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Structure and Dynamics of Metalloproteins in Live Cells

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Abstract

X-ray absorption spectroscopy (XAS) has emerged as one of the premier tools for investigating the structure and dynamic properties of metals in cells and in metal containing biomolecules. Utilizing the high flux and broad energy range of X-rays supplied by synchrotron light sources, one can selectively excite core electronic transitions in each metal. Spectroscopic signals from these electronic transitions can be used to dissect the chemical architecture of metals in cells, in cellular components, and in biomolecules at varying degrees of structural resolution. With the development of ever-brighter X-ray sources, X-ray methods have grown into applications that can be utilized to provide both a cellular image of the relative distribution of metals throughout the cell as well as a high-resolution picture of the structure of the metal. As these techniques continue to grow in their capabilities and ease of use, so too does the demand for their application by chemists and biochemists interested in studying the structure and dynamics of metals in cells, in cellular organelles, and in metalloproteins.

I. Introduction

Metalloproteins, or proteins that bind metal cofactors, make up a substantial portion of the human proteome and are essential for the viability of both individual cells and of the overall organism. By some estimates, as many as one-third to one-half of all proteins are metalloproteins (Ascone *et al.*, 2003). Both the reactivity and, indeed, even the structure of a protein can change when a metal binds to the organic protein peptide chain. These changes often allow the resulting metalloprotein to accomplish unique chemistry and maintain unique folds that could not be accomplished in the absence of metal. For example, the oxygen binding ability of hemoglobin and the CO₂ hydration activity of carbonic anhydrase have an absolute dependence on the presence of, respectively, heme iron and a divalent cation (typically zinc). Given the versatility in chemistry that each metal can perform, it is no surprise that metalloproteins are selective in the metal they accept, and different metals are utilized to a greater degree in different regions of the cell and in the organism. However, the chemical reactivity provided by metals can be both a blessing and a curse, especially when cellular pathways for metal homeostasis fail. Friedreich's ataxia and Wilson's disease are just two examples of hereditary disorders that are linked to failures in metal homeostasis: iron in the case of Friedreich's ataxia and copper in the case of Wilson's disease. In both diseases, a breakdown in a single component of the complex cellular metal-processing machinery has highly deleterious consequences to the organism.

Cells have, therefore, developed complex and comprehensive protein controlled mechanisms to ensure that metal homeostasis proceeds in an unhindered fashion. At the molecular level, placement of a specific metal, and only the appropriate metal, in the correct protein binding site is essential. At the same time, the protein-based ligand environment surrounding the metal helps to control the chemical reactivity of the metal (e.g., the porphyrin ring and hydrophobic ligand binding pocket in hemoglobin help promote reversible O₂ binding and release in favor

of CO binding and/or formation of μ -oxo bridged hemes, two reactions that are preferred in solution). The individual components of the cellular homeostasis machinery include metal specific pumps to bring the metal into the cell or cellular organelle, metal chaperones that selectively deliver metal to the metalloprotein partner and transcriptional regulation proteins that control the process at the genetic level. To fully understand and appreciate this machinery, it is necessary to understand both the details of the individual protein components, especially at the metal center, and the ways in which these parts interact to form the whole.

II. Rationale

Although genetic studies have proven to be highly effective in helping to determine the global role of metalloproteins within the complex machinery of cellular metal homeostasis, a complete understanding of how this mechanism functions requires knowledge of how the individual components operate. For metals, functional characterization begins with a thorough understanding of the metals themselves, over a range of distance scales from cellular to atomic. At the cellular level, studies of the distribution of each metal within the cell provide a general blueprint of cellular metal homeostasis. Characterization of metal site structure at the cellular organelle level allows one to focus on how metals are compartmentalized and generally utilized within unique regions of the cell. Finally, characterization of metals at the atomic level provides a high-resolution picture of how metalloproteins utilize each metal. While no single technique can answer all of these questions, modern X-ray methods have provided valuable insights at all of these dimensional scales. The goal of this report is to describe two X-ray-based techniques currently employed to characterize metals in cells at different degrees of resolution: X-ray absorption spectroscopy (XAS) and X-ray fluorescence (XRF) imaging.

The extremely high flux of tunable X-rays available at synchrotron radiation laboratories has dramatically expanded the range of applications that can be utilized to characterize metals in cells at different degrees of resolution. XAS is an element-specific probe of the local structural environment of a metal (Scott, 1985). XAS includes both extended X-ray absorption fine structure (EXAFS), which gives information on the bond-lengths, and X-ray absorption near-edge structure (XANES), which gives information on metal geometry and oxidation state. By making measurements on intact whole-cells, these properties can be exploited to characterize the average structural environment for each of the metals in a cell. In those cases where average information is not sufficient, it is necessary to enhance the resolution, either by imaging or by purification. XRF imaging can provide nanometer scale detail regarding the distribution of metals within cells, but only rarely provides the spectroscopic detail available from XAS. Alternatively, XAS can be used to provide high-resolution details for purified metalloproteins. This provides the highest accuracy structural information, but with the loss of detail regarding spatial distribution within a cell. In the following, we provide a general description of each technique, outline what is required to collect and analyze each type of data and finally provide specific examples of how each technique has been employed to directly address questions regarding metals that are predominately bound to proteins within cells.

III. Methods

A. XAS

XAS can be utilized to determine the high resolution electronic and structural details of metals bound to a single protein sample under a variety of different conditions (substrate vs. inhibitor bound, different metal oxidation states, etc.) or for each metal-bound molecule in a multiprotein complex. Sample constraints for the technique require metal concentrations of at least 0.1–0.3 mM (although higher is better) and solution volumes generally between 50 and 150 μ L. Solution samples must be frozen quickly, typically with glassing agents present, in order to avoid ice formation and the absorbing element's atomic number, in most cases, must be bigger

than ~ 19 (i.e., first transition series elements or heavier), although some very nice work has been done on lighter elements, such as sulfur. Questions typically addressed using this technique include: (1) what is the average oxidation state of the metal(s) in the sample, (2) is the metal site affected by the presence of substrates, inhibitors, redox agents, etc., (3) what is the ligand environment around the metal, and (4) is the metal part of a multinuclear cluster in the protein under investigation?

The XAS method utilizes intense monochromatic X-rays from a synchrotron light source to excite the core electrons in the absorbing atom (A) of interest. When the electron is excited to the continuum, the resulting photoelectron wave is scattered by the neighboring atoms (S, the scattering atom). For iron, the electron excitation edge occurs at ca. 7130 eV, as shown in the XANES region of Fig. 1. Information regarding the average oxidation state, ligand coordination geometry, and symmetry of the absorbing atom can be obtained from analysis of the XANES region of the XAS spectrum. In Fig. 1, the EXAFS refers to the modulations in the absorption coefficient on the high energy side of the absorption edge. EXAFS oscillations occur as a consequence of the fact that, depending on the wavelength of the photoelectron wave, the outgoing and backscattering waves can overlap either constructively or destructively to give maxima (E_1) and minima (E_2), respectively, in the absorption coefficient. Simulations of the EXAFS region can be used to determine the average metal–ligand bond distances with good resolution (~0.1 Å), very good accuracy (~0.02 Å), and outstanding precision (~0.004 Å). Metal–ligand coordination numbers can be determined to ± 1 and ligand type can be determined to within a row of the periodic table (i.e., oxygen can be distinguished from sulfur, but not from nitrogen atoms). The information from XAS is thus, similar to that obtained from protein crystallography, but without the need for diffraction quality crystals. The overall information content of protein crystallography is much higher, but even when a crystal structure is available, it is often the case that XAS can nevertheless provide unique information regarding the metal site structure (Tobin *et al.*, 2003).

B. Purified Protein XAS

Historically, almost all biological XAS studies have been performed on purified proteins. This is because XAS is a “bulk” spectroscopy (i.e., it is sensitive to all of the forms of an element that are present in a sample). If the element of interest is present in two different forms, the resulting XAS spectrum will be the weighted average of these two. Thus, a protein that contains one ZnCys₂His₂ site and one ZnCys₄ site would be indistinguishable from a protein containing two ZnCys₃His sites. A particularly problematic case is protein samples that are heterogeneous, for example, with a mixture of active site metal and adventitious metal.

C. Whole Cell XAS

Despite the limitations inherent in averaging, there has been growing use of XAS to characterize the average coordination structure and electronic properties of heterogeneous samples. Whole cell XAS can be utilized as a direct method for determining the average metal chemistry within the entire cell. The element specificity of X-ray absorption allows one to answer specific questions regarding the form of each metal in the cell. Virtually any form of sample can be used, with examples including whole blood from tunicates, intact biological tissue, isolated bacterial cultures, or even soil samples. Measurements are made in the same way as conventional X-ray absorption. Because the resulting data represents a weighted average of all of the chemical environments of the element of interest, it can be difficult to use with complex mixtures, although there has been some success in modeling the resulting spectra as linear combinations of reference spectra. This is particularly true for elements such as sulfur (Gnida *et al.*, 2007; Pickering *et al.*, 1998), where there is dramatic spectral variation for different chemical species. One common application of whole-cell XAS is for samples where an exogenous metal (perhaps a toxin or a drug) has been added to the sample. In such cases,

the absence of an endogenous form of the metal can simplify the analysis since the element of interest may be present in only one predominant form. In this case, whole-cell XAS can be used to determine the chemical state of the added metal, i.e. the form of the metal stored in a tolerant organism.

D. XRF Imaging

With modern, so-called “third generation” synchrotron sources, it is possible to focus high-flux, high-energy X-ray beams to very small spot sizes (~150 nm or smaller). This beam can be raster scanned across a sample in order to obtain spatially resolved data (Paunesku *et al.*, 2006). The simplest experiment is to record the intensity of X-rays emitted from each point in the sample. Since each element emits XRF at a characteristic energy, the resulting emission spectrum permits direct determination of the composition of a sample. By recording XRF spectra at each point in a sample, it is possible to construct a map of the spatial localization of each element heavier than phosphorus. Lighter elements can, in principle, also be imaged; however, this generally requires that samples be mounted in a vacuum chamber. With proper calibration, XRF images can provide absolute elemental concentrations. Most XRF imaging has been performed on thin samples, but it is also possible to extend the technique to three dimensions, using tomographic reconstructions to obtain the complete three-dimensional (3-D) distribution of an element.

E. Combined Spectral and Spatial Resolution

XRF is essentially an atomic phenomenon—that is, the XRF signal is virtually independent of the chemical environment of the atom. Thus, an iron XRF map provides information on the local Fe concentration, but no insight as to whether this is due to a heme protein, a Fe/S protein, or the iron-oxide core of ferritin. To get around this limitation, it is possible to combine XRF imaging with XAS using either microXAS or XANES imaging (Pickering *et al.*, 2000). If one measures the complete XAS spectrum for a spatially resolved portion of the sample (so-called microXAS), it is possible to determine the chemical form (or at least the average chemical form) of a particular element at a distinct point in the sample. Alternatively, one can take advantage of the fact that the excitation profile for each chemical form of an element has a slightly different energy dependence (that is, as noted above, each chemical form has a different XANES spectrum). In simple XRF imaging, samples are illuminated with high-energy X-rays, so that all chemical forms are excited with equal probability. In XANES imaging, an XRF image is excited using several different X-ray energies in the XANES region. Each chemical form of an element will have a slightly different excitation probability at each wavelength. By measuring multiple maps at several different excitation energies, it is possible to tease apart the spatial distributions of each of the chemical forms of an element. Differences in the diverse imaging methods are illustrated schematically in Fig. 2.

IV. Materials

A. Purified Protein XAS

Samples for single molecule XAS metalloprotein studies should be homogeneous with no unbound metal, since the data being collected is the average of protein sites that utilize the excited metal. The technique is buffer and pH independent, so samples can exist under a variety of solution conditions. Samples are generally measured at low temperature as frozen solutions in order to minimize thermal disorder and, more importantly, to limit the extent of X-ray induced radiation damage. As mentioned earlier, agents such as glycerol are generally added at ca. 30% by volume to ensure homogeneous sample glassing and prevent ice formation. Samples can have multiple different elements, as the technique is element specific, however, the same metal at two independent sites in the metalloprotein will appear as the average. Sample cell dimensions are specific for the cryostat being used and cryostats differ at different X-ray

facilities. Sample cells are generally constructed from metal free materials, such as Lucite (Bencze *et al.*, 2007). Cells can be a variety of shapes and sizes, although they generally have one long open window that is placed at a 45 degree angle to the incident beam. This exposed window is usually wrapped in metal free Kapton tape to prevent sample loss.

Because they are so dilute, biological XAS spectra are almost always measured as fluorescence excitation spectra. In this experiment, the characteristic XRF intensity is monitored as the excitation energy is scanned. In the limit of thin or dilute samples, fluorescence intensity is proportional to X-ray absorption cross-section. Most modern XAS beam lines are equipped with a solid-state energy-resolving XRF detector.

B. Whole Cell XAS

Many strategies have been utilized to prepare whole-cell XAS samples. In order to achieve good signal to noise ratios, the concentration of the metal within the cell must first be considered. In some cases, it may be sufficient to prepare a monolayer of confluent cells or directly use bacterial cells in a cell culture. If metal concentrations within the cells are low (submicromolar range), one may consider harvesting a population of cells into a small volume to increase the amount of sample being exposed to the X-ray beam and hence, increase the signal intensity. This has been done simply by filtration of a bacterial culture using a 0.2 μm filter, sandwiching the filter paper between layers of Kapton tape followed by flash freezing in liquid nitrogen. Others have harvested cells by centrifugation and placed the collected cells directly into a 140 μL Lucite XAS sample cell using a customized setup.

C. XRF Imaging

Perhaps, the most important part of an imaging experiment is the focused X-ray beam. Reflective optics (e.g., Kirkpatrick–Baez mirrors; Kirkpatrick and Baez, 1948) work well for beam sizes down to $\sim 1 \mu\text{m}$ and are thus well suited for XRF imaging of millimeter scale sample (e.g., intact tissue samples). Better spatial resolution, down to $\sim 100 \text{ nm}$ or better, can be achieved with zone-plate optics (Cai *et al.*, 2003; Schroer, 2006). Samples for XRF imaging are typically placed on an X-ray transparent substrate such as ultra thin ($\sim 100 \text{ nm}$) silicon nitride. The mechanical systems that are used to position the sample relative to the X-ray beam need to have precision and stability that is better than the desired spatial resolution. Fortunately, most of the synchrotron laboratories have one or more beam lines dedicated to XRF imaging, including all of the necessary focusing and sample positioning hardware, together with the standard XAS hardware for tunable X-ray excitation and energy-resolved XRF detection.

V. Discussion

Examples of how these X-ray techniques have been applied in order to address specific questions regarding the cellular utilization of metals are provided in the following section. These applications include specific examples that cover excitation of a variety of different elements including both metals and nonmetals.

A. Purified Protein XAS

Numerous laboratories have utilized XAS to answer structural and functional questions regarding many different metalloproteins. We have chosen three specific examples from the long library of protein XAS studies that illustrate how the technique was used to address specific questions regarding metal(s) bound to a protein. These examples are outlined below in detail. In the first example, superoxide reductase (SOR) from *Pyrococcus furiosus* was used to determine mechanistic issues regarding the enzyme's active site. This is an excellent example where characterization of the structural and electronic properties of the bound metal by XAS helped elucidate the enzyme's reaction mechanism. In the second example, the

multimetal/multinuclear sites in particulate methane monooxygenase (pMMO) from *Methylococcus capsulatus* were characterized by XAS to provide the high resolution details of the metal center that could not be obtained by X-ray crystallography. In the third example, the Zn finger domain of the ubiquitin binding protein Np14 was characterized by XAS; the Zn structural parameters were then utilized in combination with NMR spectroscopy to provide the high-resolution structural details of the protein. In addition to structural insight, XAS can in some cases also provide insight into reaction dynamics. As an example, we discuss the time resolved XAS of the Zn site in alcohol dehydrogenase (ADH), an excellent example of how structural changes during catalysis provide insight into the dynamic nature of an enzyme.

Although the *in vivo* function of this protein is unknown, SOR is believed to provide a mechanism to combat against oxidative stress in anaerobes, which typically lack superoxide dismutase, by reducing superoxide to hydrogen peroxide (Imlay, 2002). To elucidate the role of iron in enzymatic activity, the mononuclear Fe metal center was characterized in both the ferric and ferrous oxidation states by XAS (Clay *et al.*, 2002). Iron in the ferric form is high spin and six-coordinate, with a coordination geometry constructed by four equatorial histidyl ligands, an axial cysteinate, and a monodentate glutamate ligand. Ferric iron EXAFS data were best fit using one Fe-S at 2.36 Å and five Fe-N/O bonds at an average distance of 2.12 Å. In the reduced state, the ferrous site of SOR was shown to have square-pyramidal coordination geometry with four equatorial histidines and one axial cysteine; Fe EXAFS data for this sample were best fit by one Fe-S at 2.37 Å and four Fe-N/O at an average distance of 2.15 Å. A ligand for the iron can be substituted in the oxidized form, and the vacant site can be populated in the ferrous form by cyanide, which acts as a molecular mimic for superoxide. The ability to bind exogenous ligands in both the ferrous and ferric sites of SOR was suggested to be consistent with an inner-sphere catalytic mechanism involving superoxide binding at the ferrous site to yield a ferric-(hydro)peroxo intermediate. Additional mechanistic insight was supplied by characterizing the sulfur K-edge XAS for the cysteine bound to the iron in SOR (Dey *et al.*, 2007). XANES analysis of the sulfur edge indicated that the thiolate is a highly anionic covalent ligand; the anionic character of the thiolate most likely increases the pK_a of the Fe(III)-OOH intermediate, facilitating protonation and eventual release. Combined, these studies paint a structural picture of how the ligand environment facilitates catalytic turnover during enzymatic activity.

pMMO is a multicopper membrane bound enzyme that catalyzes the oxidation of methane to methanol in methanotropic bacteria. Prior to complete structural characterization of this multicomponent enzyme, Cu K-edge XAS studies were utilized to determine the average oxidation state and provide structural details regarding copper bound to pMMO. Initial Cu XANES spectra of as isolated Bath pMMO indicated a near equal distribution of Cu(I) and Cu(II) for the ca. four coppers bound to the protein, based on general edge features and the observation of both $1s \rightarrow 3d$ and $1s \rightarrow 4p$ electronic transitions apparent in the edge (Lieberman *et al.*, 2003). Subsequent reports confirmed the protein could be prepared in a homogeneous metal oxidation state (Lieberman *et al.*, 2006). EXAFS analysis of the as isolated protein confirmed the presence of a dinuclear Cu center, with a copper separation of 2.51 Å, and showed that the Cu-Cu distance increases to 2.65 Å when the metal is fully reduced. The remaining ligands were completely oxygen/nitrogen based and located at an average distance of 1.97 and 2.22 Å in the as isolated protein and 1.98 and 2.14 Å in the reduced sample. The high-resolution structural details provided by XAS helped contribute to the correct assignment of the high-resolution crystal structure of pMMO (Lieberman and Rosenzweig, 2005).

Protein ubiquitination plays an important role in a variety of cellular processes. Ubiquitinated proteins are directed into the different cellular pathways through interactions with effector proteins that contain conserved ubiquitin binding motifs. The solution structure of one such motif, NZF, was solved utilizing the metrical parameters of the metal center provided by Zn

K-edge XAS studies (Wang et al., 2003). Simulations of the Zn EXAFS showed Zn was bound to four sulfur atoms at an average bond length of 2.33 Å. The Debye–Waller factor, a measure of metal–ligand bond disorder was 0.00454 Å² indicating the average Zn–ligand environment was most likely distorted. During the solution structure calculations, the Zn bond lengths obtained by XAS were used as constraints while the value for the Debye–Waller factor was utilized to generate limits for these distance constraints. XAS is, therefore, an effective tool that can be used to provide unique structural details not attainable by other techniques, especially in the case of the generally spectroscopically silent metals like Zn.

ADH catalyzes the oxidative conversion between alcohols and aldehydes or ketones. The catalytic active site of *Thermoanaerobacter brockii* ADH contains a Zn ion bound to a single cysteine, histidine, aspartate, and a glutamate. Prior to this study, two catalytic mechanisms were proposed for the protein: the first required a four-coordinate Zn intermediate generated from water displacement at the Zn site, the second mechanism involved a five-coordinate Zn site where the water, rather than being displaced acts as a site for transient proton transfer. In a very interesting application of XAS, Sagi and coworkers utilized time-resolved freeze-quench XAS to trap TbADH during multiple stages within a single catalytic turnover cycle, hence providing snapshots of the Zn site during turnover (Kleinfeld *et al.*, 2003). Two distinct intermediates were detected during the mixing time range of 3 and 70 ms, and bound Zn was pentacoordinate at each step. Intermediate 1 (IM1) detected between 3 and 5 ms, and IM2, detected between 15 and 19 ms after mixing, both had 4 O/N and 1 S ligands bound to Zn; however, there was a distinct ca. 0.15 Å expansion in two Zn–O/N bond lengths relative to the starting Zn–ligand complex. Results from this study allowed the authors to select the more appropriate proposed catalytic mechanism in agreement with the structural details regarding the catalytic Zn site in the enzyme.

An additional emerging application of the technique involves the detailed characterization of the near edge XANES data for metal containing systems using the multiple scattering edge simulation package MXAN. Utilized recently by the Hodgson and Hedman groups, MXAN analysis was applied to characterize the mononuclear copper complex [Cu(TMPA)(OH₂)](ClO₄)₂ (Sarangi *et al.*, 2005). When applied to this copper complex, that authors nicely illustrated the utility of the technique for accurately abstracting geometric information of the metal center from an XAS spectrum. When coupled with the highly accurate bond lengths obtained from EXAFS analysis, MXAN can be incorporated to provide an accurate metal–ligand geometric picture of homogeneous metal centers in biomolecules.

B. Whole Cell XAS

Numerous published reports utilized whole-cell XAS to determine the overall metal speciation within different cell types. The following section highlights three examples of how the technique was applied at the cellular level and in the process, nicely illustrate the range of applications for this method towards addressing diverse biological and environmental questions. In the first report, whole-cell studies were used to investigate the high concentration of vanadium in the blood of subtidal marine organisms. In the second report, whole-cell XAS studies were utilized to determine metal chelation strategies for five different Zn tolerant microbes found in a highly Zn-contaminated lake. In the final report, whole-cell XAS was used to quantitate the generally spectroscopically silent sulfur contained within equine blood.

Tunicates are fascinating subtidal marine organisms in that some species, such as *Ascidia nigra* and *Ascidia ceratodes*, have the ability to accumulate and concentrate vanadium to levels over a million fold higher than found in seawater. Although the reason for this unusual storage ability remains unknown, much work has been done to determine the method of uptake and storage of metal in each system. Vanadium resides in multiple cell types within the blood of these organisms and interestingly the V-containing cells and the chemical speciation of metal

differ even between closely related organisms. In the report by Frank and coworkers, whole-cell XAS of blood cell samples from *A. nigra* and *A. ceratodes* were used to investigate species dependent differences for V distribution, which may have possible taxonomical implications (Frank *et al.*, 1998). Subtle differences between XANES spectra for each sample were identified, confirming a unique global V environment in each cell type. A shoulder in the XANES region of intact *A. ceratodes* blood cells occurred at 5476 eV, closely matching the feature found for V(III) in *aquo-VSO₄⁺*. In contrast, the large 1s→3d pre-edge transition of *A. nigra* indicated ~25% of the V in this species closely matches the vanadyl ion ([V(IV) = O]²⁺), which has a characteristic feature at 5468.6 eV. The edge energies for these two species differ by approximately 1 eV (5480.5 eV for *A. ceratodes* vs. 5479.5 eV for *A. nigra* samples), suggesting the remaining V(III) found in *A. nigra* has a different ligation environment from that in *A. ceratodes*. These differences suggest unique vanadium storage distributions between species, despite the fact that the biochemical mechanisms responsible for V uptake and storage appear to be very similar.

The rise in industrialization over the past few centuries has led to environmental contamination of areas surrounding large manufacturing facilities. Operations, such as smelting, introduce high levels of Zn into local water supplies. Decontamination of these areas requires an understanding of metal mobility, toxicity, and reactivity beginning with an understanding of the Zn speciation in metal-contaminated areas. In one such study, Webb and coworkers identified five bacterial anaerobes that were able to thrive in the Zn contaminated waters of lake DePue (IL) (Webb *et al.*, 2001). These microbes were collected and grown in a laboratory setting using high Zn media. Whole cell XAS was used to determine the peptide coordination of the metal. Fitting analysis revealed unique global Zn coordination in all five species. One isolate showed Zn ligation solely by sulfur (4 S at an average bond distance of 2.34 Å), two had mixed sulfur and oxygen/nitrogen coordination (3 S at average bond distances of 2.34 Å and 1 O/N at 1.97 Å, or 2 S at 2.34 Å and 2 O/N at 1.96 Å), while the final two showed all oxygen/nitrogen ligation (4 O/N at 1.96 or 5 O/N at 1.97 Å). The data were consistent with the low-Z (i.e., “O”) ligands coming from phosphoryl groups. Given the number of phosphate groups located outside the cell (i.e., at the level of the cell membrane), these results may suggest that O/N dominated microbes may bind/store Zn on their outer cell surface while the S ligand dominated microbes may store Zn within the cell.

Although not a metal, sulfur is one of the essential elements most easily studied by XAS, since there are dramatic spectral variations between different chemical forms of the element. Relatively little is known about the chemical reactivity of sulfur *in vivo* due to the limited number of direct methods that can be used to study it. Its ability to cycle between different redox states makes it useful in numerous life processes towards regulating oxidation chemistry, as well as for serving as a soft base for metals themselves. Its high abundance in the cell make it perfectly suited for XAS studies, as this technique is extremely sensitive for detecting structural and redox state changes. As a proof of concept, Pickering and coworkers were able to quantitate sulfur speciation in intact equine blood cells without chemical manipulations of the sample using K-edge XAS (Pickering *et al.*, 1998). By fitting empirical data with linear combinations of spectra from a model library, they were able to determine the relative quantities of different sulfur species within samples. The authors observed sulfur within red blood cells to be 21.4% disulfide, 54.4% thiol, 21.3% thioether, 2.1% sulfoxide, and 0.8% sulfate. In contrast to the intact erythrocytes, the sulfur present in the plasma had drastically different speciation: 76.5% disulfide, 20.6% thiol, 0% thioether, 0% sulfoxide, and 2.9% sulfate. These results provide confirmation of the conventional wisdom regarding the chemical form of intra and extracellular sulfur in blood, namely that extracellular cysteine is predominately in a disulfide bond while intracellular sulfur is much more likely to be present as the reduced cysteine.

C. XRF Imaging

As noted above, XRF imaging is conveniently divided into macroscopic (micrometer scale imaging using mirrors) and microscopic (nanometer scale imaging using zone plates). Macroscopic studies have examined a variety of tissues. Given the available resolution, these studies generally do not provide cellular detail, but can be used to correlate metal concentrations with histologically identifiable regions of tissue. There have been several recent reviews of XRF imaging (e.g., Lankosz *et al.*, 2007; Paunesku *et al.*, 2006), including a careful analysis of the relative benefits of particle beam versus X-ray excitation (Petibois and Cestelli Guidi, 2008), and consequently, only the key points will be summarized here. Much of the interest in macroscopic-scale XRF imaging derives from attempts to correlate metal levels with disease states. One of the more interesting tissues to study is brain tissue. For example, there is substantial evidence that metals are associated, in some way, with Alzheimer's disease. Recent work combining XRF imaging with IR microscopy succeeded in showing that Cu and Zn colocalize specifically with the β -sheet form (i.e., the "misfolded" form) of the amyloid β protein that is the principle component of senile plaques in Alzheimer's disease (Miller *et al.*, 2006). A key to this study, and to all such studies, is the development of appropriate methods (IR imaging in this case) that allow the investigator to correlate the metal levels, as seen by XRF, with specific features in the tissue.

A second area that has received significant attention is cancer, where there have been numerous attempts to correlate changes in metal levels (either essential metals such as Fe, Cu, and Zn, or toxic metals such as Cd, Cr, and As) with disease state. Although this work remains largely phenomenological at this point, it is clear that there exist many tantalizing differences in metal status between diseased and healthy tissue. The challenge, and also the promise, of such studies will lie in sorting out cause and effect. That is, is a low or high level of a particular metal a cause or a symptom of the disease?

One approach to disentangling cause and effect is to treat samples with different concentrations of the element of interest (e.g., a toxic element) and follow the distribution of this element through the sample in both space and time (Isaure *et al.*, 2006; Mesjasz-Przybylowicz *et al.*, 2007). Much of this sort of work has been done on plants because of their ease of manipulation. One of the first such examples was an elegant study by Pickering *et al.* (2000) that used XANES imaging to study the distribution and speciation of selenium in plants that had been exposed to 5 mM selenate. By measuring the absorbance, the X-ray scatter, and the XRF at two different excitation energies, they were able to determine that Se is present predominantly as selenate in the mature leaf tissue while young leaves and roots contain almost exclusively organoselenium. This observation may be helpful in understanding the biological basis of selenium hyperaccumulation in some plants.

XRF imaging can, in principle, be used for elements as light as phosphorus (or even lighter with the use of vacuum sample chambers). For heavy elements (e.g., first transition series metals and higher), X-ray absorption by the sample is relatively unimportant, at least for samples that are no more than a few hundred μm thick. However, attenuation by the sample can be quite important for thick samples, leading to apparent heterogeneities in composition (i.e., light elements appear to be depleted from the far side of the sample because the fluorescence is absorbed by the intervening sample (Mesjasz-Przybylowicz *et al.*, 2007). A second difficulty with XRF studies of light elements is that, even if the sample is thin and dilute on an average, the local concentration of the element of interest may still be large enough to significantly distort the spectrum. This is important, for example, in studies of sulfur metabolism by bacteria where sulfur is present as globules of essentially pure sulfur, which can significantly distort the resulting XANES spectrum (Pickering *et al.*, 2001).

Most XRF imaging studies have used thin, flat samples and have thus obtained only two-dimensional (2-D) data. For 3-D samples, one can measure data at several different angles and use the resulting data to infer localization (Young *et al.*, 2007), and complete 3-D information is available if full tomographic measurements are done. A beautiful demonstration of the ability of the latter to provide biologically relevant information was a study by Kim *et al.* (2006) showing that iron is specifically accumulated in the provascular system of *Arabidopsis* seeds. Many studies have been carried out on chemically fixed samples, but recent work on cryogenically fixed samples suggest that this may be a viable approach (Kanggiesser *et al.*, 2007) for preparing samples that are closer to biologically-relevant wet conditions.

The discussion above focused on macroscopic XRF imaging. Increasingly, however, additional studies have focused on nanometer scale imaging. With this resolution, it is possible to determine directly the intracellular variations in metal concentration. Once again, there are a variety of possible biological applications (Fahrni, 2007; Paunesku *et al.*, 2006). A representative example is the study by Finney and co-workers of the role of copper in angiogenesis (Finney *et al.*, 2007). It has long been known that copper is important in angiogenesis, and there have been suggestions that by modifying Cu metabolism (e.g., with Cu specific chelators), it may be possible to inhibit angiogenesis and thus interfere with tumor growth. X-ray nanoprobe images of individual cells showed that during angiogenesis, the bulk of the intracellular Cu is relocated to the tips of nascent endothelial cell filopodia and across the cell membrane, providing direct evidence for the role Cu plays. Interestingly, Cu chelation, while disrupting angiogenesis, had little effect on the observed Cu relocation. The ability to determine metal concentrations with subcellular resolution was critical to this work. A variety of other groups have used analogous studies to explore the effect of metals (both toxins and metallodrugs) on cells, exploring both the localization of the added compound and the effect that it has on cellular metal metabolism. Although the available X-ray resolution is ~100 nm, and even better resolution should still be possible, it can nevertheless be challenging to determine the precise localization of a particular element. The reason is cell thickness is typically much more than 100 nm, and therefore, 2-D metal distributions are, in reality, the average of the spatial distribution across the cell. In practice, this means that it is often only possible to distinguish between cytoplasm and nucleus, but not to identify finer scale structures, unless one looks at thin sections cut from a cell (Dillon *et al.*, 2002).

The lower flux and smaller sample volumes in nanoprobe studies mean it is difficult to perform full XANES imaging studies analogous to those discussed above for microprobe imaging. However, even here some spectra resolution is possible, demonstrating, for example, that most of the copper in NIH 3T3 fibroblasts is present as Cu(I) (Yang *et al.*, 2005).

VI. Summary

As synchrotron capabilities have developed, the applications of XAS to biology have grown to the point that this is now a mature field. For most purified proteins, it is now (relatively) straightforward to obtain direct structural information for the metal site using XAS. This information is complementary to that available from crystallography, and is often critical to understanding the structural and chemical properties of the protein. It is possible, but more challenging, to extend these studies to more complex samples, for example, heterogeneous whole-cell samples or time-dependent samples, but even these studies are becoming more common. Focused X-ray beams can also be used for elemental imaging, providing information similar to that available from particle beam microscopy, but with greater sensitivity to heavy elements, and much greater tolerance for “wet” samples. While far from routine, X-ray microprobe studies of macroscopic samples (e.g., biological tissues) and X-ray nanoprobe studies of microscopic samples (e.g., single cells) are increasingly being used to interrogate subcellular distributions of elements. Much remains to be learned about the normal variability

of metal concentrations within healthy cells before it will be possible to interpret the effect of drugs and disease on metal distributions. Nevertheless, it is clear that XRF imaging has the potential to revolutionize the study of metal ions in biology.

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Fig. 1. Iron K-edge XAS spectrum from ferrous ammonium sulphate. Spectra are divided into the XANES region (-10 to $+40$ eV relative to the large excitation signal) and the EXAFS region (>40 eV above the excitation edge). In the EXAFS region, absorption maxima (E_1) and minima (E_2) are highlighted. Inset: constructive and destructive overlap of photoelectron wave generated from excitation of the iron 1s core electron to the continuum.

XRF Imaging

Excite at E_4

Measure $I(E_F)$ as a function of x, y

XANES Imaging

Excite at E_1, E_2, E_3 & E_4

Measure $I(E_F)$ as a function of $E_{\text{excitation}}$

Calculate C_1, C_2 & C_3 (l to r) as a function of x, y

MicroXANES

Scan E from E_0 to E_4 at same x, y

Measure $I(E_F)$ as a function of $E_{\text{excitation}}$

Fig. 2.

Schematic illustration of XRF imaging experiments for a sample that has the *average* absorption spectrum shown in the top/right panel. Because all forms of the element emit with nearly the same emission, there is only a single resolved X-ray fluorescence peak, shown in the top/left panel. For XRF imaging, the sample is excited well above the edge (E_4) and the XRF intensity is measured as a function of the location (x, y) that is excited. The resulting pattern gives an elemental map with no chemical information. If the peaks at E_1, E_2 , and E_3 are due to different chemical species (**1–3**), it is possible to perform XANES imaging by measuring XRF at each energy and calculating the relative concentration of each: C_1, C_2 , and C_3 (typically measurements at several additional energies are necessary to obtain a robust solution). In this

illustration, each species is well separated spatially (middle image). Finally, by measuring a full XAS spectrum with a microbeam, it is possible to obtain spatially resolved XAS spectra. In this simplified example, spectra measured at the point marked by the three arrows are the spectra for pure **1–3**. (See Plate no. 34 in the Color Plate Section.)