

Structural study of the copper and zinc sites in metallothioneins by using extended X-ray-absorption fine structure

Ian L. ABRAHAMAS,* Ian BREMNER,† Gregory P. DIAKUN,‡ C. David GARNER,* S.Samar HASNAIN,‡|| Ian ROSS* and Milan VAŠÁK§

*Department of Chemistry, University of Manchester, Manchester M13 9PL, U.K., †Rowett Research Institute, Aberdeen AB5 9SB, U.K., ‡Synchrotron Radiation Source, SERC Daresbury Laboratory, Warrington WA4 4AD, Cheshire, U.K., and §Biochemisches Institut der Universität Zürich, CH-8032 Zürich, Switzerland

Zn-metlothionein 1 from rabbit liver was investigated by means of Zn *K*-edge extended X-ray-absorption fine structure (e.x.a.f.s.). Also, the Cu and Zn *K*-edge e.x.a.f.s. were measured for two samples of mixed Cu Zn-metlothionein 2, with Cu/Zn ratios of 5:2 and 6:3, from pig liver. Detailed simulation of the Cu sites shows a primary co-ordination with three sulphur atoms, presumably from cysteine residues at $0.225 \text{ nm} \pm 0.001 \text{ nm}$ ($2.25 \pm 0.01 \text{ \AA}$). The data for the Zn sites are best reproduced by four Zn–S separations at $0.233 \pm 0.001 \text{ nm}$ ($2.33 \pm 0.01 \text{ \AA}$). The Zn *K*-edge e.x.a.f.s. recorded for rabbit metallothionein 1 at 77 K shows, in addition to the primary co-ordination shell, evidence for two Zn–Zn separations at approx. 0.50 nm (5.0 \AA). This latter result provides the first information concerning the internal arrangement of zinc atoms in Zn₇-metlothionein.

INTRODUCTION

A number of structural studies have been carried out to elucidate the structure of metal-binding sites in MT, a low- M_r (approx. 6000) cysteine-rich metal-binding protein (Vašák & Kägi, 1983). The majority of mammalian MTs may be separated by ion-exchange chromatography into two main isoforms, designated MT-1 and MT-2, which differ in their overall negative charge and detailed amino acid composition; however, the number and position of the cysteine residues is highly conserved (Kägi & Nordberg, 1979). Recent notable achievements include the crystal-structure study of rat liver Cd₅Zn₂MT (Stout *et al.*, 1986) and the solution-structure determination by two-dimensional n.m.r. of rabbit liver Cd₇MT (Wagner *et al.*, 1986). Crystal structure and the two-dimensional-n.m.r. solution structure are at variance with each other in detail of the organization of both 3 and 4 metal–thiolate clusters in this protein, but both agree that the metal atoms are co-ordinated exclusively by the cysteine-residue sulphur atoms, as previously determined by the Zn *K*-edge (Garner *et al.*, 1982), Cd *K*-edge (Abrahamas *et al.*, 1985) and Pb and Hg *L₃*-edge e.x.a.f.s. data (Hasnain *et al.*, 1986). The crystal structure is presently refined to 0.23 nm (2.3 \AA) resolution with $R \approx 33\%$, and shows that the Cd₅Zn₂MT is arranged as two globular domains of roughly equal dimensions stacked end-to-end with slight side-to-side contact. One domain (α) enfolds a Cd₄ cluster, the other contains the fifth Cd and two Zn sites in the protein. At present, the resolution is not sufficient to distinguish between the Zn–S and Cd–S separations or define these distances accurately.

The technique of e.x.a.f.s. is complementary to crystallography of metalloproteins in that it can accurately determine the local environment of the co-ordinated metal (Bianconi *et al.*, 1983; Blackburn *et al.*,

1983, 1984). We have therefore measured the e.x.a.f.s. spectra for a number of MTs and report here the results on the Cu and Zn environment in Cu + ZnMTs from pig liver and the Zn environment in Zn₇MT from rabbit liver.

MATERIALS AND METHODS

MT samples were prepared and assayed by procedures described by Bremner & Davies (1975) and Vašák *et al.* (1980). For X-ray-absorption measurements the finely ground freeze-dried samples were pressed into aluminium sample holders with Sellotape windows. E.x.a.f.s. spectra were recorded in the transmission mode at the Daresbury Synchrotron radiation source. The Zn₇MT and 6Cu:3ZnMT samples were measured at 77 K, whereas the data for 5Cu:2ZnMT was obtained at 300 K. Data analysis utilized the single-scattering spherical-wave method for calculating e.x.a.f.s. with phase shifts derived from ‘*ab-initio*’ calculations as described previously (Lee & Pendry, 1975; Perutz *et al.*, 1982). The quality of fits was assessed by using criteria described by Perutz *et al.* (1982) and Blackburn *et al.* (1983).

RESULTS AND DISCUSSION

Fig. 1 shows the X-ray-absorption spectrum of 6Cu:3ZnMT from pig liver, at both the Cu and Zn *K*-edges. The data for the other sample of Cu + ZnMT, with a Cu/Zn ratio of 5:2, are essentially the same, although this latter spectrum has a higher signal-to-noise ratio. The fine structure following each of the absorption edges contains local structural information about the specific element, i.e. Cu or Zn. Both of these spectra show fine structure typical of that expected from the back-scattering from sulphur atoms (Hasnain *et al.*, 1985). The differences in terms of the position of minima

Abbreviations used: e.x.a.f.s., extended X-ray-absorption fine structure; MT, metallothionein.

|| To whom correspondence should be addressed.

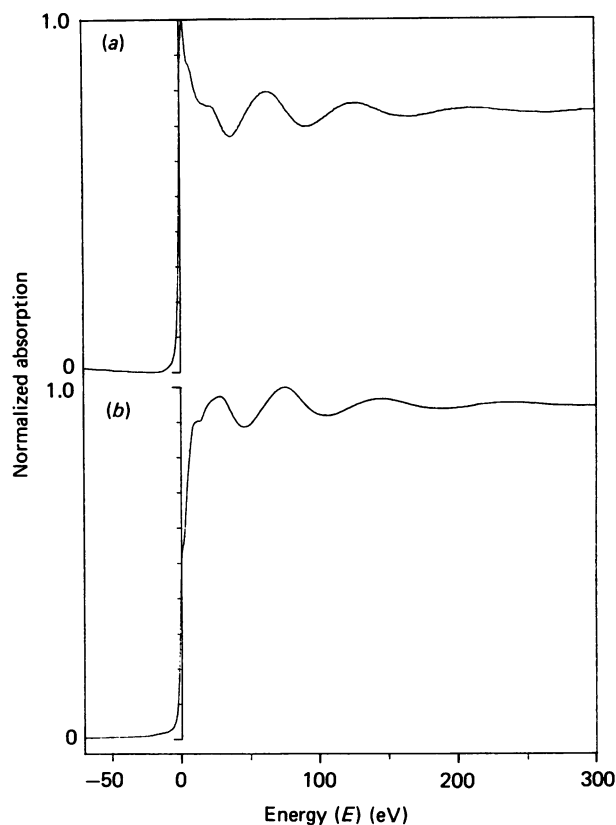


Fig. 1. Normalized e.x.a.f.s. spectra for Cu (b) and Zn (a) in 6Cu:3ZnMT

and maxima are due primarily to the difference in the metal–sulphur separations in the two cases. Also, it is clear from the profile at the top of the X-ray-absorption edge that the nature of the bound-state transitions, from the 1S level to virtual orbitals of the metal centre, are different from Cu as compared with Zn.

Zn environment in MTs

The Zn *K*-edge e.x.a.f.s. of the two Cu+ZnMT samples and Zn₇MT are superimposable except that the data quality of Zn₇MT is statistically better and is available for an extended range 30–740 eV [i.e. $k = 35\text{--}140\text{ nm}^{-1}$ ($3.5\text{--}14\text{ \AA}^{-1}$)]. E.x.a.f.s. spectrum of Zn₇MT was recorded at 77 K, and its k^2 -weighted experimental spectrum and its Fourier transform are shown in Fig. 2. The Fourier transform shows peaks at around 0.4 nm (4 Å) and 0.5 nm (5.0 Å) in addition to the primary peak at about 0.23 nm (2.3 Å). To assess the significance of these relatively smaller peaks in the Fourier transform the Fourier filtering technique was employed. The Fourier-transformed data in the ranges of 0.09–0.28 nm (0.9–2.8 Å) and 0.09–0.56 nm (0.9–5.6 Å) were back-transformed to isolate the e.x.a.f.s. components corresponding to the peaks that occur in these regions of the Fourier transform. The Fourier-filtered spectrum with the extended range [0.09–0.56 nm (0.9–5.6 Å)] shows deviation from the sinusoidal behaviour, particularly in the k range $90\text{--}140\text{ nm}^{-1}$ ($9\text{--}14\text{ \AA}^{-1}$). E.x.a.f.s. simulation of both the raw normalized data and Fourier-filtered data with one shell of sulphur atoms gave the same result, i.e. Zn is co-ordinated to four sulphur atoms at a distance of

$0.233 \pm 0.001\text{ nm}$ ($2.33 \pm 0.01\text{ \AA}$) with a Debye–Waller term, σ^2 , of 0.00005 nm^2 (0.005 \AA^2). This result is in accordance with the structural data for zinc–thiolates (Watson *et al.*, 1985). The single-shell fit is compared with the Fourier-filtered 0.09–0.56 nm (0.9–5.6 Å) data (Fig. 3), where departures from the single scattering distance is evident in the data range $k = 90\text{--}140\text{ nm}^{-1}$ ($9\text{--}14\text{ \AA}^{-1}$). To analyse the higher-shell contribution, data were back-transformed in the range (R) 0.46–0.58 nm (4.6–5.8 Å), which is shown in Fig. 4 with the best stimulation obtained by using two Zn–Zn separations at about 0.50 nm and 0.52 nm (5.0 Å and 5.2 Å). Attempts to simulate this Fourier-filtered spectrum by using one Zn–Zn and one Zn–S separations or a Zn–S separation were not successful. It is important to note that the strong

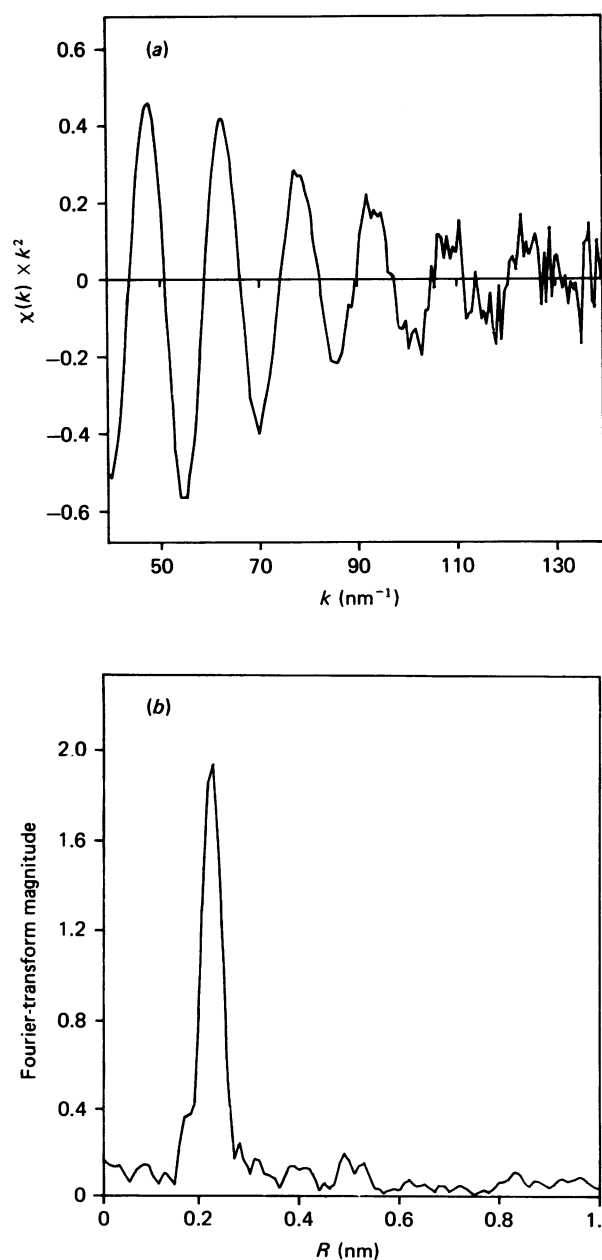


Fig. 2. (a) Experimental e.x.a.f.s. spectrum and (b) Fourier transform of Zn₇MT, recorded at 77 K

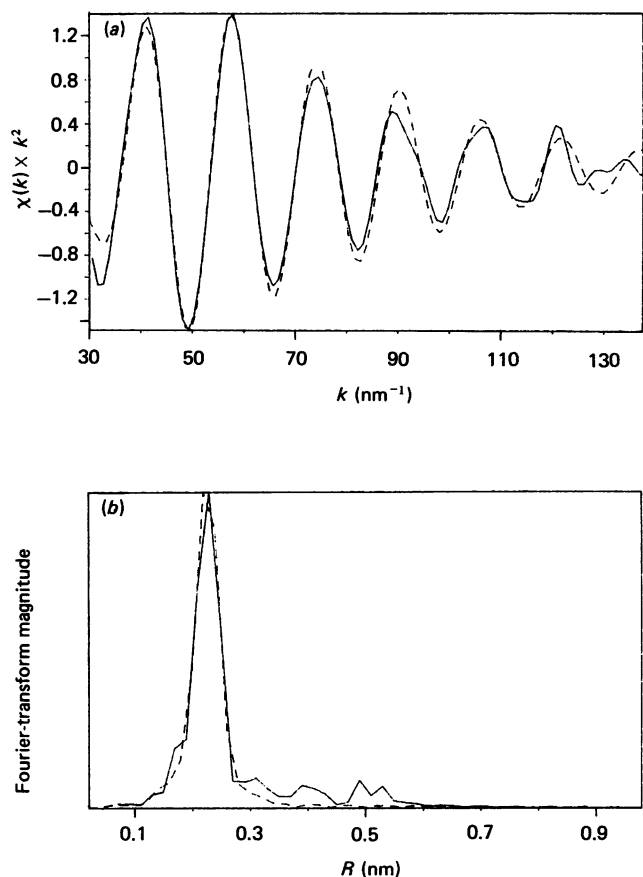


Fig. 3. (a) Fourier-filtered e.x.a.f.s. data [$R = 0.09\text{--}0.56$ nm ($0.9\text{--}5.6$ Å)] (—) and a single-shell fit with 4(Zn-S) separations at 0.233 nm (2.33 Å) and σ^2 of 0.00005 nm² (0.005 Å²) (---) and (b) Fourier transform for Zn₇MT

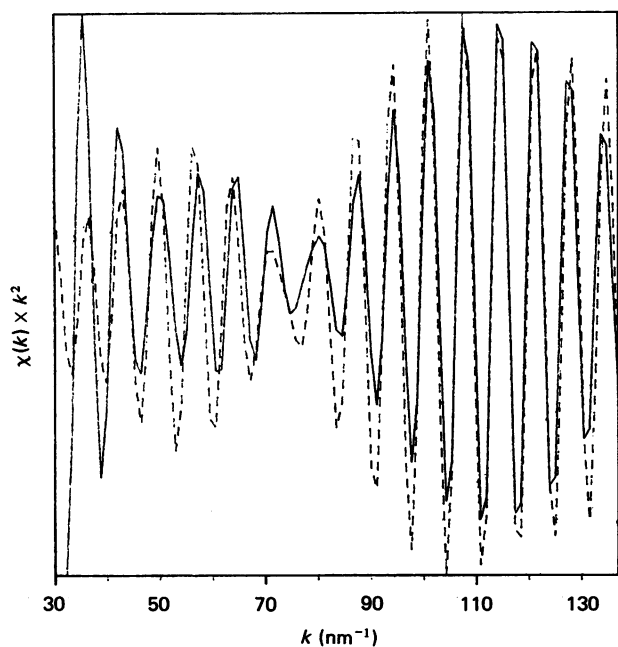


Fig. 4. Fourier-filtered e.x.a.f.s. data [$R = 0.46\text{--}0.58$ nm ($4.6\text{--}5.8$ Å)] (—) and a theoretical simulation with two Zn separations at 0.50 nm (5.0 Å) and 0.52 nm (5.2 Å) (---) for Zn₇MT

beating of the two waves results in cancellation of contribution from two distances over a significant range $k = 60\text{--}90$ nm⁻¹ ($6\text{--}9$ Å⁻¹). Thus, in order for such contributions to be detectable, e.x.a.f.s. data of very high quality over an extended range are essential. Further improvement was obtained with the inclusion of back-scattering from a sulphur atom at approx. 0.41 nm (4.1 Å). Additional confidence on these distal separations was obtained when an excellent simulation of the extended-range Fourier-filtered data was obtained with these contributions included. Fig. 5 shows the final stimulation with the parameters given in Table 1.

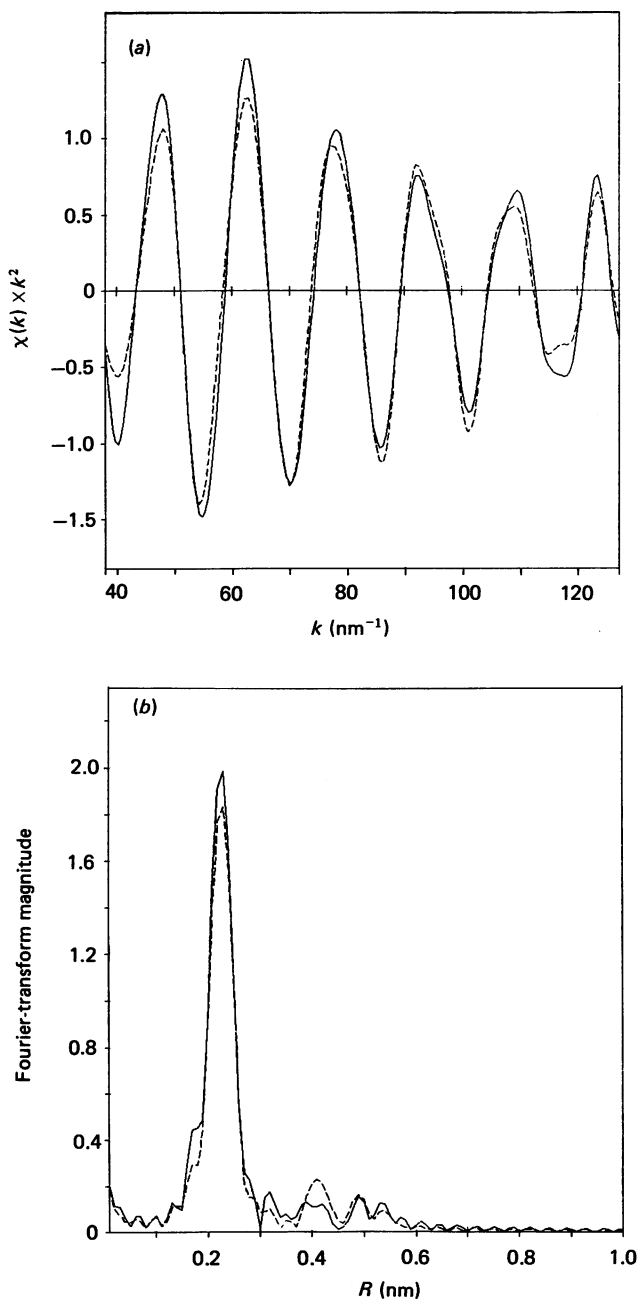


Fig. 5. (a) Four-shell fit (---) to the Zn *K*-edge Fourier-filtered e.x.a.f.s. data (—) [$R = 0.09\text{--}0.56$ nm ($0.9\text{--}5.6$ Å)] and (b) Fourier transform with parameters given in Table 1 for Zn₇MT

Table 1. Parameters used to simulate the e.x.a.f.s associated with the Zn *K*-edge of rabbit Zn₇MT and Cu *K*-edge of pig liver Cu + ZnMT

R denotes the distance of scattering atoms from the Zn atom in Zn₇MT and Cu atom in Cu + ZnMT. σ^2 is a Debye-Waller factor and is equivalent to ΔR_{rms}^2 .

Zn ₇ MT*			6Cu:3ZnMT*			6Cu:2ZnMT		
Atom	σ^2 (nm ²)	<i>R</i> (nm)‡	Atom	σ^2 (nm ²)	<i>R</i> (nm)‡	Atom	σ^2 (nm ²)	<i>R</i> (nm)‡
4S	0.0005	0.233	3S	0.0005	0.225	3S	0.0007	0.225
1S†	0.0001	0.41	—	—	—	—	—	—
1Zn	0.0002	0.50	—	—	—	—	—	—
1Zn	0.0003	0.52	—	—	—	—	—	—

* Data for these were obtained at 77 K.

† Contribution due to this shell is relatively small.

‡ The estimated error for Cu-S and Zn-S distances is less than 0.002 nm (0.02 Å). This has been assessed by comparison with the crystallographically characterized chemical systems. Error for higher shells is more difficult to assess in view of their weaker contributions, but is not expected to be greater than 0.01 nm (0.1 Å).

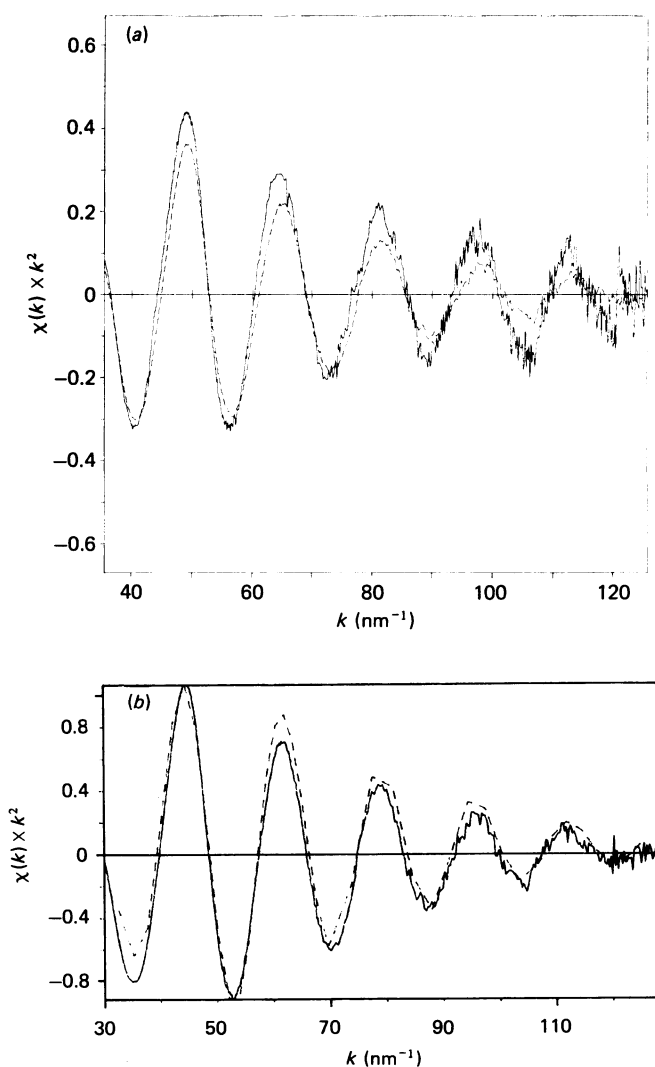


Fig. 6. (a) E.x.a.f.s. spectra at Cu *K*-edge of 6Cu:3ZnMT (—) and 5Cu:2ZnMT (---) and (b) Cu *K*-edge e.x.a.f.s. spectrum of 5Cu:2ZnMT from pig liver (—) and a theoretical simulation with three Cu-S separations at 0.225 nm (2.25 Å) and σ^2 of 0.00007 nm² (0.007 Å²) (-----)

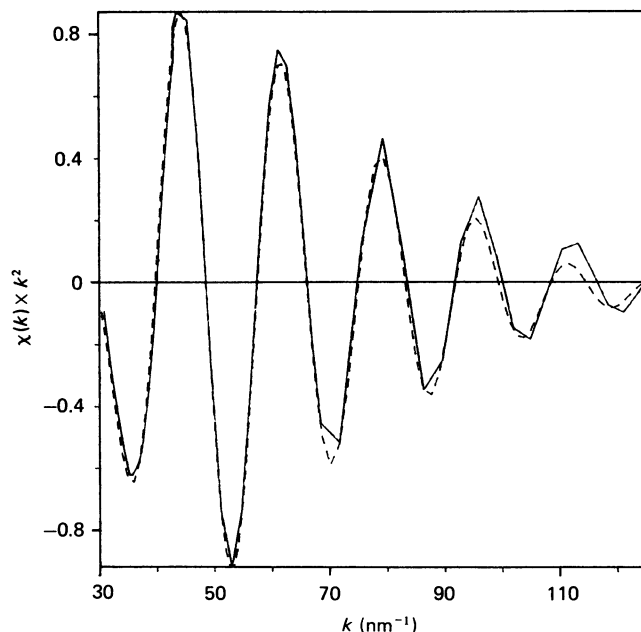


Fig. 7. Comparison of best theoretical simulations obtained with models with three Cu-S components at 0.225 nm (2.25 Å) (—) and with one Cu-S component at 0.217 nm (2.17 Å) and two Cu-S components at 0.229 nm (2.29 Å) (-----)

Cu in Cu + Zn metallothionein

Cu *K*-edge e.x.a.f.s. data have been obtained for two Cu + ZnMTs, 6Cu:3ZnMT and 5Cu:2ZnMT from pig liver. The data range available for the Cu *K*-edge e.x.a.f.s. is limited to 600 eV [$k = 120 \text{ nm}^{-1}$ (12 \AA^{-1})] by the presence of Zn *K*-edge. The data obtained from the latter are of better quality. The two e.x.a.f.s. spectra are very similar except that the spectrum of the 6Cu:3ZnMT sample shows an increase in amplitude typical for a decrease in σ^2 . This is to be expected as the data for the 6Cu:3ZnMT was recorded at a lower temperature, 77 K. Fig. 6 shows a comparison of the two sets of data. For both data sets the best simulation is obtained with three Cu-S separations of $0.225 \pm 0.001 \text{ nm}$ ($2.25 \pm 0.01 \text{ \AA}$).

For 5Cu:2ZnMT, the Debye–Waller term, σ^2 , is refined to 0.00007 nm² (0.007 Å²), which is reduced to 0.00005 nm² (0.005 Å²) for 6Cu:3ZnMT.

Two other models were investigated, keeping the total co-ordination of sulphur atoms to three since simulations with more or less than three sulphur atoms were unsatisfactory. We attempted refinement starting with (a) one Cu–S component at a distance of 0.21 nm (2.1 Å) and two Cu–S components at 0.22 nm (2.2 Å) and (b) two Cu–S components at the shorter distance and one Cu–S component at the longer distance. Both of these lead to equally good simulations of the e.x.a.f.s. spectrum. For model (a), the shorter Cu–S distance refined to 0.217 nm (2.17 Å) and the longer distance to 0.229 nm (2.29 Å). The σ^2 for both shells was almost equal [0.00002 and 0.00003 nm² (0.002 and 0.003 Å²) respectively]. For model (b) the two distances refined to 0.221 nm (2.21 Å) and 0.232 nm (2.32 Å) with σ^2 for the two Cu–S components being 0.00005 nm² (0.005 Å²), twice as large as that of the contribution of the single Cu–S component [0.00002 nm² (0.002 Å²)]. Fig. 7 compares the best fit obtained with three sulphur atoms at 0.225 nm (2.25 Å) to the one obtained with model (a), i.e. one Cu–S component at 0.217 nm (2.17 Å) and two Cu–S components at 0.229 nm (2.29 Å). It is evident that differences occur in the k range 95–130 nm⁻¹ (9.5–13 Å⁻¹). The present quality and range of data do not allow us to distinguish between the two cases.

Our data do not support the co-ordination of Cu to four sulphur atoms, as was reported for yeast Cu-thionein (Bordas *et al.*, 1982), where the Cu was suggested to be co-ordinated to four sulphur atoms, two at 0.216 nm (2.16 Å) and two at 0.228 nm (2.28 Å). An attempt to analyse the present 5Cu:2ZnMT data on the basis of the yeast Cu-thionein model was unsuccessful, as it gave extremely large errors [> 0.05 nm (0.5 Å)] in the two distances after refinement, and also the Debye–Waller term, σ^2 , increased by more than 4-fold. This may reflect the fact that mammalian Cu+ZnMT and yeast Cu-thionein are quite different proteins, with different amino acid compositions. Their only similarities are their size, their high cysteine content and the binding of Cu to these residues. However, we note that the data range for yeast Cu-thionein was much limited and did not have sufficient resolution to define two distances within 0.012 nm (0.12 Å). It will be of interest to compare e.x.a.f.s. data obtained under identical conditions for the two proteins to establish whether there are indeed major differences in the co-ordination of the Cu atoms.

These results have provided direct structural informa-

tion about the Zn and Cu sites of MTs. Cu is three-coordinate, whereas Zn is four-coordinate. In the case of the metal-homogeneous system studied here, Zn₇MT, evidence for higher shells was obtained.

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