial ligand in galactose oxidase model compounds as seen with high resolution X-ray emission experiments and FEFF simulations

X-ray Emission Spectroscopy (XES) crossover peaks combined with FEFF8 simulations reflect the changes in the protonation state of the phenolate ligand for the Cu(II) center in models of Galactose Oxidase.

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PAPER

# Protonation of the oxygen axial ligand in galactose oxidase model compounds as seen with high resolution X-ray emission experiments and FEFF simulations

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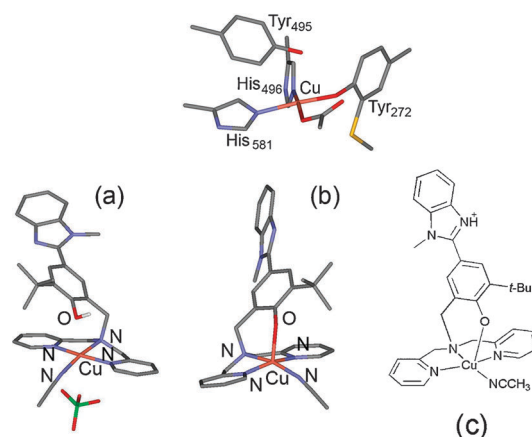
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X-ray Emission Spectroscopy (XES) crossover peaks were shown to be sensitive to the protonation state of solvent molecules in the Zn protein carbonic anhydrase and its model compounds. Here we extend such studies to galactose oxidase models *i.e.* Cu(II) open d-shell systems, illustrating that XES combined with FEFF8 simulations reflect changes in the protonation state of the phenolate ligand for the copper center.

## Introduction

Nature has invented many efficient enzymes able to perform high turnover rates. These biological macromolecules have inspired chemists for the development of new catalyst materials. *Galactose Oxidase* (GO) is a copper enzyme of fungal origin that catalyzes the oxidation of primary alcohols into aldehydes, with reduction of dioxygen into hydrogen peroxide.<sup>1,2</sup> The protein ligand provides a non-square-planar N<sub>2</sub>O<sub>2</sub> donor set for a single copper ion (Fig. 1). The three in-plane coordinating atoms are two N from histidines and one O from a tyrosine (Tyr<sub>272</sub>). As observed in some polypeptides the former residues help in maintaining the copper ion coordinated<sup>1,2</sup> in the active site, especially during turnovers. Tyr<sub>272</sub>, in its radical form Tyr<sub>272</sub><sup>•</sup>, acts as organic redox center to perform an H-abstraction from the substrate. The fourth protein ligand is an axial tyrosine residue (Tyr<sub>475</sub>) that manages the proton transfer occurring during catalysis.<sup>2,3</sup> The non-square-planar N<sub>2</sub>O<sub>2</sub> coordination polyhedron is completed by the substrate or an acetate molecule (from the crystallization buffer) that binds in the basal plane.<sup>2</sup>

Aiming to better understand the properties of the enzymes active sites and reproduce their high reactivity, bio-inspired catalysts have been developed. For example, Cu–histidine (His) complexes in aqueous solutions and in zeolites have been studied with a multi-technique approach<sup>4,5</sup> (EPR, ENDOR



**Fig. 1** Active site of GO and crystal structures of Cu(H<sub>2</sub>L<sup>Me</sup>) (a) and Cu(HL<sup>Me</sup>) (b). In (a) one perchlorate counter ion is included. Hydrogen atoms (except the phenolic one) are omitted for clarity. (c) Chemical formula of Cu(HL<sup>Me</sup>).

with DFT)<sup>†</sup> to shed light on the coordination modes between His and Cu(II). The results suggest the coexistence of several species in different proportions for different pH values, which also was confirmed by a later study<sup>6</sup> including IR, Raman, UV/VIS/NIR, ESR, and XAS.<sup>‡</sup> The imidazole binds at low pH in a monodentate manner to the metal, from the carboxylate end, and with increasing pH the coordination turns to

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<sup>†</sup> Acronyms: CA: Carbonic Anhydrase; DFT: Density Functional Theory; DOS: Density Of States; EXAFS: Extended X-ray Absorption Fine Structure; EPR: Electron Paramagnetic Resonance; ESR: Electron Spin Resonance; IR: Infrared spectroscopy; KIE: Kinetic Isotope Effect; NMRD: Nuclear Magnetic Relaxation Dispersion; UV/VIS/NIR: Ultraviolet-Visible-Near Infrared spectroscopy; XANES: X-ray Absorption Near Edge Spectroscopy; XAS: X-ray Absorption Spectroscopy; XES: X-ray Emission Spectroscopy.

bidentate. This is, however, rather different from the normal binding mode of Cu–His in proteins (by any of the nitrogens of the imidazole ring). In another study on enzyme mimicry, Cu–His was immobilized within the cages of an inorganic porous zeolite host, and the species appearing for different pH values have been investigated using UV/VIS, ESR, IR, and XAFS.<sup>7</sup> The modeled compounds are proposed to have a tridentate His coordination, at difference of the bidentate coordination proposed for Cu–His in aqueous solution.<sup>6</sup>

One of the first GO models that mechanistically mimics very well the protein activity has been reported by Wang *et al.*<sup>8,9</sup> Using combined EXAFS, XANES, EPR, crystallography, and KIE† experiments they showed that the non-square-planar geometry of the N<sub>2</sub>O<sub>2</sub> moiety is as a key feature to get a high catalytic activity. More recently the model compounds depicted in Fig. 1 were developed in order to analyze finely the structural changes resulting from deprotonation of the axial phenol.<sup>3</sup> It has been seen that formation of a stronger axial copper–phenolate bond forces the metal to move out of the square plane towards the oxygen. The ordering of the p*K*<sub>a</sub> in these complexes (benzimidazolium/phenol/coordinated water) was determined since it is important for understanding the proton transfer and ultimately, the determinants of the mechanism of GO.

XAS has proved to be a very powerful tool to determine the structural features of complexes from coordination chemistry and biology.<sup>10–13</sup> EXAFS studies on GO<sup>14,15</sup> agree with the crystal structure at a resolution of 1.7 Å.<sup>2</sup> They are consistent with a single shell of low *Z* scatterers (N or O) at about 2 Å distance to the metal, presumably in an equatorial position. A solvent or acetate molecule would be present, depending on the pH, which was identified using NMRD measurements.<sup>15</sup> This is not resolvable by EXAFS due to the similar scattering distances with higher shell atoms, and the similar phases of N and O ligands. The same problems avoid the identification of the long axial tyrosine by EXAFS. XANES has shown to be more sensitive than EXAFS to bond angles when used in combination with XANES simulations in modern codes (MXAN<sup>16</sup> and FEFF8<sup>17–21</sup>). A lot of electronic structure information is contained in the pre-edges, that is, transitions from the 1s to 3d that in lower symmetries, has also contributions from the 3d with 4p hybridization. DFT<sup>10</sup> has been used to explain the pre-edges. A more accurate description is obtained with multiplet simulations, which take into account all intra-atomic interactions.<sup>22</sup> However the Cu(II) compounds of this paper are 3d<sup>9</sup>, thus without 3d–3d interactions.

Some of the limitations presented by XAS are surmounted in emission experiments.<sup>23</sup> The peak at the lower energy side of the emission K<sub>β2,5</sub>, the so called “crossover peak”, has been assigned to the transition of the metal p character of the 2s ligand orbital to the empty 1s metal orbital.<sup>24–26</sup> That X-ray Emission Spectroscopy (XES) should be sensitive to the protonation state of the ligand was first hypothesized by Bergmann and Penner-Hahn. The first experimental proof<sup>27</sup> for the sensitivity of the crossover peak to the ligand protonation state comes from studies on the zinc enzyme carbonic anhydrase (CA) and its model complexes.<sup>28,29</sup> Indeed a solvent molecule bound to Zn<sup>27</sup> is deprotonated during turnover. Most importantly, it has been shown that the energy

position of the crossover peaks depends not on the atomic levels but on molecular levels. Consequently, different elements, such as N and O, could have contributions to the same peak. Smolentsev *et al.*<sup>21</sup> also related the crossover peaks to molecular orbitals for transition metal complexes involving an open d-shell Mn ion, showing that XES is sensitive to protonation and ligand type. While in oxides<sup>26</sup> the crossover peaks of elements with different *Z* value have unique energy positions, in molecular compounds this is not granted.

In this work XES data was collected for the K<sub>β2,5</sub> and crossover peak for two models of GO with the aim of monitoring changes in the protonation state of the axial phenol moiety. From simulations using FEFF8.2<sup>30</sup> the emission spectra and the local densities of states (LDOS) were obtained. The analysis of the LDOS showed the influence of the proton at the O axial ligand. In this work we demonstrate the sensitivity of XES to the protonation state of the axial ligand for the square pyramidal copper ion.

## Experimental

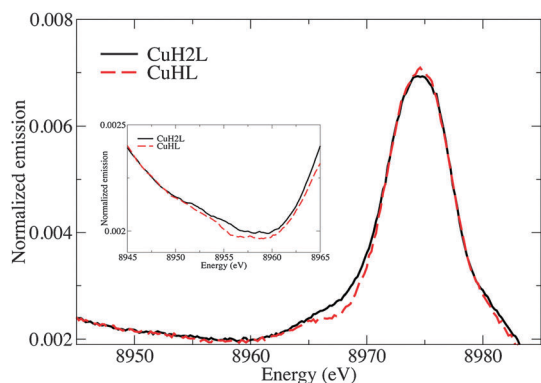
Synthesis of the copper(II) complexes of the tripodal ligand HL<sup>Me</sup> containing two pyridyl and one 2-*tert*-butyl-4-(*N*-methylbenzimidazol-2-yl)phenol unit under different protonation states have been described.<sup>3</sup> In Cu(H<sub>2</sub><sup>LMe</sup>) the phenol is protonated, whereas in Cu(HL<sup>Me</sup>) it is deprotonated (in both cases the benzimidazole is protonated, *i.e.* in its benzimidazolium form). The perchlorates, being potentially explosive, have been substituted by triflates for the experiments (structures of the perchlorate compounds are shown in Fig. 1). The samples were measured at the Taiwan IXS beamline, BL12XU in SPring8 (Japan). The samples were encapsulated in a metal holder with polyimide windows and placed in a cryostat (CRYOMECH 4 K pulse tube cryorefrigerator) at 7 K. The light source is an in-vacuum undulator providing an incoming beam of 10<sup>13</sup> phs/sec/eV. The beamline optics comprises a Si(111) double crystal monochromator, a cylindrical Si collimating mirror, and a Pt toroidal focusing mirror.<sup>31</sup> The beam size was 120 μm (H) \* 80 μm (V). Data was collected using a spherically bent crystal analyzer (Si553) and an AMPTEK X-ray detector (XR-100CR) all mounted in the IXS spectrometer with sample-detector and analyzer distance of about 1 m. A box filled with helium gas occupied all spaces between the sample and the analyzer-detector, to avoid absorption on air. Total counts per second at the maximum of the K<sub>β2,5</sub> were about 1000. Tests showed that photoreduction was negligible up to 27 min. However, in order to avoid any chance of radiation damage only one scan on each spot was collected. An excitation energy of 9200 eV was used. Data for the crossover and K<sub>β2,5</sub> region were collected. Sample duplicates were measured in a second experiment to ensure data reproducibility. The spectra were averaged and normalized to the intensity of the K<sub>β2,5</sub> for comparison.

## Simulations

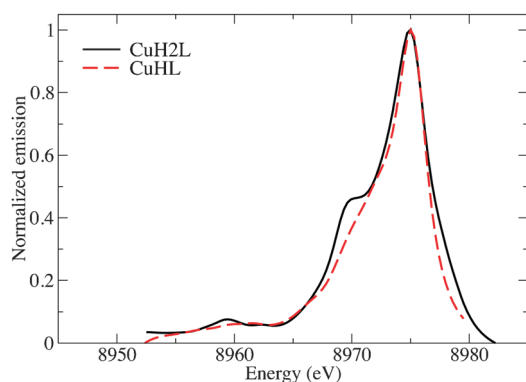
XES was simulated using FEFF8.2.<sup>30</sup> Since the crystal structure for the compounds using triflates is not available, the structure of the compounds synthesized with perchlorate

**Table 1** First shell ligand distances (Å) for the GO model compounds Cu(H<sub>2</sub>L<sup>Me</sup>) and Cu(HL<sup>Me</sup>)<sup>3</sup>

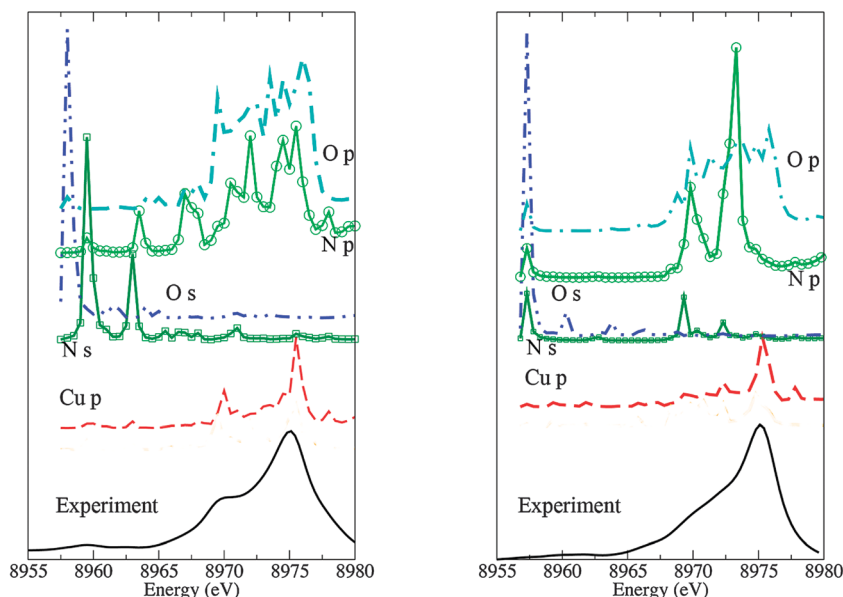
	N	N	N	N	O	O
Cu(H <sub>2</sub> L <sup>Me</sup> )	1.967	1.972	1.983	2.031	2.4	2.698
Cu(HL <sup>Me</sup> )	1.979	1.995	2.0	2.04	2.134	



**Fig. 2** XES K<sub>β2,5</sub> and zoom of the crossover region for the GO models compounds Cu(H<sub>2</sub>L<sup>Me</sup>) (solid) and Cu(HL<sup>Me</sup>) (dash).



**Fig. 3** FEFF8 simulations of XES for the GO model compounds Cu(H<sub>2</sub>L<sup>Me</sup>) (solid) and Cu(HL<sup>Me</sup>) (dash).



**Fig. 4** XES simulation from FEFF8 (solid line at bottom) and densities of states (upper lines) of Cu(H<sub>2</sub>L<sup>Me</sup>) (left) and Cu(HL<sup>Me</sup>) (right).

salts<sup>3</sup> was used as a starting point. The perchlorates were replaced by triflates using Spartan,<sup>32</sup> preserving the orientations and bond distances (Table 1). Also a simulation without any counter ion was performed, in order to analyze its influence. Full multiple scattering within a single molecular unit was included and a self consistent field calculation was performed before calculating XES and the LDOS. The ground state and the Hedin Lundqvist potential were used. A small broadening of 0.5 eV was added to account for experimental broadening.

## Results and discussion

The experimental XES spectra of the GO model compounds Cu(HL<sup>Me</sup>) and Cu(H<sub>2</sub>L<sup>Me</sup>) are very similar. The K<sub>β2,5</sub> peak differs in the feature at the low energy side of the peak in Cu(H<sub>2</sub>L<sup>Me</sup>), and in the broadening. The crossover peak for Cu(HL<sup>Me</sup>) is not visible in the experiment, while for Cu(H<sub>2</sub>L<sup>Me</sup>) a tiny peak can be seen (Fig. 2).

The XES simulations using the real space multiple scattering theory using FEFF8.2 calculate the spectrum considering a dipole transition. The simulations reproduced fairly well the distinct features observed for the two compounds (see Fig. 3). The tiny peak at about 8958 eV for the protonated compound Cu(H<sub>2</sub>L<sup>Me</sup>) appears in the simulation as well.

XES measures the occupied density of states (Fig. 4). These are calculated in FEFF8 using a self-consistent field calculation. Though the “muffin-tin” potential used in FEFF lacks the accuracy of a full potential calculation, the agreement of the XES simulation with experiment is good, also taking into account the large spectral broadening due to the 1s core hole. Within these limits the calculated LDOS can shed some light on the origin of the observed spectra:

(a) The crossover peak has been formerly assigned to the transition from the 2s level in the ligand to the 1s level in the metal.<sup>24–26</sup> This transition is allowed due to the presence of copper p-DOS in the 2s level of the ligand. A shorter Cu–ligand distance increases the overlap of the p-orbitals with



the ligand 2s orbitals, thereby yielding more copper p-character into the ligand 2s-band and as such a larger intensity.<sup>22,33</sup> In Cu(H<sub>2</sub>L<sup>Me</sup>) this interpretation is compatible with the calculated DOS: the Cu p DOS has a good match with N s at about 8959 eV and 8963 eV. The O sDOS has a much lower match to the Cu s and p DOS than the N sDOS, due to the longer O–Cu bond, thus giving a reduced contribution to the lower energy side of the crossover peak in the protonated sample. In Cu(HL<sup>Me</sup>) the “flat” crossover area could be assigned to the tiny Cu p DOS at about 9859 eV and 9862.5 eV.

(b) Deprotonation of the O ligand leads to a stronger Cu–O bond with the Cu going out of the square plane and towards the oxygen. This change in the geometry affects mostly the closest ligand, that is the N. Thus, the effect on the XES spectrum of the protonation of the oxygen seems to be indirect. However more details calculations using *ab initio* DFT would be needed to confirm a more important role of the Cu p DOS with respect to the N ligand s DOS in the crossover peak.

## Conclusions

XES is shown here to be sensitive to the protonation of coordinating ligands, which is not achievable by EXAFS. The X-ray emission experiment on model compounds for the galactose oxidase active site shows the sensitivity of the technique to the protonation of the axial phenolate. The tiny experimental crossover peak for the Cu(H<sub>2</sub>L<sup>Me</sup>) and the differences at the K<sub>β2,5</sub> peak could be successfully simulated using the real space multiple scattering theory as in FEFF8.2. Interestingly, protonation of the coordinated phenolate of Cu(HL<sup>Me</sup>) induce changes in the planarity. The Cu atom is shifted out of the basal plane, whereas the Cu–O axial bond distance dramatically increases (see Table 1). Although the protonation state of the phenolate oxygen cannot be tested directly by the crossover peak (due to the long oxygen-metal distance), the changes induced in geometry of the surroundings of the closest ligand DOS, the nitrogen, seem to be reflected in the tiny crossover peak. FEFF reproduces the main features of the XES spectra, which in this case come from strong changes in the geometry. However fine details of the electronic structure (like an accurate description of the hybridization) from the DOS require *ab initio* DFT calculations.<sup>33,34</sup> This is a first example of a more systematic study that needs to be done on molecular compounds where changes in the geometry induced by changes in the electronic structure of ligands far from the metal center, are reflected “indirectly” in the crossover peak from the closest ligand.

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