Chemical modifications of metallothionein

Preparation and characterization of polymers

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Reaction of rat liver metallothionein-II with two bifunctional cross-linking reagents, glutaraldehyde and dimethyl suberimidate, produces high yields of polymeric species. It is argued that cross-linking is trapping preformed aggregates of the protein, which therefore represent a stabilized quaternary structure of metallothionein. The two polymeric species differ in a number of respects. With dimethyl suberimidate, the polymer retains all metal-binding sites of the monomer, and has an unaltered isoelectric point. Reaction with glutaraldehyde causes loss of one or two $\text{Cd}^{2+}/\text{Zn}^{2+}$ -binding sites and elevates the pI. Both species are nearly spherical aggregates, in contrast with the highly asymmetrical metallothionein. Both polymers are linked through lysine residues, and the thiol groups remain reduced. The biological significance of these aggregates is discussed.

Metallothioneins (MT) are a class of low-molecular-mass metal-binding proteins with a high cysteine content, which have been isolated from a wide variety of organisms (Kojima & Kägi, 1978; Norberg & Kojima, 1979). The biological functions of these proteins remain to be precisely defined, although a role in zinc and/or copper metabolism is likely (Vallee, 1979; Nordberg & Kojima, 1979). In mammalian systems, MT synthesis is inducible by metals such as cadmium, and the toxicity of some heavy metals is altered by the protein (Cherian & Goyer, 1978; Cherian & Nordberg, 1983).

Mammalian MT binds 7g-atoms of Cd^{2+} or Zn^{2+} /mol, and ^{113}Cd -n.m.r. studies suggest that metals in MT are bound in two distinct polynuclear clusters, one containing three and one containing four metal ions (Otvos & Armitage, 1980). The two clusters are spatially separated and non-interacting (Winge & Miklossy, 1982), and display different affinities for Cd^{2+} , Zn^{2+} and Cu^{2+} (Boulanger *et al.*, 1983). The significance of the existence of these two clusters is unknown. Each Cd^{2+} ion is bound by two bridging and two terminal thiolate ligands

in a tetra-co-ordinate geometry (Otvos & Armitage, 1979), and Zn^{2+} (Garner *et al.*, 1982) and Co^{2+} and Ni^{2+} (Vasak *et al.* 1981) are similarly bound. Thus stringent criteria for tertiary structure must be met to provide these multiple similar metal-binding sites, and to avoid formation of disulphide bonds, but information concerning the role of a quaternary structure is lacking.

Dimeric forms of MT have been shown to occur in the livers of rabbits given large amounts of cadmium (Suzuki & Yamamura, 1980), and also accumulate as degradation products *post mortem* (Suzuki *et al.*, 1983) or on air oxidation of MT. Higher-molecular-mass polymers have been proposed to function as foetal copper-binding proteins (Porter, 1974). Each of these dimers or higher-order polymers appears to arise from intermolecular disulphide-bridge formation. Since all cysteine residues are involved in Cd^{2+} or Zn^{2+} binding, a loss of binding sites must accompany these polymerizations.

We have undertaken a study of chemically modified forms of MT with two aims in mind. First, we wish to probe the structural features that determine metal-binding affinities and specificities for various bivalent metal ions. Secondly, we hope to use chemically modified forms of MT as novel chelates of Cd^{2+} , to investigate the behaviour of this ion in biological systems. In the present paper we report the preparation and characterization of two cross-linked MT polymers.

Abbreviations used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); pHOHgBzO^- , *p*-hydroxymercuribenzoate; MT, metallothionein; GA-MT, glutaraldehyde-cross-linked metallothionein; DMS-MT, dimethyl suberimidate-cross-linked metallothionein.

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These are produced under non-oxidizing conditions, and retain in whole or in part the metal-binding capacity of native MT.

Materials and methods

Metallothionein was isolated from rats injected with CdCl_2 labelled with ^{109}Cd , at 18 mg of Cd/kg body wt., in six divided doses. Animals were killed 1 day after the last injection, and the livers were homogenized in 25 mM-Tris/HCl buffer, pH 7.4 (1:1, w/v), containing 10 mM- MgCl_2 , 25 mM-KCl and 1 mM- CaCl_2 at 4°C. After centrifugation at 12000g for 10 min, the pellet was rehomogenized, and the pooled supernatants were heated at 80°C for 2 min. After centrifugation at 27000g for 10 min, the supernatants were applied to a Sephadex G-75 column (5 cm × 90 cm) and eluted with 10 mM-Tris/HCl buffer, pH 8.6. The MT thus obtained was further purified by elution from a DEAE-Sephadex column (2 cm × 7 cm) with a linear gradient of 10–250 mM-Tris/HCl buffer, pH 8.6. The MT-II was the second of two fractions eluted, at higher ionic strength (0.15 M), and showed an A_{254}/A_{280} ratio of 20. Protein concentrations were determined gravimetrically after extensive dialysis against water, and checked by the Coomassie Blue colorimetric method (Bradford, 1976). An absorption coefficient $A_{254}^{1\%} = 13.1$, thus determined, was subsequently used to check protein concentration.

Cross-linking with glutaraldehyde was carried out by addition of a 10-fold excess of glutaraldehyde (5%, v/v; Eastman Chemicals, Rochester, NY, U.S.A.) to 0.5–5 mg of MT-II in 1.0 ml of 0.1 M-sodium phosphate buffer, pH 6.2, with stirring at room temperature. At 2 h lysine was added to 0.1 M, and after a further 30 min a 20-fold excess of NaBH_4 (to protein) was added in five divided portions as the solid. Reaction with dimethyl suberimidate (Pierce Chemical Co., Rockford, IL, U.S.A.) was optimal when similar quantities of protein in 0.5 ml of 0.1 M-sodium borate buffer, pH 8.9, were mixed with 0.5 ml of a solution of reagent in the same buffer, to a final concentration of 0.5 mg/ml of reagent, at room temperature. Both polymers were separated from lower-molecular-mass species by elution with 10 mM-Tris/HCl buffer, pH 8.6, from Sephadex G-75 columns (0.9 cm × 30 cm). Metals were determined by atomic absorption by using a Jarrell Ash 810 spectrophotometer in flame (Cd, Zn) or flameless (Cu) mode. An LKB 1270 Rackgamma γ -radiation counter was used to determine ^{109}Cd , with 70% counting efficiency. Amino acid analysis was performed on a Beckman Auto Programmed 120C amino acid analyser, and cysteine was determined after performic acid oxidation. Standards of the glutaraldehyde- or dimethyl suberimidate-cross-

linked bis-lysines were prepared from *N*- α -t-butoxycarbonyl-L-lysine (Sigma, St. Louis, MO, U.S.A.). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on 10%-acrylamide gels by the procedure of Weber *et al.* (1972), and gels were stained with Coomassie Blue G-250 or Violet R-250. To avoid aggregation of thioneins on the gels, *S*-carboxymethyl derivatives were prepared (Boulanger & Armitage, 1982).

Stokes radii were determined by gel filtration on a Sephadex G-100 column (0.9 cm × 58 cm) by using the relation (McGuinness, 1973) $R_0 \propto (-\log K_{av})^{1/2}$, where the partition coefficient $K_{av} = (V_e - V_0)/(V_t - V_0)$. V_e is the elution volume of the protein, V_0 the void volume determined with Blue Dextran, and V_t the total column volume. The column was calibrated with proteins of known Stokes radii as follows: ribonuclease A (1.65 nm), chymotrypsinogen A (2.25 nm), ovalbumin (2.73 nm) and fructose biphosphate aldolase (4.65 nm). Frictional coefficients ($R_0 = f/f_0$) were then calculated from the relation (McGuinness, 1973):

$$R_0 = (f/f_0)(3\bar{v}M/4\pi N)^{1/2}$$

where the partial specific volume $\bar{v}$ is taken as 0.72, and M and N are the relative molecular mass and Avogadro's number respectively. Isoelectric focusing was performed in an LKB 110 ml column with a glycerol gradient and 1% Ampholine solutions (LKB), with pH ranges 4–6 or 3.5–10. Samples were focused at 1.6 kV with constant power (1.5 kW) maintained by an LKB 2103 power supply, at 4°C for 18 h. Samples were applied to the middle of the density gradient.

Titration of protein thiol groups with Nbs_2 was performed by addition of 80 μg of Nbs_2 in 0.020 ml of phosphate buffer, pH 7.0, to a solution of 20–40 μg of protein in 1.98 ml of 10 mM-HCl buffer, pH 8.6. All solutions were de-aerated and kept under N_2 in sealed cuvettes. The change in absorbance at 412 nm was read against a blank containing 80 μg of Nbs_2 in Tris/HCl buffer. Stepwise titration with pHOHgBzO^- was carried out by the procedure of Boyer (1954), in 2.00 ml of 0.1 M-Tris/HCl buffer, pH 7.5. Absorbance was corrected for dilution after addition of 10 μl portions of reagent.

Results

Cross-linking reactions

Cross-linking with glutaraldehyde proceeds over several hours until all protein is converted into insoluble material. To avoid this over-polymerization, the reaction mixture was quenched with lysine at 2 h, at which time all material remained soluble. Denaturing sodium dodecyl

sulphate/polyacrylamide-gel electrophoresis of a dialysed sample of this reaction mixture (Fig. 1a) demonstrates that at 2 h most protein is in the form of a single species with a molecular mass of 58900 Da. A smaller amount of unmodified MT-II remains, but only traces of intermediate polymers (dimer, trimer) are observed. This electrophoretic pattern is unaltered when the reaction mixture is treated with NaBH_4 after quenching with lysine. Reaction of MT-II with dimethyl suberimidate similarly gives a high yield of a single high-molecular-mass species of 49000 Da, determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1a). A small amount of unmodified MT-II remains after prolonged reaction (18 h), and again only minor amounts of lower-order polymers are detected. Treatment of a freeze-dried sample of the major high-molecular-mass product of the dimethyl suberimidate reaction, with an NH_3 /acetic acid (15:1, v/v) mixture, results in substantial conversion of this polymer into material with a molecular mass similar to that of monomeric MT-II (Fig. 1b). For all subsequent

studies, the major glutaraldehyde- and dimethyl suberimidate-cross-linked polymers were separated from lower-molecular-mass species by Sephadex G-75 gel-permeation chromatography (Fig. 2). These polymers are denoted GA-MT and DMS-MT respectively.

Amino acid composition

Results of amino acid analyses of MT-II, GA-MT and DMS-MT (Table 1) reveal that both cross-linking agents modify primarily lysine residues. The decrease in lysine in both polymers, as compared with monomer, is not fully accounted for by the appropriate bis-lysines. Standards were not prepared to allow detection of monofunctionalized or otherwise modified lysine residues, which are presumed to result from non-cross-linking modifications. Thus two lysine residues per monomer are

Fig. 1. Denaturing sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of MT-II and polymers thereof

(a) Electrophoresis of dimethyl suberimidate/MT-II reaction mixture (i), glutaraldehyde/MT-II reaction mixture (ii) and standards (iii) of known molecular masses [from the top: phosphorylase B (94000 Da), bovine serum albumin (67000 Da), ovalbumin (43000 Da), carbonic anhydrase (30000 Da), soybean trypsin inhibitor (20100 Da) and α -lactalbumin (14400 Da)]. (b) Electrophoresis of MT-II (i), DMS-MT purified by Sephadex G-75 (ii), and DMS-MT after treatment with NH_3 /acetic acid (15:1, v/v) for 8 h at 4°C.

Fig. 2. Gel chromatography of MT-II and its polymerizing reaction mixtures

MT-II (a) and the glutaraldehyde/MT-II (b) and dimethyl suberimidate/MT-II (c) reaction mixtures were eluted from a Sephadex G-75 column (0.9 cm $\times$ 58 cm) with 10 mM-Tris/HCl buffer, pH 8.6. Column eluent was monitored for absorbance at 254 nm (----), and individual fractions were taken for counting of γ -ray emission from ^{109}Cd (●—●). The large absorbance at the penetrating volume in (b) is due to a coloured glutaraldehyde-lysine product formed when the reaction is quenched with lysine.

Table 1. *Amino acid analysis and metal content of rat liver MT-II and two polymers thereof*

Cysteine was determined after performic acid oxidation. Polymers are normalized to 52 amino acids, excluding lysine. The appropriate bis-lysines are determined based on standards prepared as described in the Materials and methods section. Residues are expressed to nearest integral values. Metals are expressed as g-atoms/molecule of monomer, on the basis of a molecular mass of 6200 Da for the monomeric subunit.

Amino acid	Composition		
	MT-II	GA-MT	DMS-MT
Asp	5	5	4
Thr	3	3	3
Ser	8	10	10
Glu	3	3	3
Pro	2	2	2
Gly	5	5	5
Ala	5	5	5
Cys	17	16	17
Val	1	1	1
Met	2	1	1
Ile	1	1	1
Lys	9	2	5
Bis-Lys	—	3	2
Total	61	57	59
Metal			
Cd	5.2	4.2	5.2
Zn	0.8	0	0.8
Cu	0.2	—	—

involved in the DMS-MT polymerization, and three in the GA-MT polymerization. More-extensive non-polymerizing lysine modifications occur with glutaraldehyde.

Molecular size and shape

Molecular masses determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of *S*-carboxymethyl derivatives are, for MT-II, 6250 Da; for GA-MT, 58900 Da; for DMS-MT, 49000 Da. From the amino acid analysis, MT-II has a molecular mass of 6200 Da. Thus DMS-MT corresponds to an octamer of MT, whereas GA-MT is somewhat more massive. Consistent with the known asymmetry of MT, a frictional ratio (f/f_0) of 1.3 and Stokes radius (R_0) of 1.60 nm was determined for MT-II, by gel-permeation chromatography. For GA-MT, the values were $f/f_0 = 1.06$ and $R_0 = 2.76$ nm, and for DMS-MT, $f/f_0 = 1.04$ with $R_0 = 2.54$ nm. Thus both polymers are more nearly spherically symmetrical than monomeric MT.

Metal-binding capacity

By atomic absorption spectrophotometry, and assuming a molecular mass of 6200 Da for MT-II, the starting material was found to contain 5.2 g-atoms of Cd^{2+} and 0.8 g-atoms of Zn^{2+} /mol, with an insignificant amount of Cu (Table 1). When the metal content of the polymers is expressed per 6200 Da of protein (i.e. per monomeric subunit), DMS-MT is seen to have a composition identical with that of MT-II. No ^{109}Cd is separated from protein on Sephadex G-75 fractionation of the DMS-MT reaction mixture (Fig. 2). Similar analysis of GA-MT demonstrates loss of one or two $\text{Cd}^{2+}/\text{Zn}^{2+}$ -binding sites in this species (Table 1), with separation of about 20% of the ^{109}Cd from protein by gel fractionation (Fig. 2). This partial loss of metal-binding capacity is due to reaction of the protein with glutaraldehyde. It persists when the borohydride reduction step is omitted. Attempts to replace lost cadmium in GA-MT were unsuccessful. Increasing concentrations of Cd^{2+} caused precipitation of the protein before significant binding occurred.

Thiol content and reactivity

Titration of MT-II, GA-MT and DMS-MT with pHOHgBzO^- detects 18 free thiol residues per monomeric subunit, in all three species, consistent with amino acid analyses for cysteine. Although the fully reacted pHOHgBzO^- derivatives of the forms of MT were soluble at pH 7.5, all species (MT, GA-MT and DMS-MT) were precipitated when approximately one-half of the thiol groups were modified at pH 4.5. In fact, each species was precipitated at its pI (see below) when the pH of solutions of fully reacted proteins was lowered stepwise. This did not represent irreversible denaturation, and all species redissolved, and showed unaltered absorption spectra, when the pH was returned to neutrality. Contrary to this insolubility of the pHOHgBzO^- derivatives under acidic conditions, all three species were fully soluble at their pI values and below.

Additional information can be gained by reaction of the proteins with Nbs_2 . Reaction of this reagent with metallothioneins is comparatively slow, requiring several hours to go to completion. Under conditions of a large excess of reagent, protein concentration is limiting, and biphasic reaction kinetics are observed (Fig. 3). Reaction of MT-II with Nbs_2 detects 16.7 thiol groups/molecule at completion, indicating that all thiol groups are reactive. When the initial rapid phase is corrected for the competing slow reaction, it is consistently found that 30–35% of the thiol groups react in this fast reaction. Both polymers also demonstrate biphasic reactions with Nbs_2 , al-

Fig. 3. Titration of thiol groups with Nbs_2 . The Figure shows the early time course of the reaction, with comparable protein concentrations and excess reagent (see the Materials and methods section). (a) MT-II; (b) DMS-MT, (c) GA-MT.

though there is less than quantitative modification of thiol groups. In GA-MT, 10.2 thiol groups/molecule are modified, and 13.2 thiol groups/molecule reacts in DMS-MT. Both reaction phases proceed more slowly with both polymers than with MT-II, and the rates are especially decreased with GA-MT. In both polymers less than one-third of the reactive thiol groups are reactive in the initial fast phase. On the basis of 18 thiol groups per subunit, the ratio of thiol groups to $Cd + Zn$ is 3.0 for both MT-II and DMS-MT. This value increases to 4.3 in GA-MT.

Isoelectric points

The isoelectric-focusing patterns of MT-II, GA-MT and DMS-MT demonstrate that both MT-II and DMS-MT have isoelectric points of 4.6, whereas the major component of GA-MT has an increased pI of 5.2 compared with the monomer. This separation technique indicates some heterogeneity in this Sephadex G-75-purified material. All samples were also focused on a pH 3.5–10 gradient to demonstrate that no material lies outside the range pH 4–6. When the major species

obtained by focusing on the pH 4–6 gradient were eluted, their apparent molecular masses as obtained by gel filtration were identical with those of corresponding samples before isoelectric focusing. Thus focusing does not cause degradation of the polymers.

Discussion

The work of Armitage and co-workers (Otvos & Armitage, 1979, 1980; Briggs & Armitage, 1982; Boulanger *et al.*, 1982), based mainly on ^{113}Cd and 1H n.m.r., has culminated (Boulanger *et al.*, 1983) in a satisfying structural description of mammalian metallothioneins. In this mode, 20 thiolate ligands provide seven binding sites for Zn^{2+} or Cd^{2+} , each metal ion being tetra-co-ordinated. These binding sites are arranged in two separate polynuclear clusters, containing three and four metal ions respectively. Seven distinct cadmium environments have been indicated for rat MT-II (Sadler *et al.*, 1978), although binding of a full complement of seven Cd and Zn atoms has been difficult to demonstrate (cf. Winge & Miklossy, 1982; Boulanger *et al.*, 1982; Kägi *et al.*, 1974). This could be due to systematic errors in determining protein concentration or metal composition, as has been suggested (Boulanger *et al.*, 1982). Quite likely some loss of metal-binding capacity results from oxidation (Minkel *et al.*, 1980) and/or loss of tertiary structure during isolation. This is supported by measurement of less than 20 thiol residues/molecule. Heterogeneity of MT preparations, either real or artifact, also remains a possibility.

Cross-linking reactions of MT provide high yields of single high-molecular-mass species. This surprising result may be accounted for in three ways. It appears unlikely that a kinetic reaction product is being observed. Sampling reaction mixtures at various times does not change qualitatively the electrophoretic pattern, only the ratio of single polymer to monomer. The polymers may represent thermodynamic products of the specific protein/reagent systems. However, similar results are seen for different reactions. A third and more likely possibility is that we are trapping pre-aggregated protein species. The estimated molecular mass of the DMS-MT polymer corresponds to that of an octamer of MT. If we postulate trapping of a pre-associated octamer, the higher molecular mass (by 9900 Da) of GA-MT could be accounted for by the addition of one or two more MT subunits, with greater heterogeneity resulting. Alternatively, non-cross-linking lysine mono-functionalizations, which occur extensively in GA-MT, could serve as sites of initiation of glutaraldehyde polymerization, a known competing side reaction (Cheung & Nimmi, 1982).

Treatment of MT with glutaraldehyde has been used to enhance protein antigenicity and allow development of a radioimmunoassay for MT (Vander Mallie & Garvey, 1978). Characterization of the GA-MT polymer in the present work reveals extensive modification of lysine residues accompanied by a decrease in metal-binding capacity. As the thiol content is preserved, loss of at least one metal-binding site is due to structural changes prohibiting the required Cd^{2+} or Zn^{2+} co-ordination. Cross-linking with dimethyl suberimidate, however, produces a polymer that retains the isoelectric point and metal content of MT-II. An oligomeric quaternary structure is therefore possible for this protein, without alteration of its metal-binding capacity. Oxidation of thiol groups is not required for oligomer formation, nor do the lysine residues appear to play a major structural role. Two classes of thiol groups remain in each polymer, as determined by Nbs_2 . Biphasic reaction of MT-II with Nbs_2 has been interpreted as resulting from differing rates of substitution of bridging and terminal thiolate ligands (Li *et al.*, 1981). One-third of the thiol groups of MT-II react in the fast phase when protein concentration is limiting, consistent with reaction of bridging thiolates in a metal cluster where each metal is bound by two bridging and two non-bridging ligands. The pH-dependent behaviour of the pHOHgBzO^- derivatives of MT is also preserved in both polymers (details of this phenomenon are not included in the present paper).

The ability of MT to dimerize through disulphide linkages is well known (Suzuki & Yamamura, 1980; Suzuki *et al.*, 1983), and higher-order polymers of MT have been postulated to function as neonatal Cu^{2+} -binding proteins (Porter, 1974). Conditions of thiol exposure (i.e. metal release) and oxidation, whether occurring *in vivo* or during protein isolation, can lead to intermolecular disulphide bond formation. However, *in vitro* air oxidation leads to the formation of intramolecular disulphide bonds (Suzuki *et al.*, 1983). Intermolecular bond formation reflects a propensity of MT to aggregate under certain conditions. The non-oxidized polymers reported in the present paper may be useful in determining the physiological role of MT aggregates (Cherian *et al.*, 1983). In view of MT's ability to dimerize, and the present observation of higher-order oligomers in solution, it is interesting to note that recently prepared crystals of rat liver Cd,Zn-MT-II have eight molecules in the unit cell (Melis *et al.*, 1983). However, the significance of MT's propensity to form these possibly octameric aggregates remains unknown. During foetal development, MT is localized in hepatocyte nuclei, as demonstrated by immunohistochemical techniques (Panemangalore *et al.*,

1983). Moreover, these techniques have revealed that, although most of the MT in adult rat liver is located in the cytosol, induction of MT synthesis by injection of Zn^{2+} or Cd^{2+} salts results in its presence in the cell nucleus (Banerjee *et al.*, 1982). On the basis of these and other results, it was speculated that the metal-free apothionein can have a translational control on the synthesis of MT (Cherian & Nordberg, 1983). However, the major regulatory control on induction of MT synthesis by metal ions has been shown to be transcriptional and involves gene amplification (Hager & Palmiter, 1981). Recently M. Karin (personal communication) has suggested that apothionein may serve as a repressor protein to the MT gene, binding to its promoter site. DNA recognition and binding is one function associated with well-defined protein aggregates. Such a role for thionein of well-defined quaternary structure is an intriguing possibility.

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