XRF and XAFS Analysis of Electrophoretically Isolated Nondenatured Proteins

K.M. Kemner,[1] S.D. Kelly,[1] E.J. O'Loughlin,[1] M.I. Boyanov,[1] T. Khare,[1] L.A. Moe,[2] B.G. Fox,[2]
M.I. Donnelly,[1] Y. Londer,[1] M. Schiffer,[1] C.S. Giometti[1]

[1]Argonne National Laboratory, Argonne, IL, U.S.A.; [2]University of Wisconsin, Madison, WI, U.S.A.

Introduction

Current methods for identifying and characterizing metalloproteins in complex mixtures are slow and laborious. Isotopic labeling of proteins with specific metal tags enables detection only of metalloproteins containing that specific metal (e.g., Fe). *In situ* labeling with radiolabeled metals can be problematic if the cell's uptake and metabolism of the radiolabeled molecules are inefficient. Staining methods for detection of metalloproteins, such as the heme stains, allow for the detection of only one type of metalloprotein. The sensitivity of these specialized stains is also limited, necessitating the use of relatively large amounts of protein (often over 100 µg of pure protein) for detection. New methods must be developed, therefore, to achieve high-throughput, global discovery and characterization of individual metalloproteins of different types in the complex mixtures of proteins produced by organisms.

The global detection and characterization of metalloproteins requires, in addition to the detection and characterization methods provided by XRF and XAFS, a method for separating complex mixtures into distinct protein components. Although many metalloproteins retain their metal moieties under denaturing conditions, we hypothesize that separation under nondenaturing conditions will preserve even the protein-metal interactions that might be disrupted by denaturation.

Methods and Results

We compared the reproducibility and accuracy of XRF elemental mapping for identifying the presence of cytochrome c7 (a triheme Fe-centered metalloprotein) overexpressed by an *Escherichia coli* culture (100 µg of total protein) on a one-dimensional (1-D) polyacrylamide electrophoretic gel strip. In addition, 50 and 100 µg of purified cytochrome c7 (as calibration standards) and 200 µg of material from an *E. coli* control culture (i.e., not overexpressing cytochrome c7) were also measured. XRF measurements of the spatial distribution of the Fe Kα fluorescent intensity on a 1-D gel were performed at the MRCAT [1] beamline (Fig. 1). A 10.5-keV x-ray beam (0.7 mm by 1.0 mm) was used as a probe, and the fluorescent x-ray intensity was monitored with three elements of a multielement solid-state detector. All measurements were made at room temperature and atmospheric pressure. Results for the relative intensities of the Fe Kα fluorescence radiation relative to the position on the 1-D gel are shown in Fig. 1. These results clearly illustrate (1) correlated elevations in Fe Kα fluorescence intensity for the cytochrome c7 standard and the *E. coli* overexpressing cytochrome c7 (at ~59,000 µm, marked "A" on the strip); (2) reproducibility (i.e., collocation of Fe at ~59,000 µm, marked "A" on the gel strip, for two replicate samples); and (3) the presence of additional, reproducible Fe fluorescence radiation in both the overexpressed cytochrome c7 and control *E. coli* samples (at ~57,000 µm, marked "B" on the gel strip). These results clearly demonstrate that this approach enables identification of overexpressed metalloproteins in cultures. Observation of Fe at ~57,000 µm in both types of *E. coli* samples further demonstrates that detection with this technique does not necessarily require overexpression of a metalloprotein.

Although identification of the presence of metalloproteins is extremely valuable, additional information concerning the structure and function of the metal center of the metalloprotein is desirable in many instances. The atoms that coordinate the metals of a protein alter their chemistry. For example, in cytochromes, Fe is bound by four equatorial nitrogen atoms from the porphyrin ring, plus axial ligands from the protein or the solvent (see Fig. 2). In some cases, a sulfur atom provides the axial ligand. XAFS spectroscopy can be an extremely valuable tool for probing the local chemical and structural environment of the metal within metalloproteins. To investigate the possibility of coupling the XAFS technique to procedures discussed above, we have made Fe XAFS measurements on a catalase protein after isoelectric focusing on polyacrylamide on a plastic backing (an IPG [immobilized pH gradient] strip). XAFS data were collected at room temperature in the fluorescence mode with an ion chamber in the Stern-Heald geometry [2] by using a Lytle detector with a Mn filter. Linearity tests [3] indicated less than 0.5% nonlinearity in the experimental setup for 50% attenuation of the incident radiation. Incident and transmitted x-ray intensities were monitored with ionization chambers with 100% free-flowing nitrogen gas at atmospheric pressures. Results of the fitting of the data (Δk = 2.5–8.0 Å^{-1}, Δr = 1–3 Å, 7 floating variables, and 2 degrees of freedom) to theoretical standards generated with the FEFF program [4] are shown in Fig. 3. Results from fitting analysis of these data are consistent with an average of ~4 N/O and ~1 N/O (at 1.98 Å and 2.05 Å, respectively) and ~8 carbons (at 3.05 Å), with additional carbons and multiple-scattering effects (at 3.2–3.4 Å), contributing to the local environment of the Fe. These results are consistent with findings of previous XAFS studies on other purified, concentrated catalase proteins [5, 6].

Discussion

In summary, we have described the results of the development of new approaches to identify and characterize metalloproteins. Specifically, we have described the integration of XRF mapping, XRF elemental analysis, and XAFS with electrophoretic methods. Further development of these integrated techniques to enable high-throughput analysis of frozen samples (to reduce the effects of radiation damage) holds great promise for the investigation of proteomic expression in a wide variety of biological systems.

Acknowledgments

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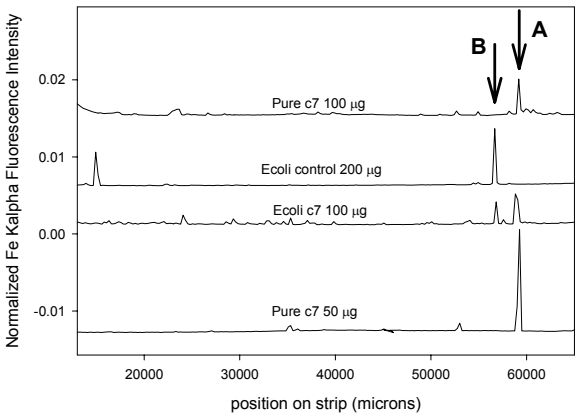

Fig. 1. XRF measurement showing the Fe Kα signal along the strip.

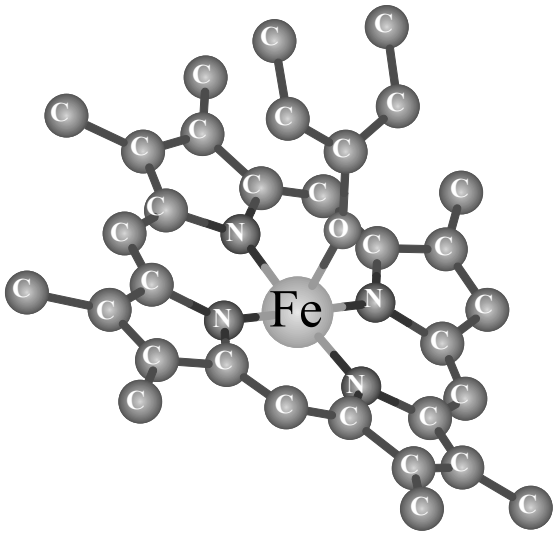

Fig. 2. Catalase structure.

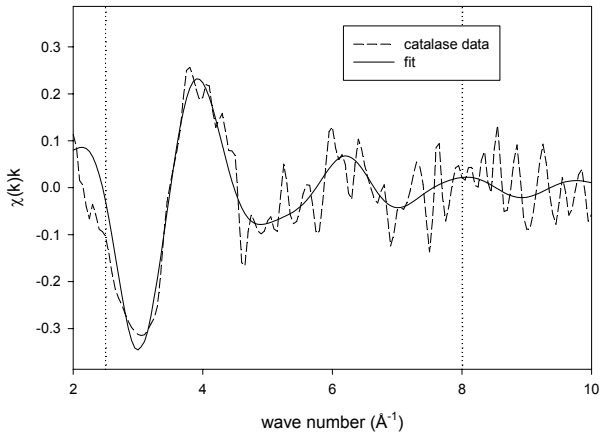

Fig. 3. χ(k) data and fit for catalase.

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