Published in final edited form as:

Chem Biodivers. 2008 October ; 5(10): 2042–2049. doi:10.1002/cbdv.200890186.

X-Ray Absorption Spectroscopy of Cuprous-Thiolate Clusters in *Saccharomyces cerevisiae* Metallothionein

Limei Zhang, Ingrid J. Pickering, Dennis R. Winge, and Graham N. George

Department of Geological Sciences, University of Saskatchewan, 114 Science Place, Saskatoon, S7N5E2, Canada (phone: +1-306-9665722; fax: +1-306-9668593; g.george@usask.ca)

Abstract

Copper (Cu) metallothioneins are cuprous-thiolate proteins that contain multimetallic clusters, and are thought to have dual functions of Cu storage and Cu detoxification. We have used a combination of X-ray absorption spectroscopy (XAS) and density-functional theory (DFT) to investigate the nature of Cu binding to *Saccharomyces cerevisiae* metallothionein. We found that the XAS of metallothionein prepared, containing a full complement of Cu, was quantitatively consistent with the crystal structure, and that reconstitution of the apo-metallothionein with stoichiometric Cu results in the formation of a tetracopper cluster, indicating cooperative binding of the Cu ions by the metallothionein.

Introduction

Proteins containing cuprous-thiolate multimetallic clusters form a large family of biological macromolecules that includes copper (Cu) metallochaperones [1], Cu transporters [2], Cu-modulated transcription factors [3], and Cu buffering systems such as Cu metallothioneins [4]. These proteins play key roles in the homeostasis of Cu ions within cells, which is critical because of copper's essentiality, and toxic nature of the metal. Key to understanding the molecular mechanisms of Cu homeostasis is an understanding of the molecular details of the binding of the metal ions in biological molecules. In many cases, structural information from protein crystallography is lacking, although some details are available from spectroscopic techniques such as X-ray absorption spectroscopy (XAS).

The metallothionein found in *Saccharomyces cerevisiae* consists of 12 cysteinyl residues within a 61 amino acid polypeptide [5]. The protein binds Cu^I ions with a solvent-shielded polymetallic core coordinated by only ten of the twelve cysteinyl residues [6] [7]. The nuclearity of the polymetallic core is not fixed, and structural data confirm that complexes with seven or eight Cu^I ions can form [6] [7]. Cooperativity in Cu^I binding is known [8], but the extent of the cooperativity is not clear. Here, we use a combination of XAS and computational chemistry to examine the Cu clusters of *Saccharomyces cerevisiae* Cu metallothionein prepared with a full complement and with stoichiometric levels of added Cu.

Results and Discussion

2.1. X-Ray Absorption Spectroscopy

Fig. 1 shows a typical Cu K-edge XAS spectrum of a low-molecular-weight cuprous thiolate cluster [4]. XAS Spectra can be divided into two indistinct regions: the near-edge spectra which comprise the structured region within *ca.* 50 eV of the absorption edge, and the extended X-ray absorption fine structure (EXAFS), which is an oscillatory modulation of the absorption on the high-energy side of the absorption edge. Near-edge spectra are comprised of transitions from the core level ($1s$ for a K-edge) to unoccupied frontier molecular orbitals of the system. Intense transitions are dipole-allowed $l = \pm 1$, and thus are to levels with a lot of p-orbital character [9]. Near-edge spectra thus provide sensitive probes of electronic structure and of coordination geometry, and cuprous thiolate systems with different coordination environments show structure that is characteristic of their geometries. A significant amount of research effort has been put into developing tools that will allow researchers to quantitatively interpret near-edge spectra of species of unknown structure, but at the time of writing, there are no universally accepted methods, and spectra are, therefore, usually interpreted by a simple comparison with spectra of samples of known structure. EXAFS Spectra are modulations in the X-ray absorption arising from photoelectron backscattering and quantum interference between outgoing and backscattered *de Broglie* waves. In contrast to near-edge spectra, the EXAFS portion of the spectrum is relatively straightforward to analyze, and can yield very accurate interatomic distances together with more approximate coordination numbers. In previous studies [4], we have suggested that measurement of Cu–S bond lengths by EXAFS could provide a tool for determining the coordination of Cu in cuprous-thiolate systems, and used this to estimate that *ca.* 30% of the Cu bound in *S. cerevisiae* metallothionein is digonally coordinated, with the rest being trigonally coordinated. We also observed that the Cu–Cu interaction is much diminished, which was attributed to EXAFS cancellation arising from a variety of Cu–Cu interatomic separations [4]. EXAFS Cancellation occurs when the EXAFS oscillations from different absorber-backscatterer pairs are wholly or partly out of phase. We have previously observed this phenomenon in systems containing tetranuclear $[\text{Cu}_4\text{S}_6]^{2-}$ clusters, and, in particular, the transcription factors Ace1 and Mac1 [3], and in the Ctr1 transporter of *S. cerevisiae* [2]. In these cases, the cluster is distorted so that partial cancellation occurs, and the presence of a tetranuclear cluster is only definitively indicated by small features arising from interactions with distant S-atoms [3].

Fig. 2 compares the XAS data for *S. cerevisiae* metallothionein prepared from apometallothionein reconstituted with stoichiometric Cu, and from fully-loaded protein, reconstituted with eight Cu ions [7]. Interestingly, the metallothionein prepared with stoichiometric Cu shows a distinct Cu–Cu peak, definitively indicating the presence of a cuprous-thiolate cluster. Thus, the binding of Cu to metallothionein must occur cooperatively, and EXAFS curve-fitting analysis (Table) indicates a best fit with the Cu–Cu interactions that might be expected from a distorted Cu_4S_6 cluster of the type established for other cuprous-thiolate systems. In general agreement with this, mass spectrometry of mammalian metallothionein indicates the presence of a tetra-metal cluster when the protein is treated with stoichiometric Cu [10]. The EXAFS spectrum of metallothionein containing a

full complement of Cu indicates a Cu cluster, but with a severely damped Cu–Cu interaction, as illustrated in the EXAFS *Fourier* transform (Fig. 2). Indeed, the damping of the Cu–Cu EXAFS is so severe that, if there were no foreknowledge concerning the presence of a metal cluster, the data could easily be misinterpreted as supporting a mononuclear site.

2.2. Computational Chemistry

The crystal structure of the Cu binding part of *S. cerevisiae* metallothionein has recently been solved to a resolution of 1.4 Å [7]. Protein crystal-structure determinations are usually insufficiently accurate to allow translation of interatomic distances to simulate the EXAFS data. Therefore, we took the cluster portion of the protein crystal structure and used density functional theory (DFT) to refine the coordinates by energy minimization of the structure, while constraining the positions of the cysteine α -C-atoms to their crystallographically determined locations. Fig. 3,a, shows the result which is very similar to the structure obtained from analysis of the crystallography data [7]. The structure shows two digonally coordinated and six trigonally coordinated Cu-atoms, which is in excellent agreement with our previous estimate of the digonal Cu content [4]. The structure shows ten Cu–Cu interatomic separations that are less than 3 Å, and these can be used to simulate the EXAFS as shown in Fig. 4, indicating that the EXAFS and the crystal structure are in good agreement. Perhaps more interesting than the structure of the cluster in fully Cu-loaded protein is the structure of the cluster that forms upon exposure to stoichiometric Cu. As mentioned above, the EXAFS of this sample are best interpreted as a tetranuclear cluster. The crystal structure of fully loaded metallothionein has a fragment which shows structural resemblance to a tetranuclear cluster. We attempted to model the metallothionein encapsulated tetranuclear cluster using DFT, and, in order to allow the cluster to form properly, the constraints on Cys16 were lifted, and the α -C-atom was allowed to move from the crystallographically determined coordinates, which it did by *ca.* 4 Å (Fig. 3). This movement of Cys16 would imply a somewhat different polypeptide fold, but this is not unexpected. We note that there may be many ways in which a tetranuclear $[\text{Cu}_4\text{S}_6]^{2-}$ cluster could form within the metallothionein polypeptide, but our modelling results do indicate that this formation of such a cluster is perfectly reasonable from the chemical point of view within the crystallographically defined framework.

Our conclusions of the formation of a tetracopper cluster are at apparent odds with earlier work which followed the cluster luminescence on titration of apo-metallothionein with Cu, and suggested that the cluster formed cooperatively in an all-or-nothing manner [8]. The results reported here are, however, quite unambiguous in demonstrating that the cluster formed with stoichiometric Cu is distinct from that formed with a full complement of eight Cu ions (Fig. 2). This apparent discrepancy could be explained, if the fluorescence quantum yield of the cluster changes with Cu loading, and our preliminary calculations of fluorescence spectra using the semi-empirical ZINDO code [11] (not illustrated) indicate that such changes are expected.

The stability of $[\text{Cu}_4\text{S}_6]^{2-}$ clusters may have an important role in Cu metallothioneins. The stability of the $[\text{Cu}_4\text{S}_6]^{2-}$ structural motif is illustrated by the fact that it is very common in

the synthetic inorganic literature, and a search of the *Cambridge* crystal structure database [12] gives 41 different examples with the $[\text{Cu}_4\text{S}_6]^{2-}$ core, which is by far the most common structural type. The $[\text{Cu}_4\text{S}_6]^{2-}$ structural motif is observed in proteins other than the Cup1 metallothionein. The tetracopper motif exists in the Cu-activated Ace1 transcriptional activator from *S. cerevisiae* and the related factor Amt1 from *Candida glabrata* [3]. The Cu-regulated transcription factor Mac1 from *S. cerevisiae* contains a related tetracopper cluster in its Cu-inactive state [3]. The cytoplasmic domain of the Cu^{I} permease Ctr1 from *S. cerevisiae* can form a $[\text{Cu}_4\text{S}_6]^{2-}$ structural motif [2]. The Cu^{I} donor in the mitochondrial intermembrane space Cox17 is capable of forming a tetracopper cluster, although its physiological state is more likely a mononuclear Cu^{I} complex [13] [14]. The stability of the $[\text{Cu}_4\text{S}_6]^{2-}$ structural motif will likely result in the future identification of additional members of this family of proteins. As we have discussed above, Cu is very tightly regulated, and the intracellular Cu concentration is, therefore, thought to be very low, and cooperative formation of the stable $[\text{Cu}_4\text{S}_6]^{2-}$ cluster at low Cu concentrations may be important *in vivo*. Cu Metallothionein is thought to have the dual roles of Cu storage and detoxification in the presence of high levels of Cu. Previous work showed that some of the Cu-atoms in metallothionein are more tightly bound than others [15]. In agreement with this, our DFT calculations indicate that the total energy decrease on binding the first four Cu ions (Fig. 3,b) is greater by 0.95eV than the decrease on binding the next four Cu ions (Fig. 3,a), to make up the full complement of eight Cu ions. These energetic differences would be reflected in the Cu-binding constants, and different affinities have previously been proposed for dissociation of the digonally and trigonally bound coppers [7]. Thus, under physiological conditions, metallothioneins may only rarely encapsulate a full complement of Cu ions, and while the ability to bind up to eight Cu ions (in yeast metallothionein) will be important, we can speculate that the predominant form of metallothionein may be the tetracopper cluster.

Experimental Part

Data Acquisition

XAS Spectra were collected at a temp. of 10 K as described in [4] using beamline 7-3 of the *Stanford Synchrotron Radiation Laboratory*. Data on low-molecular-weight compounds were collected in transmittance mode using N_2 -filled ionization chambers, while protein data were collected by monitoring the CuK_α X-ray fluorescence excitation spectrum using a 13-element Ge array detector [16].

Sample Preparation

Cu Metallothionein from *Saccharomyces cerevisiae* was prepared as described in [8]. The apo-metallothionein [8] was reconstituted with different mol-equiv. of CuCl soln. under anaerobic conditions, and loaded into 2-mm-thick *Lucite* sample cuvettes closed with adhesive metal-free mylar tape.

Data Analysis

Extended X-ray absorption fine structure (EXAFS) oscillations $\chi(k)$ were analyzed using the EXAFSPAK suite of computer programs as described in [17] [18], and employing the *ab*

initio theory code FEFF (Version 8.25) to compute the amplitude and phase functions required for curve-fitting [19] [20].

Computational Chemistry

DFT Molecular modeling used the program Dmol³ Materials Studio Version 2.2 [21] [22]. The *Becke* exchange [23] and *Perdew* correlation [24] functionals were used to calculate both the potential during the self-consistent field procedure and the energy. Double numerical basis sets included polarization functions for all atoms. Calculations were spin-unrestricted, and all electron core potentials were used. No symmetry constraints were applied (unless otherwise stated), and optimized geometries used energy tolerances of 2.0×10^{-5} Hartree. The effects of solvation were simulated by using the Conductor-like Screening Model (COSMO) [25], with a dielectric constant of 78.54.

Acknowledgments

Portions of this work were carried out at the *Stanford Synchrotron Radiation Laboratory* which is funded by the *Department of Energy Offices of Basic Energy Sciences and Biological and Environmental Research*, with additional support from the *National Institutes of Health, National Center for Research Resources*. Work at the University of Saskatchewan was supported by *Canada Research Chair awards (G.N.G. and I.J.P.)*, the *Natural Sciences and Engineering Research Council* and the *Canadian Institute of Health Research*. Work at the University of Utah was supported by a grant from *NIH* (ES03817) to *D.R.W.*

References

1. Rigby K, Zhang L, Cobine PA, George GN, Winge DR. J Biol Chem. 2007; 282:10233. [PubMed: 17237235]
2. Xiao Z, Loughlin F, George GN, Howlett GJ, Wedd AG. J Am Chem Soc. 2004; 126:3081. [PubMed: 15012137]
3. Brown KR, Keller GL, Pickering IJ, Harris HH, George GN, Winge DR. Biochemistry. 2002; 41:6469. [PubMed: 12009910]
4. Pickering IJ, George GN, Dameron CT, Kurtz B, Winge DR, Dance IG. J Am Chem Soc. 1993; 115:9498.
5. Butt TR, Sternberg EJ, Gorman JA, Clark P, Hamer D, Rosenberg M, Crooke ST. Proc Natl Acad Sci U S A. 1984; 81:3332. [PubMed: 6374656]
6. Bertini I, Hartmann HJ, Klein T, Liu G, Luchinat C, Weser U. Eur J Biochem. 2000; 267:1008. [PubMed: 10672009]
7. Calderone V, Dolderer B, Hartmann HJ, Echner H, Luchinat C, Del Bianco C, Mangani S, Weser U. Proc Natl Acad Sci U S A. 2005; 102:51. [PubMed: 15613489]
8. Byrd J, Berger RM, McMillin DR, Wright CF, Hamer D, Winge DR. J Biol Chem. 1988; 263:6688. [PubMed: 3283130]
9. Pickering IJ, George GN. Inorg Chem. 1995; 34:3142.
10. Jensen LT, Peltier JM, Winge DR. J Biol Inorg Chem. 1998; 3:627.
11. Zerner, MC. Semi Empirical Molecular Orbital Methods. In: Lipkowitz, KB.; Boyd, DB., editors. Reviews of Computational Chemistry. Vol. 2. VCH Publishing; New York: 1991. p. 313
12. Allen FH, Kennard O, Watson DG. Struct Correl. 1994; 1:71.
13. Heaton DN, George GN, Garrison G, Winge DR. Biochemistry. 2001; 40:743. [PubMed: 11170391]
14. Voronova A, Meyer-Klaucke W, Meyer T, Rompel A, Krebs B, Kazantseva J, Sillard R, Palumaa P. Biochem J. 2007; 408:139. [PubMed: 17672825]
15. Weser U, Hartmann HJ. Biochim Biophys Acta. 1988; 953:1. [PubMed: 3277670]
16. Cramer SP, Tench O, Yocum M, George GN. Nucl Instrum Methods, Sect A. 1988; 266:586.

17. George GN, Byrd J, Winge DR. J Biol Chem. 1988; 263:8199. [PubMed: 3286647]
18. George GN, Hilton J, Temple C, Prince RC, Rajagopalan KV. J Am Chem Soc. 1999; 121:1256.
19. Rehr JJ, Mustre de Leon J, Zabinsky SI, Albers RC. J Am Chem Soc. 1991; 113:5135.
20. Mustre de Leon J, Rehr JJ, Zabinsky SI, Albers RC. Phys Rev. 1991; 44:4146.
21. Delley B. J Chem Phys. 1990; 92:508.
22. Delley B. J Chem Phys. 2000; 113:7756.
23. Becke AD. J Chem Phys. 1988; 88:2547.
24. Perdew JP, Wang Y. Phys Rev B. 1992; 45:13244.
25. Klamt A, Schüürmann G. J Chem Soc, Perkin Trans 2. 1993; 2:799.

Fig. 1. Typical Cu K-edge XAS spectrum of a cuprous-thiolate cluster, bis(tetramethylammonium)hexakis(μ -benzenethiolato)tetracuprate(I), $(\text{Me}_4\text{N})_2[\text{Cu}_4(\text{SPh})_6]$ [4] The inset shows the near-edge portion of the spectrum, which is characteristic of the trigonally coordinated Cu site in this molecule.

Fig. 2. a) EXAFS Spectra and b) corresponding Fourier transforms (Cu-S phase-corrected) of 1) Cu metallothionein prepared with a full complement of eight Cu ions, and 2) with stoichiometric levels of Cu and protein

The solid lines show experimental data while the broken lines show the best fits.

Fig. 3. DFT Energy-minimized structures for Cu metallothionein fully loaded with a) Cu and b) Cu metallothionein containing a tetracopper cluster
Cu- and cysteinyl S-atoms are indicated by green and yellow spheres, resp. Only the α -C- and side-chain C-atoms were included in the computations, and the α -C-atoms were constrained at the crystallographic locations, with the exception of Cys16 in *b*.

Fig. 4. Simulation of EXAFS spectrum of Cu metallothionein containing a full complement of eight Cu-atoms showing cancellation of Cu–Cu EXAFS

The top-most pair of traces show the experimental data (solid line) and best fit (broken line), with the traces below indicating the Cu–S, and the ten different Cu–Cu interactions, with the latter using interatomic distances obtained from computational chemistry (Table 1 and Fig. 3). The overall effect is that the Cu–Cu interactions mostly cancel. The vertical bar indicates the scale for the ordinate $\chi(k) \times k^3$ of $10 \text{ \AA}^{-3}$.

Table

EXAFS Curve-Fitting Parameters^{a)}

Sample	Cu-S			Cu-Cu		
	N	R	σ^2	N	R	σ^2
1	2.75	2.241(2)	0.0031(1)	1/8	2.263 ^{b)}	0.0073(6) ^{c)}
				1/8	2.623	0.0073
				1/8	2.640	0.0073
				1/8	2.688	0.0073
				1/8	2.693	0.0073
				1/8	2.695	0.0073
				1/8	2.696	0.0073
				1/8	2.736	0.0073
				1/8	2.739	0.0073
				1/8	2.845	0.0073
2				1/8	2.895	0.0073
	3	2.256(3)	0.0049(2)	2	2.714(4)	0.0073(4)
				1	2.900(11)	0.0077(4)

^{a)} N is the mean coordination number, R the interatomic distance in Å; σ^2 , the Debye-Waller factors (the mean-square deviation in interatomic distance). The values in parentheses are the estimated standard deviations (precisions) obtained from the diagonal elements of the covariance matrix.

^{b)} Values for Cu-Cu interatomic distances not refined, instead obtained from DFT.

^{c)} A common σ^2 value for the Cu-Cu interaction was refined.