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**Biological x-ray absorption spectroscopy (BioXAS): a valuable tool for the study of trace elements in the life sciences**

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**Abbreviations**

BioXAS	biological x-ray absorption spectroscopy
DFT	density functional theory
EM	electron microscopy
EXAFS	extended x-ray absorption fine structure
MAD	multiple wavelength anomalous dispersion
NMR	nuclear magnetic resonance
PSII	photosystem II protein
PX	protein crystallography
QM/MM	quantum mechanics / molecular mechanics
SAD	single wavelength anomalous dispersion
XANES	x-ray absorption near edge structure
XAS	x-ray absorption spectroscopy
XRF	x-ray fluorescence

**Abstract**

Using x-ray absorption spectroscopy the binding modes (type and number of ligands, distances and geometry) and oxidation states of metals and other trace elements in crystalline as well as non-crystalline samples can be revealed. The method may be applied to biological systems as a ‘stand-alone’ technique, but it is particularly powerful when used alongside other x-ray and spectroscopic techniques, and computational approaches. In this review we highlight how biological x-ray absorption spectroscopy is being used in concert with crystallography, spectroscopy and computational chemistry to study metalloproteins in crystals, and report recent applications on relatively rare trace elements utilised by living organisms and metals involved in neurodegenerative diseases.

## **Introduction**

There is increasing interest in the biochemistry of metals and other trace elements in biosystems [1] and in the high-resolution structures of biomolecules containing such elements. Biological x-ray absorption spectroscopy (BioXAS) brings its own unique contributions to the list of experimental methods used by structural biologists and, while it may be used as a 'stand-alone' technique, it is best used in combination with or as a complement to other spectroscopic or structural methods. For example, interactions of chaperone proteins with their physiological partners are often studied by NMR with a role for XAS in identifying metal environments [2], such as in the Fe receptor pyoverdine [3]. Similarly, a combination of XAS and isothermal calorimetry was used to examine metal and anion binding affinities of the NikR receptor [4]. BioXAS (see Figure 1) has developed as an ideal synchrotron based technique to obtain accurate and precise atomic ('small molecule') resolution information on the chemical environments and oxidation states of trace elements in biological systems.

## **Combined methods**

*Identification of metal redox states in crystals.* Correct assignment of the oxidation states of metals is critical to our understanding of metalloprotein structure-function. The ability to distinguish between one-electron changes of metal atom oxidation states is a fundamental property of XAS and BioXAS is an important tool for identifying metal oxidation states in metalloprotein crystals during crystallographic experiments. In recent years single crystal microspectrophotometry has also been used on synchrotron x-ray beamlines [5,6] to enable *in situ* observations of the chemical states of metals during crystallographic experiments, for example on haem proteins

[7] and methylamine dehydrogenase-amicyanin complexes [8]. Recently, single crystal XAS was allied with both PX and *in situ* optical measurements to help unravel the different oxidation states of the two distinct copper centres in nitrite reductase [9]. Figure 2 illustrates this multi-technique application, which provided firm evidence for an ‘ordered’ mechanism for this class of enzyme. BioXAS may also usefully combine with on-line Raman spectroscopy [10] and PX, for example in ligand binding and identification [11].

Einsle et al [12] have pointed out how information normally ignored in SAD/MAD experiments, namely the detailed structures and positions of the XANES, may be exploited to allow individual metal redox states in metalloproteins to be assigned. Provided photoreduction effects can be minimised, such an approach is applicable to metalloprotein crystals and is particularly useful for proteins containing multiple metal sites and oxidation states.

*Radiation damage to metal sites in crystals.* X-ray damage to metalloprotein active sites has been a major concern in BioXAS experiments ever since the earliest synchrotron experiments were made, while more recently [13,14] XAS has been used to examine specific damage to anomalously scattering atoms used in SAD/MAD experiments to help devise suitable crystallographic data collection strategies.

The consequences of x-ray induced damage have been clearly illustrated by the Mn<sub>4</sub>Ca cluster that is at the heart of the oxygen-evolving centre of photosystem II (PSII). Crystal structures have been reported for PSII at modest resolution (3.0 – 3.8 Å), where interpretation of electron density at the metal cluster has been aided by XAS data [15-19]. However, radiation damage to the metal cluster is significant, as shown using XAS on PSII membrane multilayers [20] and PSII single crystals [21].

The latter showed that the functional oxo-bridged  $\text{Mn}_4\text{Ca}$  cluster in the S1 dark state was reduced from  $\text{Mn}_4(\text{III}_2\text{IV}_2)$  to a  $\text{Mn}(\text{II})$  aqueous form with an order of magnitude lower x-ray dose than that used in the determination of the crystal structure. These results have clear implications for structural work on metalloproteins, where crystallographic and XAS data collection strategies must be chosen to minimise or eliminate radiation damage. In addition to on-line spectroscopy, one approach is to use liquid helium cryo-cooling on PX and XAS beamlines. The rate of photoreduction at a metal site is in general strongly dependent on temperature, as shown in a single crystal XAS study on the  $\text{Fe}_2\text{S}_2$  site of putidaredoxin [22], where damage was observed at 110 K but not at 40 K even for an eight-fold increase in dose. The crystal structures obtained at 110 K suffered similar damage. Liquid helium cryo-cooling has been recommended as a means of reducing x-ray damage to PSII crystals [21], although XAS studies on PSII membrane particles [20] and on a di( $\mu$ -oxo) $\text{Mn}(\text{III})\text{Mn}(\text{IV})$  model compound [23] showed rapid reduction to  $\text{Mn}(\text{II})$  even at 20 K.

These studies show that whenever possible steps should be taken to monitor the extent of x-ray damage during PX experiments. SAD/MAD beamlines are equipped with x-ray fluorescence detectors and frequent in-situ ‘sampling’ of the state of the metal site should be made during PX experiments by measuring the XANES at a fixed crystal orientation. This approach, also incorporating additional on-line spectroscopic methods, is becoming indispensable as synchrotron sources become more intense.

*BioXAS and computational chemistry.* By providing sub-atomic resolution information that is local to the metal environment, BioXAS is a valuable ally of

computational chemistry. There has been progress in recent years in developing theoretical methods to study protein structures locally at metal binding sites and at locations of catalytic activity [24,25]. Recent applications combining XAS with computational chemistry have included studying the unusually high redox potential of copper in rusticyanin [26], the geometric and electronic properties of the red copper site in nitrocyanin [27] and the properties of an Fe-Fe hydrogenase active site in four protonation states involved with hydride binding [28]. Polarised single crystal XANES and time dependent DFT were used to examine the origins of electronic transitions of high valent Mn relevant to the catalytic cycle of PSII [29]. XAS has also been used with MD to identify high affinity  $Mn^{2+}$  binding sites on the extracellular region of bacteriorhodopsin near the retinal pocket [30]. Sulphur K-edge XAS was used with DFT to probe ligand-metal bond covalency and the electronic structure and reactivity of metal-sulphur sites in proteins [31-33]. In these types of studies, XAS provides the accurate metrical data that can be used to both gauge the success of the theoretical treatments and to develop combined XAS-MM/QM refinement methods [34,35]. Crystal structures alone are less accurate for these purposes unless atomic resolution ( $< 1.2 \text{ \AA}$ ) data are used; however, such data comprise less than 1 % of the metal containing protein structures deposited in the Protein Data Bank. A possible advance here would be to marry BioXAS, including polarized XANES [36] with crystallography [37] to generate the accurate three dimensional atomic resolution structures of metal centres in proteins that are required by computational chemistry.

### **Exploring the Periodic Table**

*Halogens.* One group of elements whose biological chemistry has only recently been

investigated with XAS is that of the halogens. Brown algae such as oarweed (*Laminaria*) accumulate iodine to concentrations  $10^6$  times that of surrounding seawater. Using I and Br model compound EXAFS (Fig. 3) [38], it was possible to discriminate between halogens bound to  $sp^2$ - and  $sp^3$ - hybridized carbons, due to shortening of the carbon-halogen bond with increasing s-character of the bonding orbital.

Iodine XAS of *Laminaria* has a weak fine structure that, by comparison to the spectrum of NaI in water (Fig. 3, left), was shown to represent iodide ions with their solvation shell displaced by H-bonding to biomolecules at a comparable distances [39]. On this basis a physiological role was proposed for accumulated iodide as an inorganic oxidant. The advantage of XAS as a non-invasive technique became obvious when these data were compared to that for lyophilized *Laminaria* treated with  $H_2O_2$ , where a stronger signal arising from covalent bonding to an aromatic residue, presumably a cell wall polyphenol, was observed (Fig. 3C).

A related spectrum (Fig. 3E) was observed for the Br EXAFS of the vanadium-containing bromoperoxidase from another brown algae, the 'knotted wrack', which showed that this enzyme halogenates one of its own surface tyrosine residues [40]. While the physiological significance is unclear, assigning the Br bond to a non-reactive aromatic ring rules out alternative interpretations involving reactive Br-C or Br-O bonds.

*Metalloids.* Arsenic is toxic to humans yet may be processed by other living organisms. The reduction of As(V) to As(III) by the bacterium *Geobacter sulfurreducens* during formation of biogenic magnetite was shown by Fe-edge x-ray magnetic circular dichroism and Fe- and As-edge EXAFS [41], to have a role in the



release of As into drinking water reservoirs, such as those in the Bengal delta. A study of As metabolism in the hyper-accumulating fern *Pteris vitata* using XAS with imaging [42] found that while As is present in the leaf vein as arsenate and in the leaf tissue as arsenite, the As directly surrounding the vein has thiolate coordination. Using As and Se edge XANES, it was shown that seleno-bis(*S*-glutathionyl) arsinium ions,  $[(GS)_2AsSe]^+$ , are formed from added arsenite and selenite in rabbit blood [43], an important process in arsenic detoxification. XAS of the Mo, Se and As absorption edges was used to study interactions between the Mo centre of the enzyme dimethyl sulfoxide reductase from *Rhodobacter sphaeroides* and the product analogues, dimethyl selenide and trimethyl arsenide [44].

*Chromium* probably enters cells in its highest oxidation state, Cr(VI), and subsequent reduction to Cr(III) is the reason for its toxicity and carcinogenicity. Cr(III) is thought to be involved in anti-diabetic activity. The proposed mediator for this activity is chromodulin, earlier characterized by EPR and EXAFS [45]. However, in a more recent XAS study on the effect of Cr(VI) on whole cells [46], chromium was found to be present exclusively as Cr(III) bound to proteins mainly by O donor ligands, while the XAS data ascribed earlier to chromodulin was found to be characteristic of compounds in which Cr-O bonds have been hydrolysed. Furthermore, chromodulin is not found in whole cells, so it is more likely to be an isolation artefact rather than an intracellular messenger.

The power of XAS as a non-invasive technique may be further exploited by using synchrotron x-ray fluorescence microscopy to quantitatively map elemental distributions at sub-micron resolution in fully hydrated biological samples, including studying metal imbalances involved in neurodegenerative diseases [47] and whole

cells [48]. Alongside  $\mu$ -XANES this approach provides information on the oxidation states and coordination environments of metals as well as the speciation of trace elements, toxic metals and therapeutic metal complexes [49].

*Metals in neurodegenerative diseases.* Metal dyshomeostasis is a significant factor in many neurodegenerative diseases, through mechanisms that include oxidative damage via abnormal protein metal chemistry and/or the formation of aggregates or fibrils. Proteins include amyloid-beta ( $A\beta$ ) in Alzheimer's disease (AD), Cu-Zn superoxide dismutase (SOD1) in amyotrophic lateral sclerosis (ALS) and prion protein (PrP) in human Creutzfeldt-Jakob disease and bovine spongiform encephalopathy in cattle. There is evidence that binding of copper to amyloid precursor protein (APP) leads to reduction of  $A\beta$  production and lowers AD progression [50]. A combination of PX and solution XAS [51] was used to examine the  $Cu^{2+}$  coordination environment in APP, which was found to be a solvent exposed type 2 copper centre capable of docking exogenous ligands or protein binding partners. Zinc is also thought to have a toxic effect through interactions with  $A\beta$  protein by inducing misfolding and aggregation. Using time-resolved freeze quench XAS and stopped-flow kinetics [52] Zn was shown to interact rapidly with  $A\beta_{1-40}$  and stabilise toxic oligomeric forms of  $A\beta$  that precede formation of more benign amyloid fibrils. Copper binds to cellular Prp and, while its role in protein function is unclear, XAS has been used extensively to characterise the copper binding sites in full length [53] and truncated protein [54,55], and used in combination with MM/QM methods [53,56], while Ni(II) has been shown by XAS to be a poor diamagnetic mimic for Cu(II) in PrP and therefore unsuitable as a substitute for NMR studies [57]. A combination of XRF, XAS and PX was used to unravel the metallation states of

human SOD1 that are relevant to understanding properties of ALS causing mutants [58].

## **Conclusions**

This review has highlighted the wide range of biological systems that are being tackled using XAS, to study trace elements and metals found in proteins, human diseases, plant biochemistry and toxic waste. The recent impact of BioXAS on structural biology has received much of its impetus from the integration of multiple experimental methods on synchrotron beamlines. This includes spectroscopic as well as x-ray methods, so that existing beamlines are being upgraded to incorporate optical and Raman instruments for on-line applications alongside XAS and PX capabilities. The future also looks to the use of imaging on microfocus BioXAS beamlines to study large molecules and whole cells. These enhancements to beamlines are bringing a wider community of structural biologists to make a critical examination of metalloprotein crystal structures and trace elements in biomolecules in terms of well-defined oxidation states, metal identification and distribution, and effects of x-ray damage. This combined approach, aided by new technological developments like rapid x-ray detectors, is almost mandatory on the latest generation of synchrotron sources.

## Figure Captions

Figure 1: Summary of the BioXAS approach showing the variety of samples that can be used, the main synchrotron beamline components, and the range of combined experiments that can be conducted, the information content available and a list of complementary methods. XAS measures the energy dependent absorption spectrum of a specific atom, which depends on the physical and chemical state of the absorbing atom and its environment. The x-ray absorption near-edge spectrum (XANES) reveals the oxidation state and symmetry of the metal atom's local environment, while the extended x-ray absorption fine structure (EXAFS) provides the number, type, and distances of other atoms bound to it. Combined with PX and *in situ* spectroscopic methods these techniques are able to yield three-dimensional metal site structures at 'small molecule' resolutions in well-defined oxidation states.

Figure 2: Electron-gating experiments in a single crystal of *Alcaligenes xylosoxidans* nitrite reductase using *in situ* optical, XAS and PX. **A**: optical spectra showing x-ray induced reduction of the type-1 Cu site using 1.38 Å radiation over a period of 8 mins; **B**: optical spectra before and after PX data collection, completed in 17 mins, using 0.97 Å radiation; **C**: XAS spectra sampled during PX data collection, showing that the type 2 Cu site remains oxidised in the period during reduction of the type-1 Cu site; **D**: 2Fc-Fo electron density map of type-2 Cu site after PX data collection. These data show that intra-molecular electron transfer from photo-reduced type-1 Cu to type-2 Cu is gated in nitrite reductase. Adapted from Hough et al. 2008 [9].

Figure 3: Phase-corrected Fourier transforms of I (left,  $k$  range 3.0-9.5  $\text{\AA}^{-1}$ ) and Br (right,  $k$  range 3.0-13.5  $\text{\AA}^{-1}$ ) K-edge EXAFS of **A**, fresh *Laminaria digitata*; **B**, 20 mM aqueous NaI; **C**, lyophilized *Laminaria* rehydrated with dilute  $\text{H}_2\text{O}_2$ ; **D**, 3,5-dibromotyrosine; **E**, *Ascophyllum nodosum* bromoperoxidase; **F**, 4-bromophenylalanine. Insets: structures with red dashed lines highlighting the structural significance of the shells resolved in the Fourier transform (solid red lines). Adapted from [38-40] with permission of the publishers (Blackwell, American Chemical Society, National Academy of Science-USA).

Fig 1

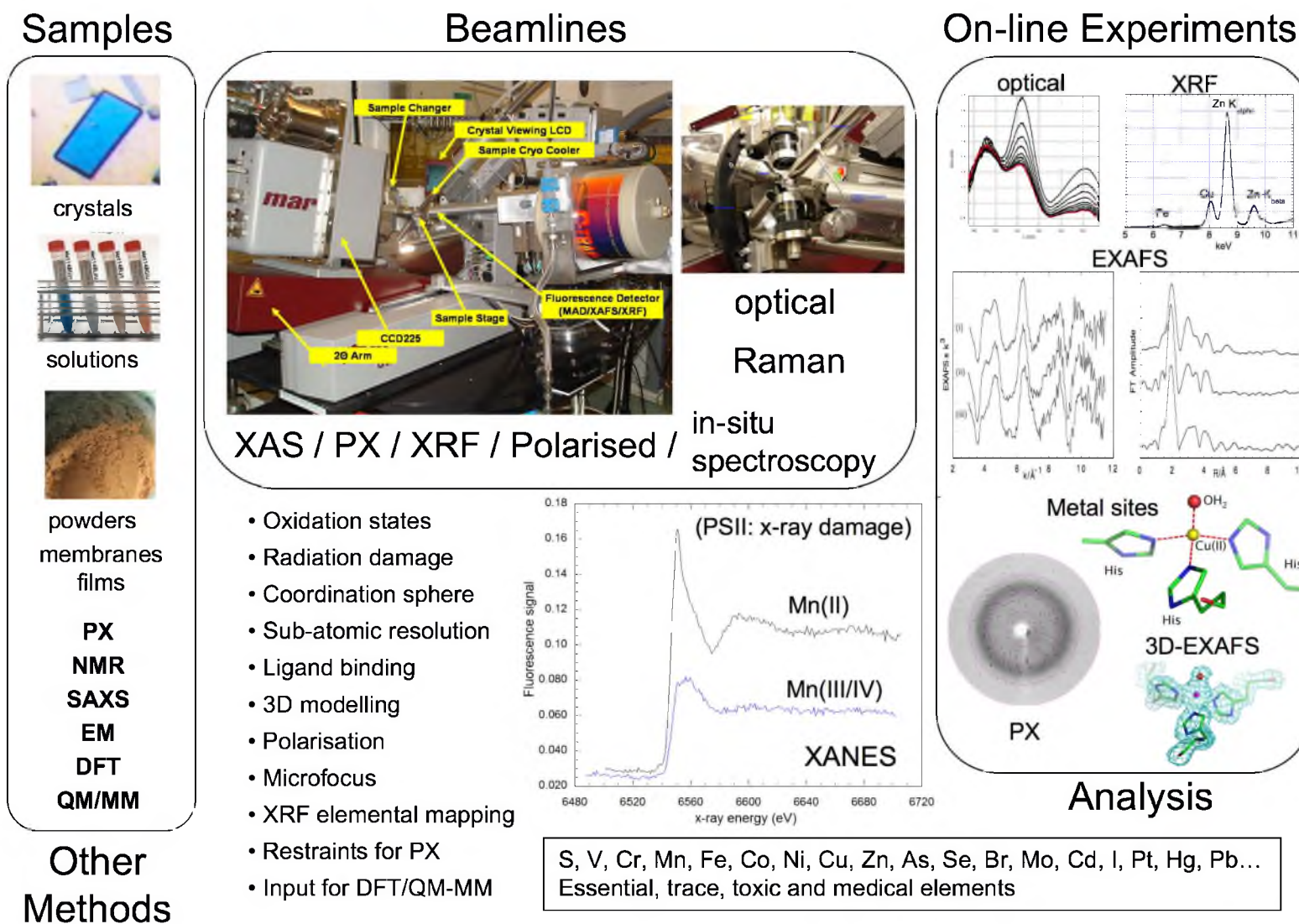


Figure 2

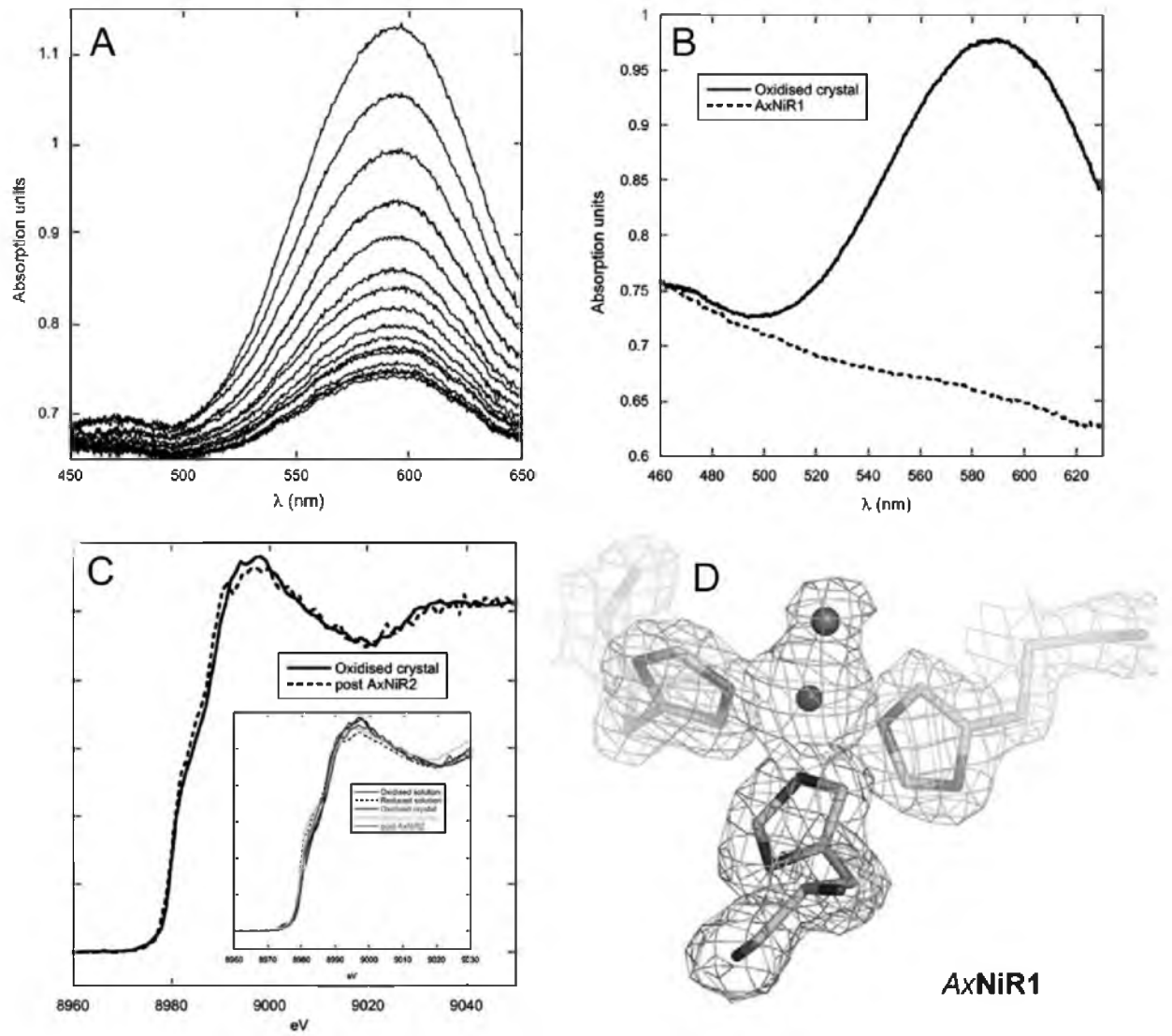
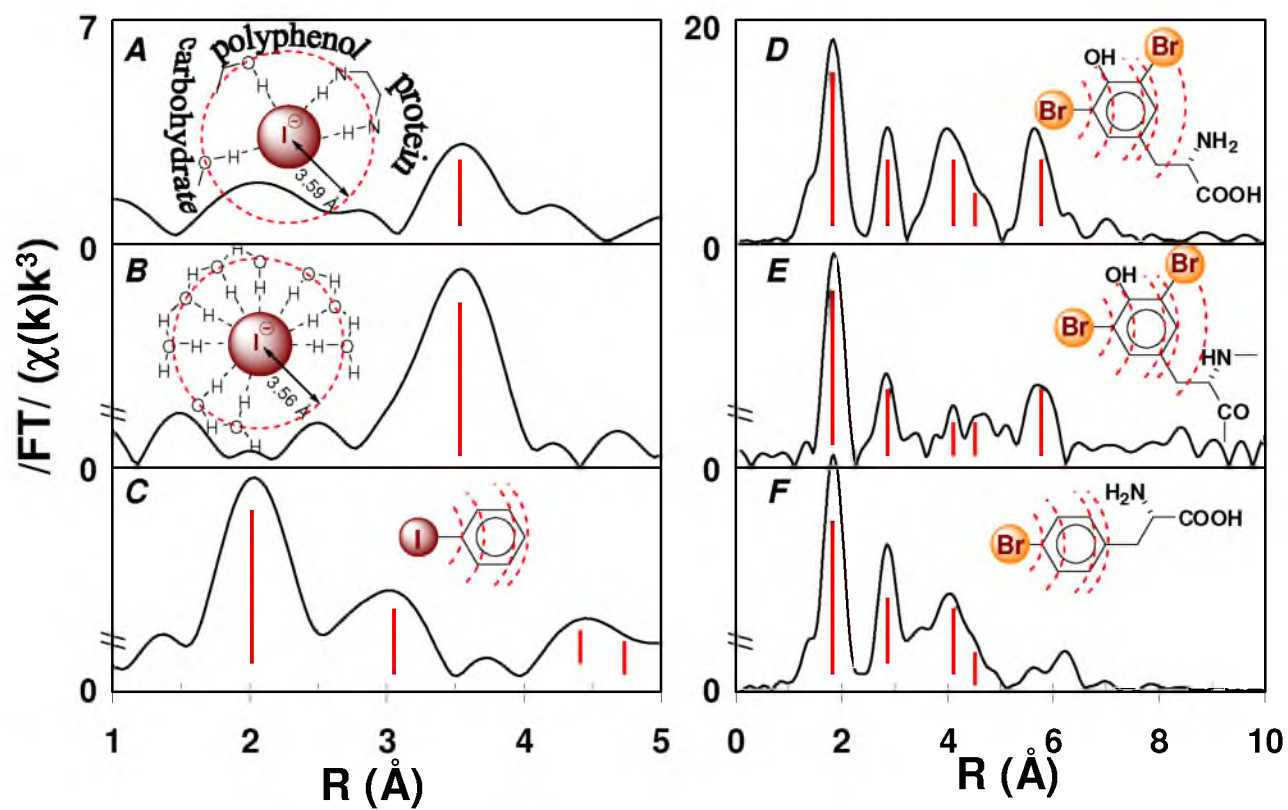


Figure 3.





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