Cadmium Ligand Coordination in the Metal Hyperaccumulating Plant *Thlaspi caerulescens*

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Introduction

Heavy atoms can be effectively localized in tissues and environmental matrices with high resolution, and the molecular environment (nearest-neighbor atoms) of those elements can be determined. Extended x-ray absorption fine structure (EXAFS) has been used to determine the speciation and coordination of numerous elements, including heavy metals (Cd, Co, Cr, Cu, Hg, Ni, Pb, Zn), metalloids (As, Fe, Mn, Se), and other elements (S, Sr, U, W). The primary benefit for plant analysis is that it provides *in situ* speciation, a result that cannot be obtained by standard analytical procedures, principally because even simple extractions distort the speciation of the element of interest. Plant researchers have used XAFS to localize Cr, Mn, Se, Ni, and Zn in order to identify the ligands involved in their transport, detoxification, and storage [1-8], but few of the researchers have used this technique to examine the ligand coordination of Cd in plants [3, 6]. In one study, the *in vitro* binding of Cd to peptides isolated from corn was examined, and those results were extrapolated to the presumed binding of Cd to phytochelatins (PCs), metal-binding peptides enzymatically synthesized by plants in response to heavy metal exposure. However, since recent results have indicated that PCs do not play a primary role in Cd tolerance in metalliferous (metal-loving) plants like *Silene vulgaris* [9-11] and *Thlaspi caerulescens* [12], there must be additional ligands involved in Cd detoxification — most likely organic or amino acids [3, 13]. *T. caerulescens*, a unique plant capable of accumulating >30,000 ppm Zn and 3,000 ppm Cd in the absence of phytoxicity [14], has been the subject of intense study in recent years as a species useful for the phytoremediation of metal-contaminated soils [15, 16] and as a model for the study of metal homeostasis in plants [17-20]. We have used x-ray fluorescence at the Cornell High Energy Synchrotron Source (CHESS) to map the distribution of Zn in leaves (unpublished results). Here, we propose a macro-XAFS analysis to investigate Cd coordination in *T. caerulescens*.

Methods and Materials

Sample Holder Construction

XAS analysis requires a suitable holder for the sample material that will not interact with the beam. Lucite® and Teflon® are two materials of choice. The latter was chosen for these experiments because it provided the most readily available and adaptable material. G. Moroz, the supervisor of the Fine Instruments Research Shop, was consulted regarding sample holder construction, and he and the author revised the design until a suitable holder was obtained (Fig. 1). The Fine Instruments Research Shop produced 50 of these sample holders for use in March 2002.

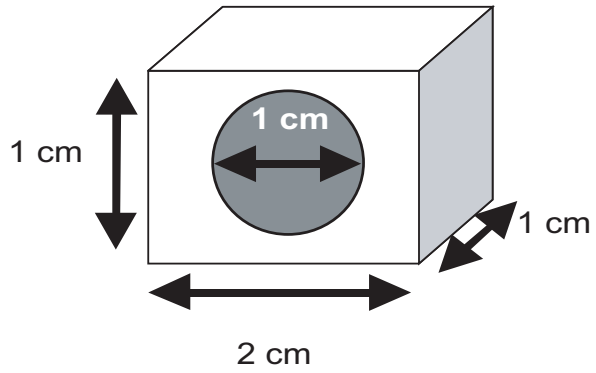

FIG. 1. Schematic diagram of the Teflon sample holder used for XAS analysis of plant tissue samples. Dimensions are not drawn to scale.

Growth and Preparation of Plants for XAS Analysis

Immediately after the inception of the project, seeds from several populations of *T. caerulescens*, along with the nonaccumulator *T. arvense*, were established in hydroponics. These plants were grown hydroponically for 50-75 days to establish biomass. Once they had reached the desired size, cadmium treatments were initiated. Plants of the hyperaccumulator were treated with 10, 50, or 100 _M of Cd, while the non-accumulator *T. arvense* plants were treated only with 10 _M of Cd. All treatments lasted for 7 days. During this time, the sensitive nonaccumulator showed signs of Cd toxicity, including leaf chlorosis, while the hyperaccumulator showed no symptoms. Previous research with this species had been used as the basis for these treatments, so these results were not unexpected.

Leaf tissues were harvested and prepared by using standard techniques. The leaves were removed and rinsed with copious amounts of deionized water. After being blotted, the leaves were weighed to obtain a total

fresh weight for each plant. Harvested leaves were frozen in liquid nitrogen and quickly ground to a fine powder in chilled mortar. This approach is a standard technique used to prepare tissue samples for XAS analysis. Tissue samples were immediately packed into Teflon sample holders and frozen at –80°C. The only use of root material in this study was to provide a standard for the association of Cd with plant cell walls. Since roots are in direct contact with the treatment solution, this interaction would predominate, and scans of root material would reveal little information concerning coordination *in vivo*. Nevertheless, the EXAFS data that were gathered could be useful in separating apoplastic binding from symplastic transport in the leaves. Root cell wall preps were prepared by soaking roots of *Thlaspi* plants in methanol:chloroform (2:1 v/v) for 3 days. This treatment strips the biotic material, leaving pristine cell wall skeletons that can be used for sorption studies [18]. The cell wall preps were immersed in a 6.7 mM solution of $CdSO_4$ for 3 days to allow adequate time for saturation of cell wall binding sites with Cd. The Cd-laden cell wall preps were thereafter treated as regular plant tissue samples.

Analysis of Samples at APS Beamline 13-BM

Beam time was obtained for the initial experiments associated with this project in March 2002. Since this was the first set of experiments, the primary goal was to develop sample spectra for standards and conduct preliminary scans to assess *in vivo* Cd coordination. Samples and standards were kept frozen or refrigerated, respectively, until analyzed. Standards consisted either of Cd solutions or Cd solids. Cadmium solids were certified American Chemical Society (ACS) chemicals purchased from chemical supply companies. All other cadmium standards were prepared in the same manner as comparable Zn and Ni standards for XAS analysis. EXAFS was performed on these samples on the bending magnet under the direction of M. Newville. The K_-edge for Cd occurs at 26.69 keV, so samples were scanned over a range from 26.55 to 28.55 keV. Two detectors were used. A Canberra Ge detector was used initially because of the low Cd levels in the plant tissues. A Lytle detector was used later for samples (cell wall preps) with high Cd concentrations. Samples were maintained at –40°C by a Peltier cooling cell supplied by J. Cross. Samples were mounted at a 45° angle incident to the beam, with the detector placed perpendicular to the beam. Teflon holders containing the tissue samples were placed directly within the Peltier cell. Solution standards were placed in XRF sample containers and held in position by a Teflon frame. Solid standards were ground to a fine powder and affixed to transparent tape and mounted on Teflon® frames. All samples were covered by a layer of Kapton film to facilitate XAS.

Results and Discussion

Initial scans of the tissue samples produced mixed results. EXAFS did show the presence of Cd in the tissue samples, but a clear signal was obtained only for the tissues treated with the highest concentration of Cd. The plants were not exposed to Cd long enough to accumulate enough to obtain a strong signal. When the data were normalized, all the spectra obtained (Fig. 2A) revealed an apparent oxygen coordination. Given the low Cd concentrations in the samples, Newville proposed that efforts should focus on an x-ray absorption near-edge structure (XANES) scan of the spectra tail from 26.1 to 26.8 keV to obtain finer detail

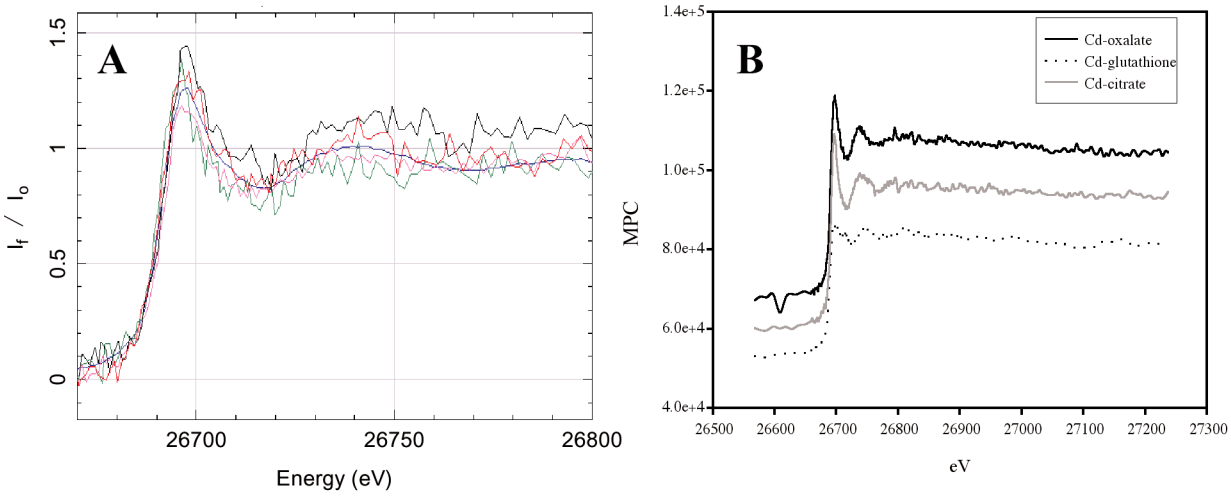

*FIG. 2. Normalized XAS spectra for tissue samples from Cd-treated plants (**A**) and three of the Cd standards (**B**). Because of the similarity in the spectra for the various samples, a specific legend identifying each curve is uncessary. The spectra for the organic acid complexes with Cd show a similar shape, which differs considerably from the K{_} edge of the Cd-glutathione (a Cd-S coordination). Data for these spectra were collected with the Lytle detector.*

with respect to the second-nearest neighbor to the Cd atom. Successful scans would potentially allow the coordination of oxygen binding with carbon to be identified, which would be more indicative of coordination with organic acids or compounds with similar functional groups. Scans were attempted on the tissue samples, but the low Cd concentrations prevented the acquisition of more detailed data.

The standards provided better results, allowing a clear distinction to be made between Cd-O and Cd-S coordination (Fig. 2B). This is an important point, since the aforementioned PCs bind Cd by using a thiol group. The Cd concentration in these three standards was clearly high enough for this discrimination. The Cd cell wall preparations also showed a strong signal and relatively clean EXAFS spectra (data not shown). This sample had a much higher Cd concentration than the leaf tissue samples. This is not surprising, given that the cell wall preps represent simple sorption and given that the treatment solution used to ensure complete saturation of the cell wall binding sites was >600-fold higher than that sites of the solution used to treat the living plants. In order to obtain meaningful data from the standards, more detailed comparisons will have to be done at a later date by using leaf tissues with higher Cd concentrations. This can be achieved rather simply by increasing the duration of Cd exposure and the Cd concentration in the exposure solution. In addition, some peculiarities were noted during the scans of the standards, and some anomalies within the tail region that would complicate interpretation of the EXAFS region were observed. These modifications will be implemented in future beam runs.

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