X-Ray Absorption Studies of the Copper-Beta Domain of Rat Liver Metallothionein

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ABSTRACT

Rat liver metallothionein contains two domains, each of which enfolds a separate metal-thiolate cluster. The binding stoichiometry of these clusters depends on the particular metal ion bound. In the amino-terminal β domain the cluster can accommodate either three Cd(II) ions or six Cu(I) ions. The Cd ions are known to be coordinated in a tetrahedral geometry. In order to better understand the binding of Cu ions in this domain, the Cu-β domain fragment of metallothionein was prepared and investigated by x-ray absorption spectroscopy. Quantitative analysis of the EXAFS data indicates copper-sulfur distances of 2.25 ± 0.03 Å. The EXAFS amplitudes and distance results are most consistent with trigonal coordination. A trigonal biprism is proposed for the Cu_6Cys_9 complex in which Cu occupies each vertex and cysteinyl sulfur bridges at each of the nine edges.

INTRODUCTION

Mammalian metallothionein contains two domains in a 61 residue polypeptide [1, 2]. Each domain enfolds a separate metal-thiolate cluster [3, 4]. The binding stoichiometry of each cluster varies depending on the particular metal ion bound [5]. Cd,Zn-metallothionein binds seven metal atoms (M_7) with a distribution of three and four metal ions in the amino-terminal β domain and carboxyl-terminal α domain, respectively [2, 4]. These ions are coordinated tetrahedrally in each cluster by the nine

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cysteinyl thiolates in the β domain and 11 cysteinyl thiolates in the α domain [6, 7]. Other metals with tetrahedral geometry in M_7-protein complexes include Bi(III), Co(II), Hg(II), Ni(II), Pb(II), and probably In(II) and Sb(III) [1, 8–11].

Unlike the former metal ions, 12 mol equivalents of Cu(I) and Ag(I) can bind to metallothionein with a distribution of six metal ions in both the α and β domains [11]. The α domain coordinates four Cd(II) or six Cu(I) ions regardless of whether the domain is part of the total protein or is studied as a separate peptide [5]. Likewise, the β domain binds three Cd(II) or six Cu(I) ions in the the intact protein and as a separate peptide suggesting that the clusters form independently [5].

Other metallothioneins have been demonstrated to coordinate Cd(II) and Cu(I) in distinct configurations. The protein from yeast binds either eight Cu(I) ions or four Cd(II) ions to the 12 cysteinyl thiolates in the molecule [12]. *Neurospora crassa* metallothionein, containing seven cysteines in a 25 residue polypeptide that is homologous to the β domain, ligates six Cu(I) or three Cd(II) ions per molecule [13].

It is important to elucidate the structure of the Cu clusters in these metallothioneins in that the conformation may differ from that of the Cd clusters. The geometry of Cu coordination in the Cu_6Cys_9 β cluster has been proposed to be trigonal with doubly bridging cysteinyl sulfurs as ligands [11]. Different coordination geometries and conformations of Zn- and Cu-metallothionein may permit the cell to discriminate between the two forms for distinct functions. In order to further characterize the Cu coordination geometry of Cu-metallothionein, we have recorded the x-ray absorption near edge structure (XANES) and extended x-ray absorption fine structure (EXAFS) associated with the Cu K-edge of the Cu-β domain of rat liver metallothionein. The β domain of metallothionein has been used for these studies since this domain exhibits preferential Cu binding in reconstitution studies and is the site of Cu binding in isolated calf Cu-Zn-metallothioncin [14]. The β domain appears to have evolved as the preferred binding domain for Cu(I).

MATERIALS AND METHODS

Sample Preparation

Apo-metallothionein from rat liver was reconstituted anaerobically with 5.8 mol eq Cu(I) stabilized in excess chloride (11). After neutralization to pH 8.1 the sample containing 18 mg protein was incubated for 1 hr at 37°C in the presence of 0.6 mg subtilisin and 2 mM dithiothreitol. The sample was then chromatographed on Sephadex G-50 equilibrated in 10 mM Tris-Cl pH 7. The subtilisin-resistant Cu-peptide was pooled and lyophilized. The sample contained a 6.5 mg Cu-β fragment with a stoichiometry of 6.3 Cu ions per molecule. It was redissolved with water to a copper concentration of 10 mM.

The compound copper (I) tris-tetramethylthiourea tetrafluoroborate ($Cu(Tm\text{-}Tu)_3BF_4$) [15] was a gift from Prof. E. Amma of the University of South Carolina.

Data Collection

The spectra were recorded at the Stanford Synchrotron Radiation laboratory using Si(220) crystal monochromaters. Bending magnet beam line II-3 was used for the model compound and wiggler beam line VII-3 was used for the protein spectra. A fluorescence detection apparatus [16] with nickel filters was used for data collection on the protein samples. During data collection samples were kept at low temperature in an Oxford Instrument CF204 continuous flow helium cryostat.

Data Analysis

The EXAFS spectra $x(k)$ were quantitatively analyzed using the following expression:

$$x(k) \cong (N/kR^2)A(k)\ \exp(-2\sigma^2k^2)\ \sin[2kR+\alpha(k)],$$

where N is the number of atoms at the distance R from the absorber atom (in this case copper), k is the photoelectron wave number, and σ^2 the mean square deviation of R. $A(k)$ and $\alpha(k)$ are the total amplitude and phase shift functions, respectively. The function $\alpha(k)$ was obtained in parameterized form from the spectrum of Cu(II) bis-diethyldithiocarbonate as previously described [17]. The function $A(k)$ was obtained by fitting the model compound $Cu(TmTu)_3BF_4$ using a theoretical sulphur amplitude function and an adjustable scale factor (18). The value of 0.533 thus obtained for the scale factor (see Table 1) was assumed transferrable to the protein.

RESULTS

Edge Spectra

The copper K-edge region for copper metallothionein and the model compound copper(I) Tris-tetramethyl thiourea tetrafluoroborate [$Cu(TmTu)_3BF_4$] are compared in Figure 1. There is no $1s \rightarrow 3d$ transition near 8980 eV, as expected since both samples contain Cu(I). The first feature in the model spectrum is a peak at 8984 eV, which corresponds to an unresolved shoulder in the protein data. The lack of similarity indicates considerable electronic differences between the two copper environments. It is also notable that the edge structure of the metallothionein is unusual for a Cu(I) species [18, 19].

EXAFS Fourier Transforms

Figure 2 shows the Fourier transformed EXAFS data for copper metallothionein at two different temperatures (50 K and 170 K), and for the model compound

TABLE 1.

Sample	Atom Number	R(Å)	σ(Å)	Fit Error[d]
Cu(I)Tris-tetramethyl Thiourea BF_4 (50 K)	3[a,b]	2.258	0.053	101
Copper Metallothionein[c] (50 K)	2[a]	2.250	0.033	208
	3[a]	2.249	0.056	208
	4[a]	2.248	0.071	302
	2.5	2.250	0.045	191
Copper Metallothionein[c] (170 K)	2[a]	2.248	0.047	46.6
	3[a]	2.247	0.067	51.9
	4[a]	2.247	0.082	122.
	2.4	2.247	0.056	36.5

[a] Atom number fixed.
[b] Scale factor of 0.533 derived from this fit.
[c] An E_0 shift of -6.91 eV was required to fit the data.
[d] Fit error defined as $\Sigma(\chi_{obs} - \chi_{calc})^2k^6$, where the summation is over all data points, χ_{obs} is the observed EXAFS and χ_{calc} is the calculated EXAFS.

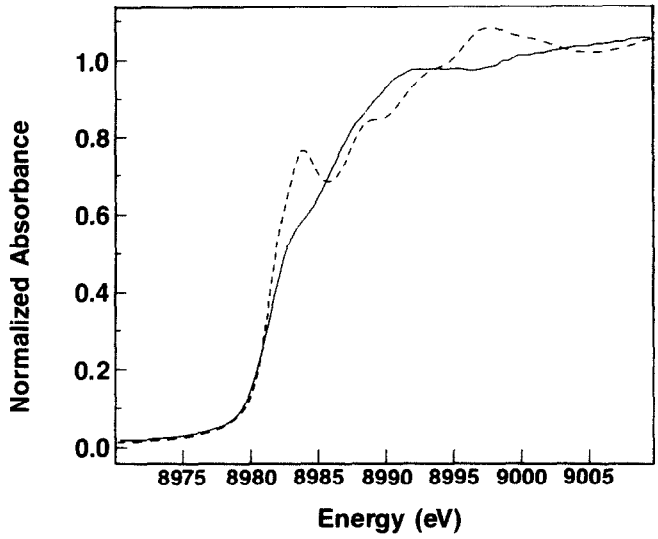
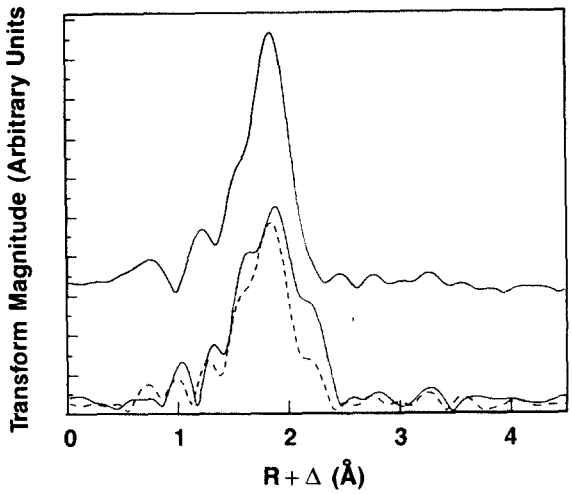


FIGURE 1. The copper *K*-absorption edges of copper metallothionein (——) vs. Cu(TmTu)$_3$BF$_4$ (---).

Cu(TmTu)$_3$BF$_4$. All of the Fourier transforms have their major peak at the same position, indicating the same average distance. It can be seen that there is a marked temperature effect on the metallothionein transform amplitude between 50 and 170 K. This is expected as, at the lower temperature, the vibrational component of the Debye-Waller term will be diminished, causing the EXAFS to be dampened out less rapidly at higher k values.

The Cu(TmTu)$_3$BF$_4$ transform has a relatively symmetric peak indicating a symmetric Cu-S distribution. The slight asymmetry on the low R side is most likely an

FIGURE 2. EXAFS Fourier transforms for Cu(TmTu)$_3$BF$_4$ (at 50 K) (upper trace), copper metallothionein at 50 K (lower trace ——) and copper metallothionein at 170 K (lower trace, ---). Transform range was 0 – 16.2 Å^{-1}, k^3 weighting.



EXAFS artifact which results from the nonlinear Cu–S phase shift. In contrast, some structure can be seen in the metallothionein transforms, and this structure is more pronounced at 50 K. This suggests an inhomogeneity in Cu–S distances.

EXAFS Curve Fitting

The fits to the model compound and metallothionein data are shown in Figure 3, and the results are summarized in Table 1. The best fit occurs with between 2 and 3 (i.e., 2.5) coordinate sulfur at a distance of 2.25 ± 0.03 Å. The uncertainty in the Cu–S coordination number is a common problem in EXAFS analysis. The coordination number and the Debye-Waller factor (σ^2) are highly correlated. This correlation is illustrated by the plot of the fit quality vs. coordination number and Debye-Waller factor in Figure 4.

DISCUSSION

The Cu–S distance of 2.25 Å found for the metallothionein β domain is compatible with a trigonal coordination of the Cu atoms. In crystallographically defined Cu_5S_7 and Cu_4S_6 complexes average bond lengths are 2.16–2.17 Å for digonal Cu–S and 2.27 Å for trigonal Cu–S bonds [20, 21]. In a Cu_4S_9 core complex prepared from thiourea, trigonal Cu–S bond lengths were 2.24 Å, whereas tetrahedral Cu–S distances ranged from 2.3–2.42 Å [22]. A variety of Cu_8S_{12} complexes prepared with D-penicillamine, β,β-dimethylcysteamine and dithiolate the Cu–S bonds were shown to have approximately equal lengths with a mean distance of 2.25 Å [23].

The crystal structure of Cd,Zn–metallothionein II has a three metal cluster in the β domain coordinated by nine cysteine thiolates, three bridging and six terminal: $Cd_1Zn_2Cys_9$ [6]. The α domain contains a Cd_4Cys_{11} cluster with five bridging and six terminal thiolates. The conformation of the β domain cluster is a chair with three axial (C_{15}, C_{21}, C_{29}), three equitorial (C_5, C_{19}, C_{26}) and three bridging (C_7, C_{13}, C_{24}) ligands. Examination of this structure shows that it cannot accommodate six three- or four-coordinate coppers without rearrangement of the structure. The EXAFS amplitudes and distance results are most consistent with trigonal coordination. A possible Cu_6Cys_9 cluster satisfying the stoichiometry and trigonal Cu(I) coordination is the trigonal

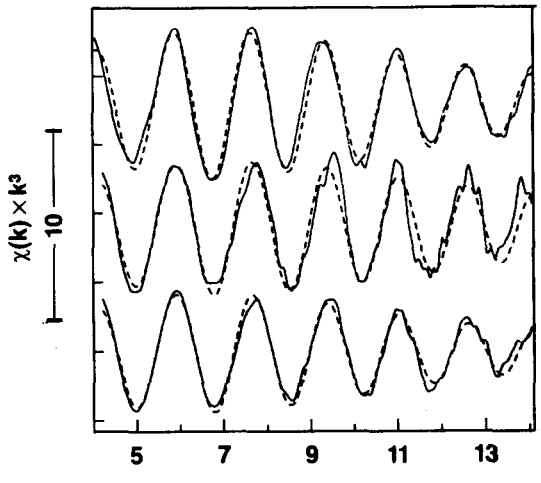


FIGURE 3. EXAFS spectra (——) and best fits (---). Top to bottom: $Cu(TmTu_3BF_4$ (50 K), copper metallothionein (50 K) and copper metallothionein (170 K). In all cases the data were smoothed using a Gaussian window of 0.1 $Å^{-1}$.

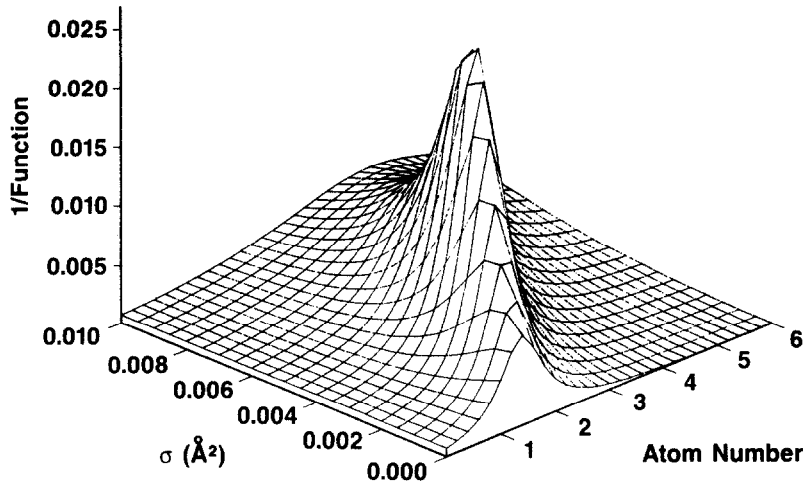
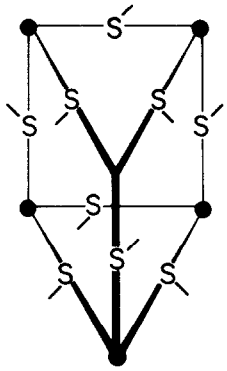


FIGURE 4. The quality of agreement between calculated and observed copper metallothionein EXAFS (at 170 K) as a function of coordination number and Debye-Waller factor σ^2. Because the reciprocal of the error is plotted the best fit occurs at a maximum rather than a minimum. The values for R and the scale factor are those described for the best fit in Table 1.

prism in Scheme 1, in which Cu occupies each vertex and cysteine sulfur bridges at each of the nine edges. This model predicts the absence of short Cu–Cu interactions in the EXAFS and requires all nine cysteines to be bridging thiolates.



Cu_6S_9

SCHEME I. Proposed trigonal Cu(I) coordination complex for the β domain of metallothionein.

The trigonal prism model can be applied to the Cu_6Cys_{11} cluster in the α domain, in analogy with the structure of the $[Cu_6I_{11}]^{5-}$anion [24]. This structure has five iodides centered on the faces of a trigonal prism, six iodides at the vertices, and six tetrahedrally coordinated Cu(I). Because the coordination number of cysteine sulfur is lower than iodide, the ligands of a $(Cu_6Cys_{11})^{5-}$ cluster could not be symmetrically face-centered. Nevertheless, a trigonal prism model for $Cu_6\alpha$ predicts no short Cu–Cu

interactions in the EXAFS, but suggests a coordination number for Cu(I) between three and four.

The Cu(I) ions bound in yeast metallothionein have been investigated by EXAFS spectroscopy [25, 26]. This molecule has only limited homology to mammalian metallothionein. The yeast protein has 12 cysteine residues unlike the 20 in the mammalian proteins and the two exhibit only 20 identities out of 48 possible matches in a computer alignment of the sequences [12]. Twelve of the identities represent the cysteinyl residues involved in metal ligation. From earlier spectra of the yeast protein and $Cu(thiourea)_3Cl$ the best fit of data predicted Cu–S distances of 2.22 Å and 2.36 Å for pairs of sulfurs. A cubane structure was proposed with a four-coordinate cysteinyl sulfurs and four three-coordinate sulfurs (25, 26). It is now apparent that the molecule used was erroneously characterized. Whereas the reported stoichiometry was Cu_4Cys_8, the actual relationship is Cu_8Cys_{12} [12]. Studies are currently under way to assess the distribution of Cu(I) ions in the molecule and to reevaluate the coordination geometry by EXAFS. Since the eight Cu ions in the protein appear to exist in a single polynuclear cluster, its structure could be that of a cube with all 12 cysteins as bridging thiolates, in analogy with the structure of the $Cu(I)_8$(D-penicillamine)$_{12}$ core of a larger $[Cu(I)–(Cu(II)]_{14}$ complex [23]. In this proposed structure each Cu(I) is formally trigonal and occupies the vertices of a cube embedded in an icosahedron of 12 thiolate sulfurs.

Zn(II) and Cd(II) ions are well established to bind to mammalian metallothionein in a tetrahedral orientation. This has been established by EXAFS and x-ray crystallography [6, 7]. Tetrahedral binding of Zn(II) ions and trigonal coordination of Cu(I) ions with different stoichiometries of binding will probably result in dissimilar structures of the polypeptide. Since metallothionein is presumed to function physiologically in some unknown aspect of zinc and copper metabolism, different conformations may impart specificity for functioning of the molecule in different reactions involving Zn(II) and Cu(I). Variants of metallothionein from *Neurospora crassa* and *Drosophila melanogaster* are truncated molecules homologous to the β domain [13, 27] and may have evolved as a region specific for Cu metabolism, whereas the α domain may be a Zn(II) specific domain. Metallothioneins with Cu in the β domain and Zn in the α domain are known [5, 28, 29].

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