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Structural model of rabbit liver copper metallothionein†

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Molecular modelling techniques have been used to calculate the structure for the primary, mammalian copper-containing protein, metallothionein Cu_{12}-MT. Structural information obtained from existing spectroscopic data and comparison with inorganic copper(I)–thiolate model compounds were employed. A two-domain structure, with stoichiometries of Cu_6S_9 and Cu_6S_{11}, was constructed in which the copper(I)–cysteine connections were based on the arrangement of the cysteines in the Zn_7-MT precursor as determined by previous ^{1}H NMR studies. In the minimized structure, in which metal–thiolate bond lengths and angles were allowed to vary, each copper(I) was trigonally co-ordinated by cysteine thiolates; the average bond length was calculated to be close to 224 pm. In the α domain of the protein both bridging (7S) and terminal (4S) thiolate ligation take part in the Cu_6S_{11} cluster structure, in the β domain all nine thiolates bridge pairs of Cu^I atoms to form the Cu_6S_9 cluster structure. Significantly, the energy-minimized structure exhibits two main crevices that are similar to those reported for the structure of Cd_5Zn_2-MT 1 obtained by analysis of X-ray diffraction data. These crevices could allow access to the otherwise embedded copper–thiolate clusters.

Metallothioneins are an important class of proteins that were first discovered in 1957 as responsible for cadmium sequestration in horse kidney.[1] Since then interest in these proteins has increased almost exponentially owing to their ability to bind such a wide range of metal ions (particularly, the toxic Group 12 metals), the multiple ways of inducing their syntheses *in vivo*, and their various physiological functions.[2] The copper(I) complexes of metallothionein are currently under intensive study because copper–metallothioneins are believed to play vital roles in (*a*) homeostasis of this metal, including absorption, storage and transport,[3–7] (*b*) reconstitution of various copper-depleted proteins and enzymes,[8] (*c*) cell proliferation[9–10] and (*d*) free-radical detoxification.[11] To date, no structures of copper-containing mammalian metallothioneins have been determined or modelled.

The key and unique properties of metallothioneins are that they are cysteine rich, low-molecular-weight proteins, with a remarkable ability to bind multiple metal ions into metal–thiolate clusters. For the Cd^{II}- and Zn^{II}-containing protein, notably Cd_5Zn_2-, Cd_7- and Zn_7-MT, these clusters dominate the tertiary structure of the peptide, see Fig. 1.[12–16] Mammalian metallothioneins (MT) are composed of 61 or 62 amino acids, of which 20 are cysteine residues in highly conserved locations along the peptide chain. Two major isoforms exist for the mammalian protein, namely MT 1 and MT 2. The nomenclature is based on the order of elution from an anion exchange column; MT 1 elutes earlier due to its lower apparent ionic charge. Within each isoform are numerous subisoforms, which differ only in their non-cysteinyl amino acid composition. Fig. 2 shows the peptide sequence for rabbit liver metallothionein, isoform 2a.[17] Metallothionein synthesis may be induced *in vivo* by administration of metals, glucocorticoid hormones, alkylating agents, as well as stress and ultraviolet or X-ray radiation.[2,18] The native protein binds Zn^{II}, Cd^{II} or Cu^I *in vivo*, although many other metal ions have been observed to bind to MT *in vitro*, including Ag^I, Au^I, Hg^{II}, Co^{II}, Ni^{II}, Pt^{II} and Fe^{II}; see, for example, the spectroscopic data for many of these proteins.[19,20]

Mammalian metallothionein is able to bind seven divalent metal ions (Zn^{II} or Cd^{II}) per protein molecule in a two-domain arrangement, see Figs. 1 and 3. The carboxy-terminal α domain contains 11 cysteine residues and binds four metal(II) ions while the amino-terminal β domain contains nine cysteines and binds three metal(II) ions. The structure and metal–cysteine connectivities for M^{II}_7-MT have been determined by ^{1}H–^{113}Cd NMR[14–16] and by X-ray crystallography.[12,13] The Cd–S_{cys} connectivities for rabbit liver Cd_7-MT 2a are shown in Fig. 3. The determination of these structures has greatly aided studies of many properties of the protein. For example, the $M_4(S_{cys})_{11}$ cluster structure is energetically more suited to bind Cd^{II}, and it is this domain that is more often observed to sequester the toxic Cd^{II} *in vivo*, resulting in a highly specific $(Cd_4)^{\alpha}(CdZn_2)^{\beta}$-MT species.[13,21]

Metal-replacement studies have shown that the seven Zn^{II} in Zn_7-MT are replaced after addition of 12 copper(I) equivalents.[22–24] Proteolytic experiments have shown that the two-domain nature of the protein is conserved in Cu_{12}-MT, with six Cu^I bound in each domain of the protein.[25] Spectroscopic studies have revealed that unique structures are formed with 9, 12 and 15 copper(I) equivalents, as well as a mixed copper(I)–cadmium(II) species with stoichiometry $(Cu_6Cd_4)^{\alpha}(Cu_6)^{\beta}$-MT.[24] The uniform increase in the absorbance due to the thiolate-to-copper(I) charge-transfer band with each equivalent of Cu^I added up to 12 indicates similar binding environments for each of the 12 Cu^I bound to the protein, most likely trigonally co-ordinated to three cysteine thiolates. Past the 12 copper(I) point, optical and chiroptical data strongly suggest a lowering of the symmetry around some Cu^I to digonal co-ordination, allowing the protein to bind the 'extra' metal ions.[24] The copper-containing metallothioneins have frequently been studied using the synchrotron-based EXAFS (extended X-ray absorption fine structure) technique as a means of overcoming the inherent silence of copper(I) as a chromophore for other structurally sensitive techniques. The EXAFS data provide averages for bond distances and co-ordination numbers of all like atoms present, so interpretation can be difficult, especially if a mixture of geometries is suspected.[20,26] Therefore, the mammalian Cu_{12}-MT represents a crossroads in the pathway between different structural species for cadmium– and copper–metallothioneins: excellent structural data are available for the M_7-MT species, excellent stoichiometric data are available for the Cu_{12}-MT species, average bond distances and co-ordination

† *Supplementary data available* (No. SUP 57208, 7 pp.): fractional co-ordinates for the molecular model. See Instructions for Authors, *J. Chem. Soc., Dalton Trans.*, 1997, Issue 1.
Non-SI unit employed: dyn = 10^{-5} N.

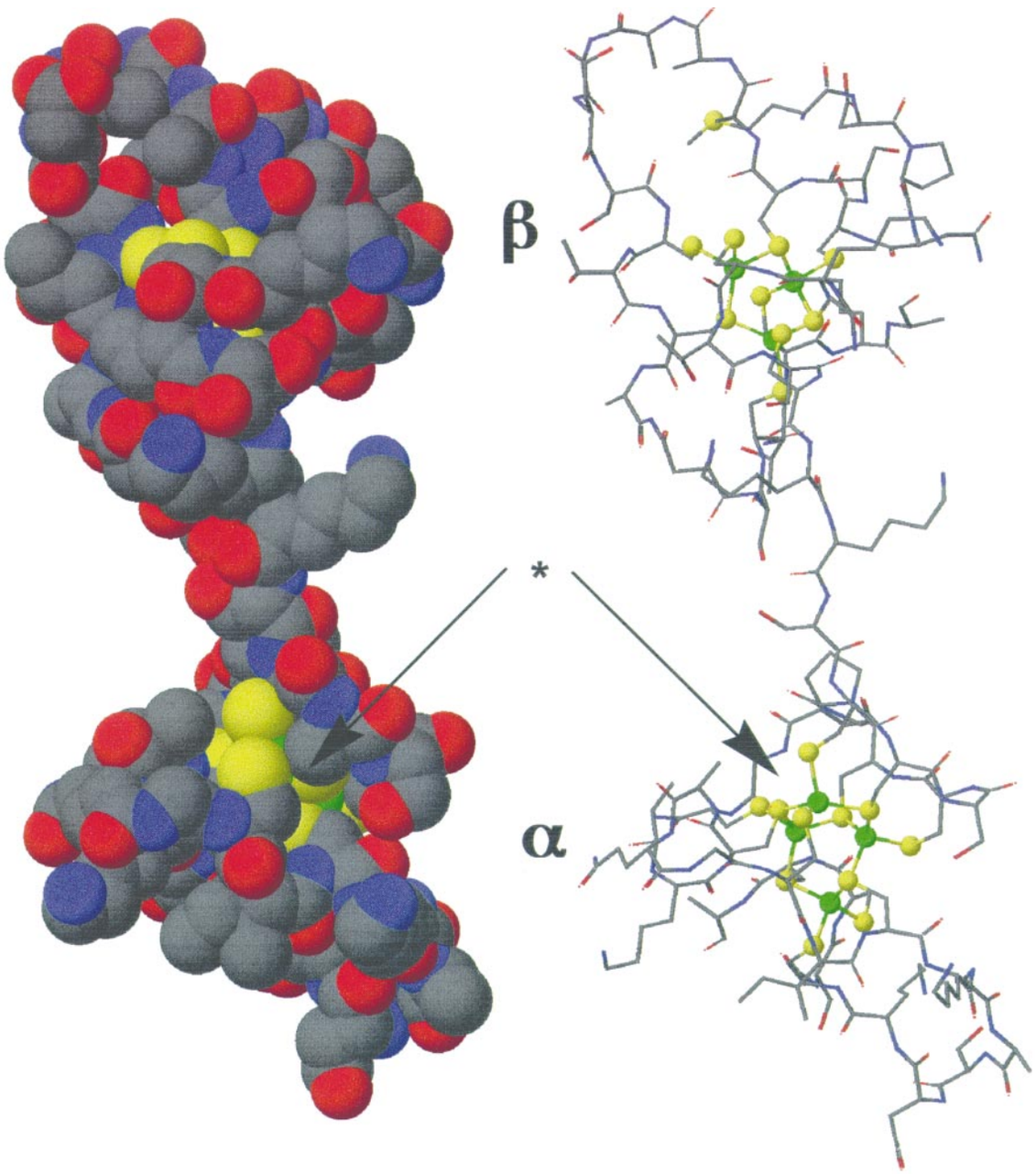

Fig. 1 Space-filling and line representation of the structure of rabbit liver Zn_7-MT 2a (methionine up), showing the cadmium(II)–cysteinyl sulfur connectivities upon which the structures are based. Key: green, Zn; yellow, S; grey, C; blue, N; red, O. The asterisk denotes the accessible sites of the zinc(II)–cysteine thiolate clusters

numbers are available for Cu_{12}-MT, but no data are available that define the three-dimensional structure. Two-dimensional ^{1}H NMR studies have provided structural information for Cd- and Zn-containing protein, unfortunately similar data cannot be obtained for the Cu^I-containing protein. While Ag^I binds to mammalian metallothionein, optical studies clearly show that the binding is not the same as in Cu^I,[27,28] so that structural information obtained by analysis of two-dimensioned NMR data from Ag-containing mammalian protein cannot be transferred directly to the copper(I) protein. At present this leaves the structural problem to be solved by EXAFS and modelling. Reports describing modelling of the three-dimensional structure of metallothionein have recently appeared.[29,30] In this paper we describe in detail results first reported in preliminary form.[24]

The structures of the metal(II) thiolate clusters in the M_7S_{20} protein (M_4S_{11} in the α domain and M_3S_9 in the β domain, for Cd^{II} and Zn^{II}) are based on the adamantanoid structure, and many synthetic analogues exist for them. Modelling the copper(I) thiolate structures of Cu_{12}-MT based on synthetic complexes also proves helpful in understanding the binding and functional properties of copper–metallothioneins. Dance *et al.*[31] have noted several recurring structural motifs in synthetic copper(I) thiolate complexes, including (*a*) $(\mu\text{-SR})_3(CuSR)_3$ rings and (*b*) $(\mu\text{-SR})_4Cu_4$ rings. These structures display trigonal co-ordination for the Cu^I atoms, the latter motif often including terminal ligation by other types of ligands such as phosphines.[32,33]

The objective of this work was to develop a molecular model specifically for rabbit liver Cu_{12}-MT isoform 2a, based on synthetic copper(I) thiolate chemistry, taking into account the constraints imposed on the latter structures by the surrounding protein, the stoichiometric ratio for copper(I) to thiolate sulfur, and the spectroscopic evidence for possible protein rearrangement as Cu^I replaced Zn^{II} in the rabbit liver protein. This model can be used to develop a clearer understanding of copper binding to metallothionein and of the spectroscopic properties of the protein. The locations of the important structural com-

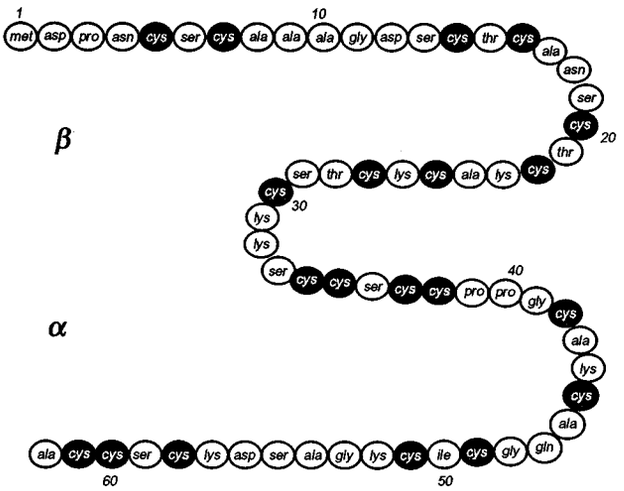

Fig. 2 The sequence of the rabbit liver metallothionein isoform 2a 62-amino acid peptide from data of Kagi.[17] Cysteine residues are represented by the dark ovals

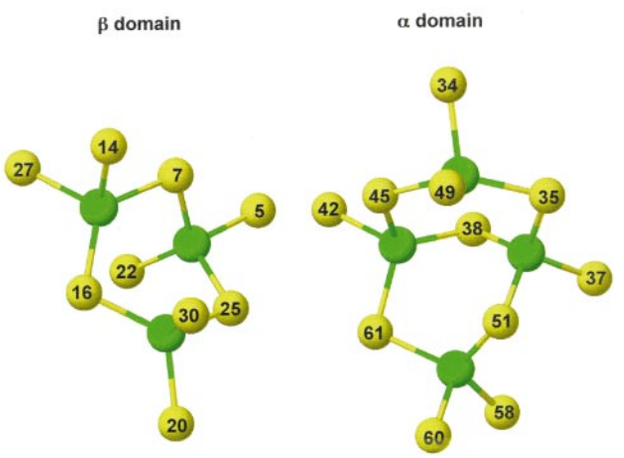

Fig. 3 A three-dimensional representation of the S_{cys}–Cd connectivities in the two domains of rabbit liver metallothionein isoform 2a 62-amino acid peptide from data of Kagi[17] and Otvos and Armitage.[14] The stoichiometries shown are Zn_3S_9 for the β domain and Zn_4S_{11} for the α domain (Zn^{II} displays the same connectivity as Cd^{II}). The numbering scheme for the S_{cys} are based on the sequence shown in Fig. 2 for the 62-amino acid peptide

ponents, the 12 Cu^{I}, the 20 cysteinyl thiolates, and the overall wrapping of the peptide chain, can all be tested in subsequent experimental studies.

Computational Details and Construction of the Model

All calculations were performed using the CACHE (Computer Aided Chemistry) three-dimensional Stereo Worksystem[34] operating on an Apple Power Macintosh 8500 computer (Macintosh OS 7.5.3) using Allinger's MM2 force field as augmented by CACHE.[35] The molecular mechanics structure was investigated by comparing the total energy of possible structures, where the energy is given by the mechanics equation $E_{total} = E_{bonding} + E_{theta} + E_{phi} + E_{improp} + E_{elec} + E_{vdW} + E_{Hbond}$, where these terms describe bond lengths, angles, dihedral angles, improper torsions, electrostatic potential, van der Waals interactions, and hydrogen bonding, respectively. The CACHE system uses the parameters published by Allinger for the MM2 force field directly for all elements except for the metals. There are no force-field parameters available for metals, rather the CACHE system provides proprietary estimates of the force-field parameters for each element in the Periodic Table. The key parameters of Cu–S bond stretching and bond bending are: (*i*) bond stretch, $E = 143.88k_s[(r - r_0)^2 - (r - r_0)^3]/2$, where k_s = 4.400 mdyn $Å^{-1}$ (bond stiffness) and $r_0 = 2.25$ Å (standard length for trigonal co-ordination); and (*ii*) bond angle, $E = 0.043\,828k_b[(\varphi - \varphi_0)^2 + 7.0 \times 10^{-8}(\varphi - \varphi_0)^6]/2$, where $k_b = 0.450$ mdyn $Å^{-1}$ (bond stiffness) and $\varphi = 120.0°$ (standard angle). In this manner the structures of metalloproteins can be calculated that include, specifically, full energy minimization of the metal and its ligands.

Like all modelling, the first guess at the structure will define completely the connectivities between each atom. For metals this means that the co-ordination number and hybridization must be known so that the correct geometry can be set. For Cd^{II} and Zn^{II} in metallothionein, tetrahedral co-ordination requires four cysteinyl groups. However, for Cu^{I}, the co-ordination number could be four, three or two. Trigonal, three-co-ordinate binding has been proposed based on the stoichiometric ratio of 12 Cu^{I} to 20 S.[25] The minimization can only show how the peptide will wrap under these constraints, not whether a co-ordination number of two would give a lower total energy. By examining the structure in detail we can determine if it is reasonable.

A possible structure for copper-exchanged metallothionein was determined using two major constraints: the primary amino acid sequence and a set of connectivities which relate cysteine residues and copper atoms. The copper–cysteine bonding was formulated so that each copper atom co-ordinates three cysteinyl residues, represented as thiolate moieties with unit negative charges on sulfur. The α and β domains were represented by residues 31–61 and 1–30, respectively. Using the three-dimensional structure of Cd_5Zn_2-MT as a guide,[12] metal-free models of the α and β domains were individually constructed and approximate structures obtained for each using a three-dimensional stereo display and manually adjusting the positions of residues and side chains in space. Six copper(I) ions of unit positive charge were then added to each domain, with each copper ion bonded to three cysteinyl residues so that the connectivity constraint was obeyed. For each domain the ions were then manipulated in three dimensions to find reasonable positions at which there was no bond interlacing. At this point the copper–sulfur bond lengths and angles were not chemically reasonable, so each domain was individually refined using steepest-descents minimization with methyl and methylene groups treated as embedded atoms. Refinements proceeded until the copper atom displacements were qualitatively small, whereupon the structure of each domain was further refined using conjugate-gradient minimization with methyl and methylene groups treated explicitly. This was continued until the energy change upon successive iterations was less than 0.001 kJ mol^{-1}. In the final step the two domains were connected and the entire structure minimized to the 0.001 kJ mol^{-1} energy tolerance using gradient minimization and explicit methyl and methylene groups. For the final structure it was concluded that: (*i*) the above constraints are consistent with each other; (*ii*) it is topologically and sterically possible, there are no interlaced bonds, and the peptide residues display no unusual bond lengths or angles which might be indicative of steric bad contacts; (*iii*) the cysteine–metal bonding is chemically reasonable as each copper(I) site can be formulated as a distorted $[CuS_3]^{2-}$ trigonal centre.

The computational details for the minimization of the model copper(I)–thiolate compounds varied from the above procedure. The model structures were entered into the CACHE Editor software manually or as fractional coordinates received from the Brookhaven Protein Database. For the structure created from fractional coordinates a molecular crystal was built up using the space group for the crystal structure described in the corresponding literature. The molecules were then inspected to observe any conflicts in the configurations of the atoms, and changes were made where appropriate.

The finalized molecules were then subjected to an optimization of geometry by molecular mechanics. The conjugate-gradient method was utilized in the minimization of the structure, using the augmented MM2 parameters and treating methylene and methyl groups explicitly. The structure was minimized and refined until the final energy change was less than 0.001 kJ mol^{-1}.

Constraints for the Copper(I)-Cysteine Connectivities in the Cu_{12}-MT 2a Model Structure

A wide range of spectroscopic studies have been reported for copper(I)-containing metallothioneins[18–20] with the sources of the protein including mammals, yeasts and fungus. Studies of the binding of Cu^{I} to mammalian metallothionein have shown that when 12 Cu^{I} are added to the mammalian protein (*i*) there are six Cu^{I} bound in each domain of the protein, to give a $Cu_6(S_{cys})_{11}$ α-domain cluster and a $Cu_6(S_{cys})_9$ β-domain cluster, and that (*ii*) all 12 Cu^{I} are trigonally co-ordinated to three cysteine thiolates. Much of the recent spectroscopic data have been summarized by Stillman.[20] The stoichiometric ratio of 12 Cu^{I} to 20 S_{cys} is directly observed from optical studies.[23,24] Furthermore, the isomorphous replacement of zinc with cadmium,[36] as well as the non-co-operative nature of zinc replacement by copper,[24] indicate that little structural change takes place during metal replacement. Therefore, (*iii*) pairs of cysteine thiolate ligands that bind the same M^{II} in the M_7-MT structure may be assumed also to bind the same Cu^{I} in Cu_{12}-MT. There will, of course, be some deviation from this since there are five more metal atoms bound per protein molecule, and each of these has one fewer thiolate ligand. Also, it is thought that the prevalence of Cys-Cys and Cys-Xaa-Cys groups in the peptide chain, unique for metallothioneins among other proteins, is of critical importance in metal–thiolate cluster formation.[37] Thus, the final constraint (*iv*) is that these proximal cysteines bind the same Cu^{I} in each domain cluster.

Discussion

The results of the molecular modelling calculation with the proposed copper(I)–cysteine connectivities for Cu_{12}-MT 2a are shown in Fig. 4, as a ball-and-stick representation of the copper(I) thiolate cores in each domain of the protein. Figs. 5 and 6 display the outline of the peptide chain in both domains and views of a space-filling model of the protein that illustrates the location of two crevices; these are unprotected regions of the Cu–S cluster structure (Fig. 6 has been rotated by 90°). The copper(I)–cysteine connectivities and the Cu–S bond lengths are given in Table 1, the nearest Cu · · · Cu distances in Table 2, and the coordinates of the 761 atoms used in the fit have been deposited (SUP 57208). The average Cu–S bond length is 224.3 pm, which is comparable to the average bond length of 225 pm as determined by XAFS for the same protein.[20,38] In terms of the constraints placed on the copper(I)–cysteine connectivities as described earlier, each bound Cu^{I} in the structure proposed for Cu_{12}-MT 2a has at least one, and in most cases two, pairs of cysteine thiolates which co-ordinate the same Cu^{I} and also bind the same Cd^{II} in Cd_7-MT 2a. For example, Cys-7 and Cys-14 are co-ordinated to Cd(2) in Cd_7-MT 2a and to Cu(3) in the proposed structure for Cu_{12}-MT 2a. Nine out of ten proximal cysteine groups along the metallothionein peptide chain co-ordinate the same Cu^{I} in the Cu_{12}-MT 2a model. The only such group chosen not to fit this criterion is the Cys48-Ile49-Cys50 group, which also does not bind to the same Cd^{II} in Cd_7-MT 2a.

The Cu_6S_{11} cluster, shown in Fig. 4, representing the α-domain cluster, is composed of two $Cu_4(S_{cys})_4$ rings, connected by a bridging S (Cys-38) co-ordinated to Cu(9) and Cu(11). There are also four terminal cysteine thiolate ligands which bind the remaining Cu^{I}, so that all copper atoms are co-ordinated to three thiolates. This structure contains the $(\mu\text{-SR})_4Cu_4$ ring motif common in several synthetic copper(I) thiolate complexes.[32,33]

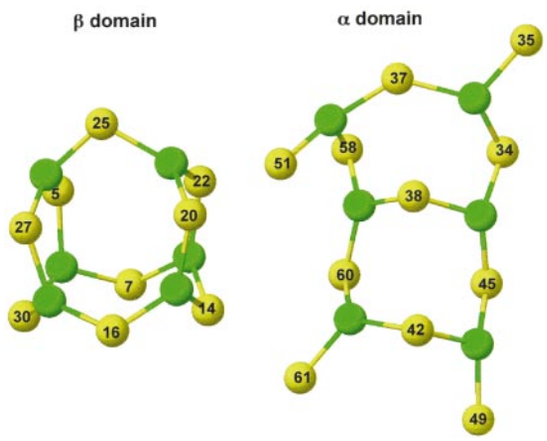

Fig. 4 Ball-and-stick representations of the copper(I) thiolate clusters in the proposed structure of Cu_{12}-MT 2a, showing the copper(I)–cysteinyl sulfur connectivities upon which the structures are based: α represents the $Cu_6(S_{cys})_{11}$ domain cluster, β the $Cu_6(S_{cys})_9$ domain cluster. The S_{cys} numberings are based on the sequences shown in Fig. 2. Key: green, Cu; yellow, S

The Cu_6S_{11} cluster representing the β-domain cluster shown in Fig. 4 is a cage displaying two six-membered rings with alternating Cu and S atoms, forming front and back 'faces' of the cage. These rings or faces are interconnected by bridging sulfur atoms between the opposite Cu atoms, such that three $Cu_4(S_{cys})_4$ rings make up the sides of the cage and all Cu^{I} have trigonal geometry. This structure is a distorted prism of copper(I) ions, has only bridging thiolate ligation, in contrast with the proposed α-domain cluster which has four terminal thiolates, and displays both the $(\mu\text{-SR})_4Cu_4$ ring and the $(\mu\text{-SR})_3(CuSR)_3$ ring motifs that are common among synthetic copper(I) thiolate complexes. Tables 1 and 2 display the Cu–S and Cu · · · Cu distances.

Agreement of the model with experimental results

The metal–thiolate bond lengths calculated and reported here are slightly short for trigonal co-ordination (typically expected to be 225 pm, but individually found to vary significantly when separate Cu–S bonds are examined in crystalline samples, see the comparison of Cu–S bond lengths in ref. 39). However, the values calculated for the model compound also appear slightly short and may represent the large difference in environment between the solid state and the gaseous state of the calculation. The mean calculated Cu–S bond length in the Cu_{12}-MT 2a model described here is 224.3 pm. X-Ray absorption fine structure (XAFS) data for Cu_{12}-MT provide an average bond length of 225 pm.[20]

We considered the question what is the effect on the bond length and geometry of the thiolates around the Cu^{I} when the thiolates are connected to a peptide chain through cysteines rather than simply being connected to small ligands. The calculation of the structure of the metal–thiolate core using CH_3SH to model the cysteinyl thiols, Fig. 7, provides that information. The bond lengths listed in Table 3 are very much more regular but are the same (at 224.6 pm) as those found for the same metal–thiolate clusters in the protein, which can be interpreted in terms of the differing effects of strain imposed on the structures by the presence of the peptide chain.

Fig. 8 shows the structure of $[Cu_4(SCH_3)_6]^{2-}$, a model compound described by Dance *et al.*,[40] that was used to calibrate the bond lengths reported by the MM2 calculation. The bond lengths reported in Table 4 are clearly shorter in our calculation

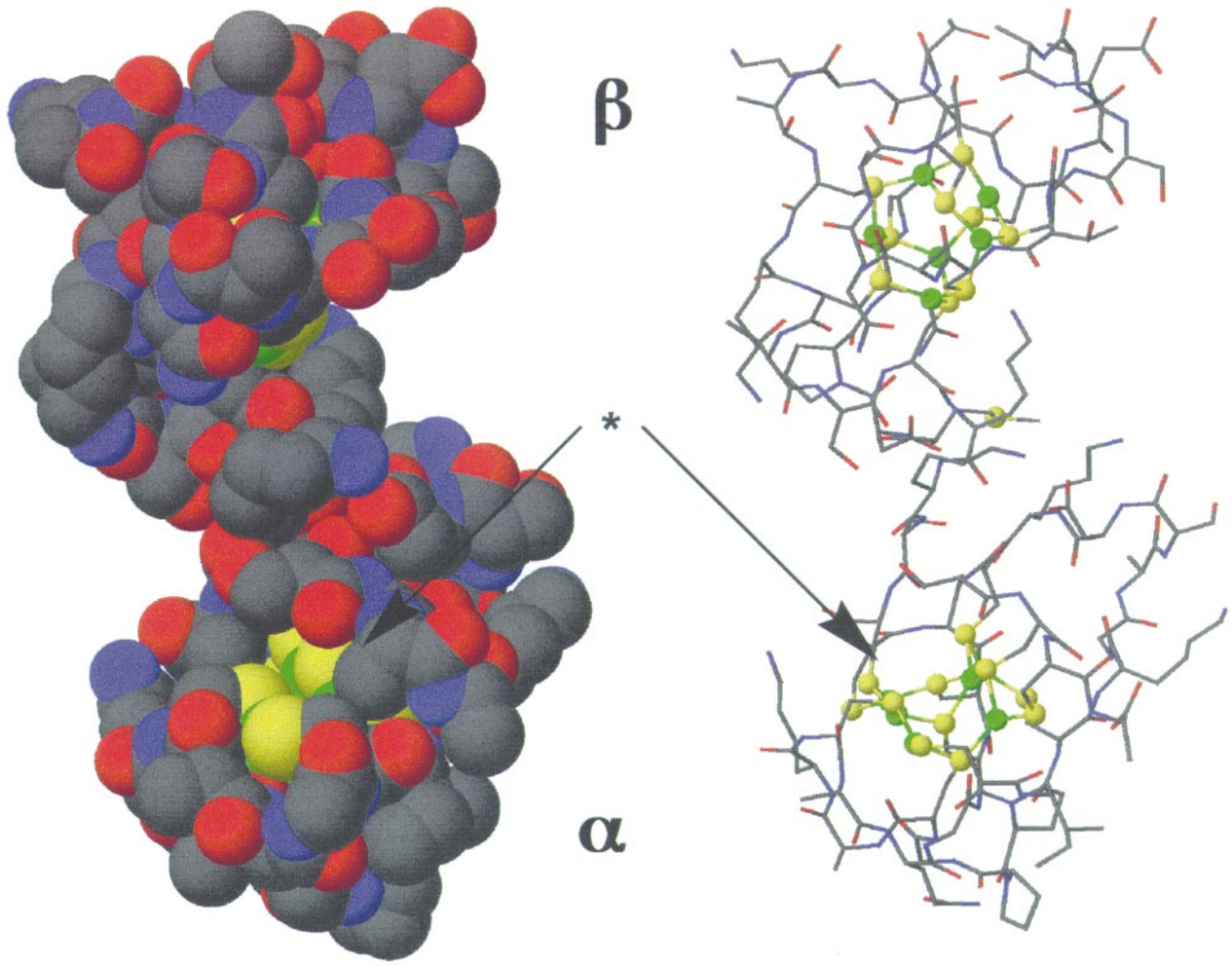

Fig. 5 Space-filling and tube representations of the proposed rabbit liver Cu_{12}-MT 2a structure (methionine behind). Although the copper(I)–thiolate clusters are embedded within the peptide wrapping, crevices visible in both domains may provide solvent access to the edges of the respective clusters. Key: green, Cu; yellow, S; grey, C; blue, N; red, O. The asterisk denotes the accessible sites of the copper(I)–cysteine thiolate clusters

Table 1 Connectivities and bond lengths (pm) in the copper(I)–thiolate clusters for each domain of the proposed rabbit liver Cu_{12}-MT 2a structure. See Fig. 4 for the numerical assignments of the Cu and S atoms

β Domain			α Domain		
Cu–S_{cys} connectivity	Bridging/ terminal S_{cys}	Bond length	Cu–S_{cys} connectivity	Bridging/ terminal S_{cys}	Bond length
Cu(1)–S(5)	Bridging	224.5	Cu(7)–S(34)	Bridging	220.7
Cu(1)–S(7)	Bridging	224.3	Cu(7)–S(35)	Terminal	228.6
Cu(1)–S(30)	Bridging	225.2	Cu(7)–S(37)	Bridging	225.9
Cu(2)–S(5)	Bridging	223.7	Cu(8)–S(42)	Bridging	222.7
Cu(2)–S(25)	Bridging	224.3	Cu(8)–S(60)	Bridging	225.1
Cu(2)–S(27)	Bridging	223.2	Cu(8)–S(61)	Terminal	224.9
Cu(3)–S(7)	Bridging	222.7	Cu(9)–S(38)*	Bridging	225.6
Cu(3)–S(14)	Bridging	223.7	Cu(9)–S(58)	Bridging	224.5
Cu(3)–S(22)	Bridging	224.0	Cu(9)–S(60)	Bridging	226.2
Cu(4)–S(16)	Bridging	225.3	Cu(10)–S(37)	Bridging	225.0
Cu(4)–S(27)	Bridging	224.4	Cu(10)–S(51)	Terminal	224.8
Cu(4)–S(30)	Bridging	226.1	Cu(10)–S(58)	Bridging	223.4
Cu(5)–S(14)	Bridging	223.0	Cu(11)–S(34)	Bridging	223.3
Cu(5)–S(16)	Bridging	222.8	Cu(11)–S(38)*	Bridging	226.0
Cu(5)–S(20)	Bridging	225.1	Cu(11)–S(45)	Bridging	223.1
Cu(6)–S(20)	Bridging	223.1	Cu(12)–S(42)	Bridging	224.5
Cu(6)–S(22)	Bridging	223.4	Cu(12)–S(45)	Bridging	226.3
Cu(6)–S(25)	Bridging	223.5	Cu(12)–S(49)	Terminal	223.4
Mean		224.02			224.67

* Atom S(38) bridges two eight-membered rings in the proposed structure of the α domain.

than the X-ray data, on average by 3 pm. However, the major differences are found in distortion within the solid-state structure that is not reproduced in the gas-phase structure. This distortion in bond lengths and angles clearly results from packing effects that are not currently calculable.

Several researchers have suggested that some of the Cu^{I} in

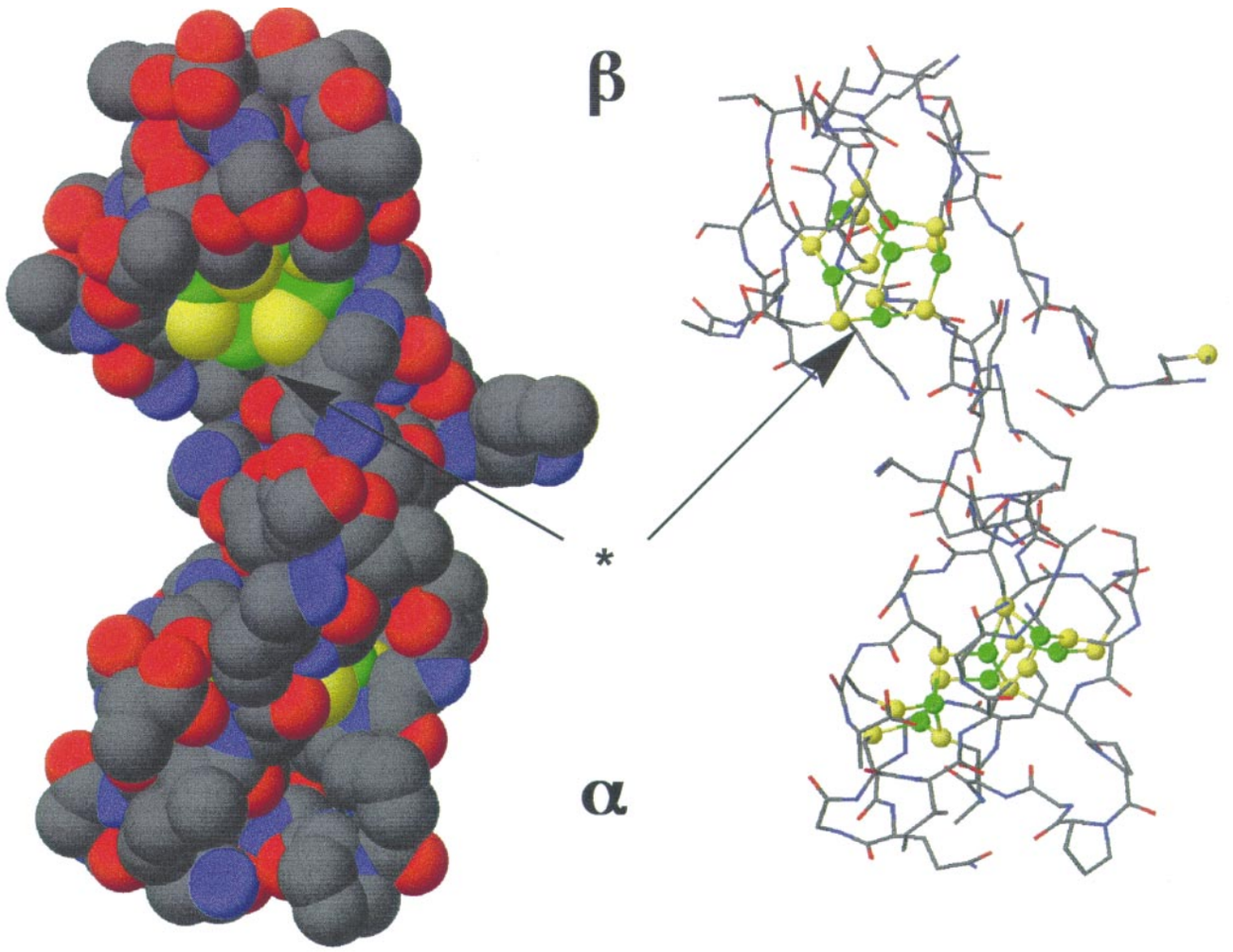

Fig. 6 Space-filling and tube representations of the proposed rabbit liver Cu_{12}-MT 2a structure (methionine right) rotated 90° from Fig. 5

Table 2 Interatomic distances (pm) of the nearest copper atoms in the copper(I)–thiolate clusters for each domain of the proposed rabbit liver Cu_{12}-MT 2a structure. See Fig. 4 for the numerical assignments of the Cu and S atoms

β Domain		α Domain	
Cu(1)···Cu(2)	371.2	Cu(7)···Cu(10)	413.4
Cu(1)···Cu(3)	359.4	Cu(7)···Cu(11)	336.9
Cu(1)···Cu(4)	363.6	Cu(8)···Cu(9)	311.3
Cu(2)···Cu(4)	361.3	Cu(8)···Cu(12)	366.0
Cu(2)···Cu(6)	355.4	Cu(9)···Cu(10)	258.6
Cu(3)···Cu(5)	376.3	Cu(9)···Cu(11)	347.1
Cu(3)···Cu(6)	369.5	Cu(11)···Cu(12)	369.7
Cu(4)···Cu(5)	365.2		
Cu(5)···Cu(6)	367.7		
Mean	365.51		343.29

Table 3 Connectivities and bond lengths (pm) in the modelled copper(I)–thiolate cluster structures containing CH_3SH instead of the cysteine in the peptide chain. See Fig. 7 for the numerical assignment of the Cu and S atoms

$Cu_6(SCH_3)_9$		$Cu_6(SCH_3)_{11}$	
Cu(1)–S(2)	224.6	Cu(1)–S(1)	224.6
Cu(1)–S(3)	225.1	Cu(1)–S(2)	224.7
Cu(1)–S(9)	224.6	Cu(1)–S(3)	224.3
Cu(2)–S(1)	224.7	Cu(2)–S(5)	224.6
Cu(2)–S(7)	224.6	Cu(2)–S(10)	224.6
Cu(2)–S(8)	224.5	Cu(2)–S(11)	224.7
Cu(3)–S(2)	225.1	Cu(3)–S(4)	224.8
Cu(3)–S(3)	224.6	Cu(3)–S(9)	224.6
Cu(3)–S(6)	224.3	Cu(3)–S(10)	224.6
Cu(4)–S(4)	224.7	Cu(4)–S(3)	224.4
Cu(4)–S(8)	224.6	Cu(4)–S(8)	224.8
Cu(4)–S(9)	224.6	Cu(4)–S(9)	224.6
Cu(5)–S(5)	224.7	Cu(5)–S(1)	224.5
Cu(5)–S(3)	224.7	Cu(5)–S(4)	224.7
Cu(5)–S(4)	224.7	Cu(5)–S(6)	224.3
Cu(6)–S(5)	224.8	Cu(6)–S(5)	224.7
Cu(6)–S(6)	224.4	Cu(6)–S(6)	224.5
Cu(6)–S(7)	224.6	Cu(6)–S(7)	224.8
Mean	224.66		224.6

Cu_{12}-MT adopt digonal co-ordination geometries based on comparisons with (*a*) the X-ray absorption fine structure parameters of three synthetic copper(I) thiolate structures,[26] and with (*b*) the structure of a silver–metallothionein species from the yeast *Saccharomyces cerevisiae* having a stoichiometry of Ag_7S_{10}.[29,41] As shown in Figs. 4–6, some of the Cu^I in the $Cu_6(S_{cys})_{11}$ structural model for the α-domain cluster do have shorter bonds to two bridging sulfurs, and a third longer interaction with a terminal thiolate. This co-ordination geometry may account for the apparent digonality obtained by XAFS measurements for some Cu^I in copper–metallothioneins.

The $Ag–S_{cys}$ connectivities in *S. cerevisiae* Ag_7-MT were determined by sequential ^1H NMR assignments.[41] These results indicate that the silver(I)–thiolate aggregate may have a structure comprised in part by two eight-membered Ag_4S_4 rings linked at a bridging cysteinyl sulfur, similar to the proposed structure for the $Cu_6(S_{cys})_{11}$ α-domain cluster. The higher $Ag^I:S_{cys}$ ratio of 7:10, compared with $Cu^I:S_{cys}$ = 6:11 or 6:9 for

Table 4 Connectivities and bond lengths (pm) in the crystallographically determined and calculated structures of the model compound $[Cu_4(SCH_3)]^{2-}$.[40] See Fig. 8 for the numerical assignment of the Cu and S atoms

	Bond length	
Connectivity	Experimental	Model
Cu(1)–S(1)	227.6	224.6
Cu(1)–S(2)	224.7	224.5
Cu(1)–S(3)	228.4	224.5
Cu(2)–S(1)	225.0	224.6
Cu(2)–S(4)	230.4	224.5
Cu(2)–S(5)	227.5	224.7
Cu(3)–S(2)	228.1	224.7
Cu(3)–S(4)	227.2	224.7
Cu(3)–S(6)	231.5	224.6
Cu(4)–S(3)	226.5	224.7
Cu(4)–S(5)	229.3	224.6
Cu(4)–S(6)	227.8	224.8
Mean	227.8	224.63

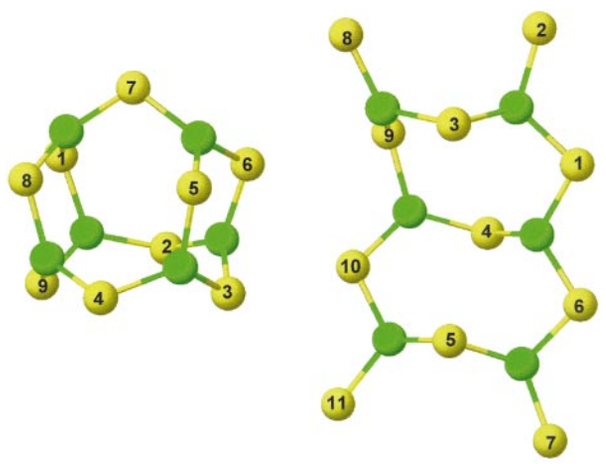

Fig. 7 The copper–thiolate cluster structures calculated for Cu_{12}-MT 2 using CH_3SH in place of cysteine in the peptide chain to investigate the effect of the latter on the structures. The $Cu_6(SCH_3)_{11}$ α domain (right) involves both bridging and terminal thiolates. The $Cu_6(SCH_3)_9$ β domain (left) involves only bridging thiolates. Methyl groups have been removed. Key: green, Cu; yellow, S

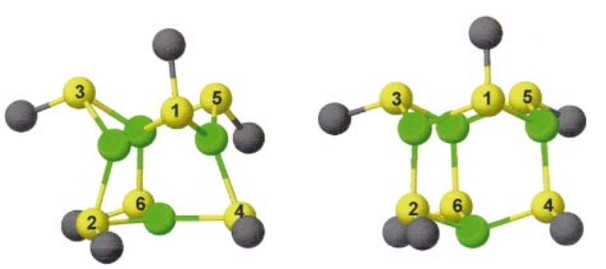

Fig. 8 The copper–thiolate cluster structure calculated for the model compound $[Cu_4(SCH_3)_6]^{2-}$.[40] The experimental crystal structure may be found on the left and the modelled compound on the right. Key: green, Cu; yellow, S

the α- and β-domain clusters respectively, requires that some Ag^{I} adopt digonal co-ordination. Optical measurements of Ag-MT from rabbit liver clearly differentiate the co-ordination geometry from that in Cu-MT.[23,24,27,28] Recent XAFS data that compare the sulfur co-ordination in Ag_{12}- and Ag_{17}-MT 1 also suggests that a high degree of digonal co-ordination exists in the Ag-MT species.[38]

The space-filling models of the protein, Figs. 5 and 6, may be used to account for several interesting properties of copper–metallothioneins. First, although the metallothionein peptide chain is rather short, it is quite efficient at embedding the copper(I)–thiolate clusters within the hydrophobic amino acids on the peptide chain. Only the crevices offer direct access to the clusters by the solvent. In effect the clusters exist in a porous, or leaky, cage through which some solvent can permeate. It has been proposed that it is for this reason that Cu_n-MT (n = 1–20) luminesces brightly at about 600 nm[19,20,23] ($\lambda_{excitation}$ = 300 nm) even in solution at room temperature (the luminescence intensity is greatly diminished by increasing the temperature and is dramatically more intense in frozen solutions).[23] Synthetic copper(I) complexes normally luminesce only in the frozen glassy state.[42–44]

Within this general encapsulation of the copper(I)–thiolate cores by the peptide chain there do exist small openings in each domain where sulfur and copper atoms are visible, much like in the structure calculated from the crystal structure data for the Cd,Zn-MT protein,[12,13,30] and also in a model of yeast Ag_7-MT.[29] These exposed edges of the copper–thiolate cluster are presumably accessible to the solvent, and by this means the excited states activated following absorption of a 300 nm photon can be deactivated by non-radiative mechanisms to greater or lesser extents depending on the porosity of the cage.[23] In addition the four terminal thiolates in the $Cu_6(S_{cys})_{11}$ α-domain cluster are available to co-ordinate extra metals; the presence of the crevice strongly suggests that these exposed sulfur atoms in the α domain may be the most susceptible to bind the metals that form the Cu_{15}-MT and $(Cu_6Cd_4)^{\alpha}(Cu_6)^{\beta}$-MT species observed from CD experiments.[24,45] The exposed Cu^{I} may also represent the sites of interactions with small ligands like glutathione (γ-glutamylcysteinylglycine),[46] which would be able to penetrate the opening and thus could be involved in metal-exchange reactions.

In conclusion, the first fully energy-minimized structural model for any copper-containing metallothionein has been calculated for mammalian Cu_{12}-MT 2a. The calculation takes into account the constraints of synthetic copper(I)–thiolate chemistry and of the protein. This model is in good agreement with the physicochemical properties of copper–metallothioneins, and may be used as a preliminary tool in understanding the binding of Cu^{I} to metallothionein. Both the Cu–S and Cu···Cu distances can be used to interpret interatomic distances determined by EXAFS techniques.

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