XRF and XAFS Analysis of Electrophoretically Isolated Nondenatured Proteins

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Introduction

Current methods for identifying and characterizing metalloproteins in complex mixtures are slow and laborious. Isotopic labeling of proteins with specific metal tags enables the detection of only the metalloproteins containing that specific metal (e.g., Fe). *In situ* labeling with radiolabeled metals can be problematic if the cell's uptake and the metabolism of the radiolabeled molecules are inefficient. Staining methods for the detection of metalloproteins, such as the heme stains, also 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 proteins (often more than 100 μg of pure protein) for detection. New methods must be developed, therefore, to achieve the 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 x-ray fluorescence (XRF) and x-ray absorption fine structure (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, Materials and Results

Identification

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 1-D polyacrylamide electrophoretic gell 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 MR-CAT beamline (Fig. 1) [1]. A 10.5-keV x-ray beam (0.7 × 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. Measurements were made on all samples at room temperature and at 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 Figure 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 the control *E. coli* samples (at ~57,000 μm, marked "B" on the gel strip). These results clearly demonstrate that this approach enables the identification of overexpressed metalloproteins in cultures. Observation of Fe at ~57,000 μm in both types of *E. coli* samples further demonstrates that this technique does not necessarily require a metalloprotein to be overexpressed in order to be detected.

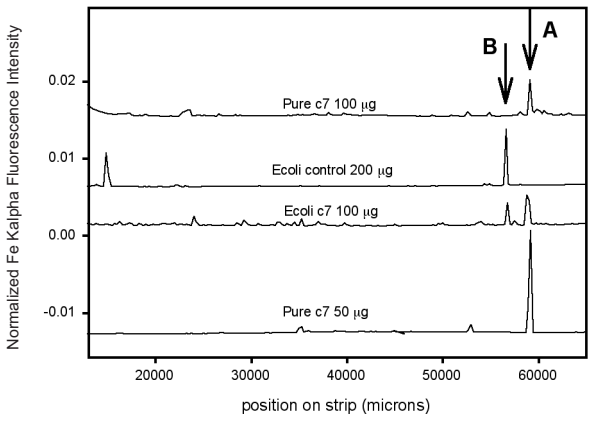

FIG. 1. XRF measurement showing the Fe Kα signal along the strip.

Characterization

In many instances, although the identification of the presence of metalloproteins is extremely valuable, additional information on the structure and function of the metal center of the metalloprotein is desirable. 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 and by axial ligands from the protein or the solvent (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 made Fe XAFS measurements on a catalase protein after isoelectric focusing on polyacrylamide on a plastic backing (immobilized pH gradient or IPG 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 an Mn filter. Linearity tests [3] indicated less than 0.5% nonlinearity in the experimental setup for a 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, seven floating variables, and two degrees of freedom) to theoretical standards generated with the FEFF program [4] are shown in Fig. 3. Results from fitting analysis of these data indicate 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), plus 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 previous XAFS studies of other purified and concentrated catalase proteins [5, 6].

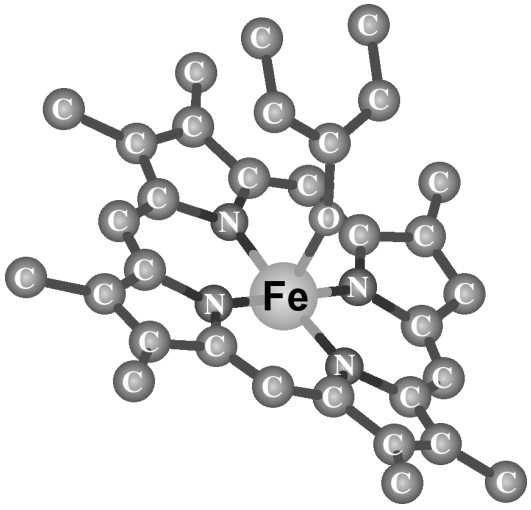

FIG. 2. Catalase structure.

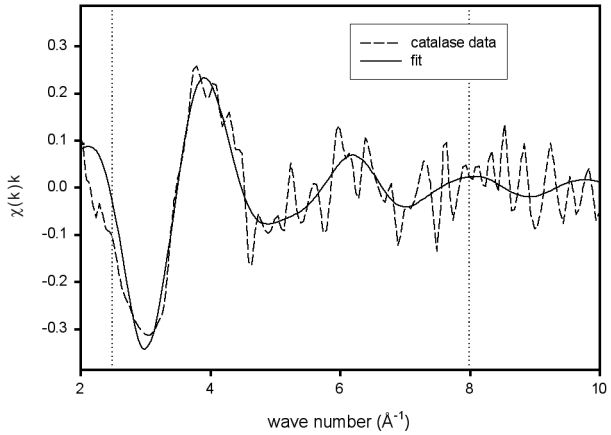

FIG. 3. χ(k) data for catalase and fit.

Discussion

In summary, we described the results of the development of new approaches to identify and characterize metalloproteins. Specifically, we 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

Use of the APS and MR-CAT was supported by the U.S. Department of Energy (DOE), Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-ENG-38. Support for this work was provided by the DOE BES Environmental Remediation Sciences Division, Natural and Accelerated Bio-remediation Research (NABIR) program.

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