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Cadmium Binding and Metal Cluster Formation in Metallothionein: A Differential Modification Study†

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ABSTRACT: Mammalian metallothioneins (MT) contain 20 Cys in a total of 61 amino acid residues and bind 7 Cd and/or Zn ions. The metal is localized in two clusters made up of three and four metal–thiolate complexes in the NH_2- and COOH-terminal half of the chain, respectively [Otvos, J. D., & Armitage, I. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7094–7098]. The formation of these oligonuclear complexes designated as Cd_4 and Cd_3 clusters has now been monitored in MT reconstituted with varying amounts of Cd by using differential chemical modification of Cys with [^{14}C]iodoacetamide. At ratios below 2–3 mol of Cd/mol of MT bound, no differential protection of Cys by the metal, and hence no preferred binding, is detectable. At Cd-to-protein ratios between 3 and 5 mol of Cd/mol of MT, the modification profiles reveal preferred and cooperative binding in the COOH-terminal half of the chain, indicating formation of the Cd_4 cluster. At still higher ratios, formation of the Cd_3 cluster is initiated in the NH_2-terminal section of the polypeptide chain. Comparison of the differential modification data of Cd_6–MT and Cd_7–MT suggests that the last Cd to be bound is coordinated to Cys ligands located mainly between positions 20 and 30 of the sequence. The extent of labeling of the different Cys in Cd_7–MT indicates that the ligands of the Cd_3 cluster are 3 times as accessible to iodoacetamide than those of the Cd_4 cluster, suggesting a greater thermodynamic or kinetic stability of the latter.

Metallothioneins (MT)[1] are widely occurring proteins characterized by a low molecular weight (6500–6800) and an extremely high metal and sulfur content. All known mammalian forms contain 20 Cys in a total of 61 amino acids. All Cys are conserved in the sequence and are serving as ligands for binding a total of seven bivalent d^{10} metal ions (Nordberg & Kojima, 1979). The synthesis of MTs can be induced by different metal ions, by glucocorticoid hormones, and by a variety of stress conditions. Zn and Cd are the major natural metallic constituents of mammalian MTs. The currently accepted model suggests that the seven diamagnetic metal ions are partitioned into two separate metal–thiolate clusters containing three and four metal ions (Otvos & Armitage, 1980) and located in domains formed by the NH_2- and COOH-terminal halves of the polypeptide chain, respectively (Winge & Miklossy, 1982).

Several lines of evidence indicate that binding of Cd to these clusters is not uniform. Thus, the observation that digestion of partially metal-depleted MT with subtilisin results in a residual fragment composed of the COOH-terminal half of the chain and containing the complete Cd_4 cluster signifies indirectly that the metal is lost preferentially from the Cd_3 cluster (Winge & Miklossy, 1982). Similarly, potentiometric titration studies (Kägi & Vašák, 1983; Avdeef et al., 1985) and the demonstration that at neutral pH EDTA removes a single Cd ion from the protein (Nicholson et al., 1986) strongly indicate the existence of at least two classes of Cd–thiolate complexes differing in thermodynamic stability.

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[1] Abbreviations: MT, metallothionein; apo-MT, apometallothionein; Cd-MT, cadmium metallothionein; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; DTE, dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; CAM-Cys, (carboxamidomethyl)cysteine; NEM, *N*-ethylmaleimide.

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Such differences in affinity of the thiolate binding sites for Cd imply the existence of a preferred pathway in the reconstitution of the holoprotein from apo-MT and Cd. Support for this view comes from a recent study of Nielson and Winge (1983), who submitted partially reconstituted Cd-MT to digestion by subtilisin and who found that irrespective of the actual Cd-to-protein ratio the COOH-terminal half-molecule containing the complete Cd_4 cluster is recovered. These data were taken to suggest that upon addition of Cd to apo-MT the Cd_4 and the Cd_3 clusters are formed in compulsary sequence and by strictly cooperative processes. However, since in these experiments a redistribution of Cd during proteolytic digestion and subsequent separation of the products could not be excluded, the validity of the conclusions drawn remains yet to be established.

In this study we employed as an alternative and more direct approach for mapping of the pathway of Cd binding to MT a method of differential chemical modification of Cys in metal-free apo-MT and in partially and fully reconstituted forms of Cd-MT using the alkylating agent iodoacetamide. The applicability of this method relies on the known protective effect of metal binding on the reactivity of the Cys of metallothionein against electrophilic reagents (Kägi & Vallee, 1961; Li et al., 1981). In principle, this procedure involves a two-step exposure of a chosen class of reactive groups of a protein to an appropriate radioactive labeling reagent (Kaplan et al., 1971). In the first step, the protein is incubated with a substoichiometric amount of the radioactive reagent, leading to partial derivatization. Subsequently, after removal of the protecting Cd ions by EDTA and denaturation of the protein, a large excess of cold reagent is added to attain quantitative modification of the remaining unmodified thiol groups. The reactivity of each group is assessed on the basis of its specific radioactivity determined after fragmentation of the protein and isolation of the peptides containing the modified groups (Bosshard, 1979).

The results obtained with apo-MT and with partially and completely reconstituted samples of Cd–MT reveal characteristic changes in the reactivity of the various Cys in function of metal content and thus allow one to trace the pathway of metal binding and metal–thiolate cluster formation in this system. The data obtained under the conditions chosen are consistent with recent UV and CD spectroscopic data, which showed the formation of Cd–thiolate clusters to set in only after about three Cd ions are bound to the apoprotein (Willner et al., 1983).

Materials and Methods

Rabbit liver metallothioneins 1 and 2 were prepared from liver of rabbits injected subcutaneously 15 times with 1 mg of $CdCl_2$/kg of body weight at 2- to 3-day intervals (Kimura et al., 1979). The protein was purified by a procedure adapted from Kägi et al. (1974) and Kimura et al. (1979). The purity of the preparation was assessed by amino acid analysis and atomic absorption spectrometry. The native protein contained a total of 7 mol of bivalent metal/mol of protein.

Apo-MT was prepared by exposure to 0.5 M HCl and subsequent removal of the metals by gel filtration on Sephadex G-25 equilibrated with 0.01 M HCl. All chemicals were of reagent grade or better. [^{14}C]Iodoacetamide was purchased from Amersham Radiochemical Center Ltd. Its specific radioactivity was 53 mCi/mmol.

All preparative work was carried out in a nitrogen-purged glovebox at room temperature. Prior to use, all solutions were degassed on a vacuum line utilizing three thaw, freeze, and pump steps. For the reconstitution of Cd–MTs, about 3 mg of apo-MT was dissolved in 3 ml of 0.1 M HCl and mixed with appropriate amounts of $CdCl_2$. Subsequently, the pH of the well-stirred solution was raised to 8.6 by slow addition of a solution of 0.1 M Tris. The final concentration of reconstituted MT was about 0.5 mg/mL. Freshly prepared samples were equilibrated in the nitrogen-purged glovebox for about 1 h prior to further use.

For the study of the modification kinetics, 1.5 mol of iodoacetamide/mol of Cys was added to apo-MT 1 and to partially or completely reconstituted Cd–MTs. Aliquots were taken at various intervals between 2 min and 5 h after addition of the reagent. The reaction was stopped by the addition of an equal volume of 1 M HCl to the aliquot, and the extent of modification was determined by automated amino acid analysis. The reaction of DTNB with apo-MT and partially or completely reconstituted Cd–MTs was followed by difference photometry at 412 nm (Birchmeier & Christen, 1971) in 20 mM Tris-HCl buffer (pH 8.6). The reference cell contained all reagents except protein. The reagent was in 12-fold molar excess over the Cys (approximately 10–40 μM) in the reaction mixture. To prevent oxidation of mercapto groups outside of the glovebox during the reaction time of 5 min, all solutions were purged by nitrogen prior to use.

For the differential modification experiments, 0.1–0.25 mol of [^{14}C]iodoacetamide (2 μCi)/mol of Cys was added to samples of native apo-MT 2 and Cd–MT 2 reconstituted with 1–7 mol of Cd/mol of MT and maintained at pH 8.6. After 10 min of incubation, the remaining [^{14}C]iodoacetamide was quenched by the addition of 10 mol of DTE/mol of MT. Twenty minutes later, the protein was denatured by the addition of 2 volumes of saturated guanidine hydrochloride containing 40 mM EDTA and 20 mM Tris-HCl, pH 8.6, and a 10-fold molar excess of cold iodoacetamide over all mercapto groups present was added to achieve alkylation of the remaining unmodified Cys. After 3 h, the reaction mixture was passed over a Sephadex G-25 column equilibrated with 20 mM NH_4HCO_3. The high molecular weight fraction was pooled and lyophilized.

In a variation of this protocol (Figure 4), differential modification by [^{14}C]iodoacetamide was carried out following partial removal of Cd by EDTA (Nicholson et al., 1986). To this end, 3 mg of Cd_7–MT 2 was dissolved in 3 mL of 50 mM phosphate buffer, pH 7.5, containing a 20-fold molar excess of EDTA over protein. After 10 min, 0.1 mol of [^{14}C]iodoacetamide/mol of Cys was added to the solution, the pH was slowly adjusted to 8.6 with Trizma base, and the sample processed further as described above.

For peptide mapping, an HPLC unit consisting of two Altex Model 110 pumps (Beckman, Berkley, CA) and a Kontron Model 200 microprocessor (Kontron Zürich, Switzerland) was used. Effluent monitoring at 220 nm was with an Uvikon 725 spectrophotometer (Kontron) with an 8-μL flow-through cell. The output of the detector was plotted by a two-channel recorder (Model 600, W+W, from Hewlett-Packard, Avondale, PA). A stainless steel pressure coil (ca. 200 cm × 0.25 mm i.d.) was connected to the detector outlet in order to prevent degassing and bubble formation in the flow-through cell. All samples were chromatographed on a C-18 reverse-phase column (Spheri 10, 10 μm) from Brownlee (Santa Clara, CA).

The modified and lyophilized samples were dissolved in approximately 2 mL of 100 mM NH_4HCO_3 and digested with 5 wt % trypsin added in six steps within 5 h. Two hours after the last addition of trypsin the samples were lyophilized. Subsequently, the digestion mixture was dissolved in 0.1 % TFA (buffer A), and a sample containing about 200 μg of

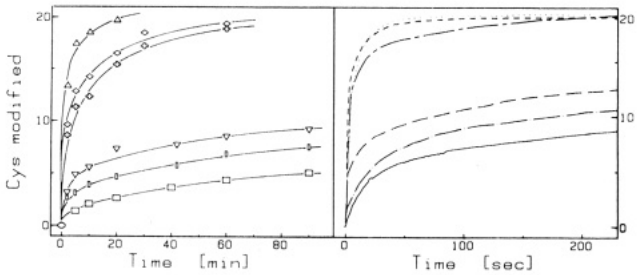

FIGURE 1: Effect of Cd binding on reactivity of Cys of MT with iodoacetamide (left) and DTNB (right). For conditions, see Materials and Methods. The Cd contents of the samples (mol of Cd/mol of MT) were as follows: none (△, ···); 2 (◇, - - -); 4 (cross, – - –); 5 (▽, – –); 6 (rectangle —·—); 7 (□, —).

protein was chromatographed by reverse-phase HPLC at a flow rate of 1 mL/min. After 5 min of isocratic elution, a linear gradient ranging from 0 to 25% volume fraction of buffer B (0.1% TFA/60% acetonitrile) was applied for 50 min (Figure 2A). The peptide fractions were collected and evaporated to dryness (Münger et al., 1985). Subsequently, the peptides were dissolved in water. One peak (no. 10) (Figure 2A) was rechromatographed in the same buffer system on the same reverse-phase column with a 5-min linear gradient extending from 0 to 20% volume fraction of buffer B and a 10-min linear gradient extending from a 20 to a 25% volume fraction of buffer B (Figure 2B, Table I). In order to determine the specific radioactivity, an aliquot of 50 μL was counted in 10 mL of scintillator liquid (Aquashure, New England Nuclear). Peptide concentrations were determined by automated amino acid analysis.

The error in the determination of specific radioactivity was estimated by analyzing corresponding fragments eluting at different positions in the HPLC chromatogram (peaks 3 and 5, peaks 4 and 6) and by comparing corresponding segments of overlapping fragments (peaks 7 and 8, peak 10b) (see Table I). The fractional standard deviation of the mean of the measured specific radioactivities in 36 samples was 7%.

RESULTS

Binding of Cd ions to the thiolate ligands of apo-MT[2] reduces their reactivity toward electrophilic agents. This effect is illustrated by the time course of the reaction of apo-MT and of partially and fully reconstituted Cd–MT with a 1.5-fold molar excess over Cys of the alkylating agent iodoacetamide (Figure 1, left) and with a 12-fold molar excess of the oxidizing agent DTNB (Figure 1, right). Comparison of the initial rates of reaction of the metal-saturated protein and of apo-MT indicates an 8-fold reduction with iodoacetamide and a 5-fold reduction with DTNB. Furthermore, with addition of metal, the extent of shielding of the thiolate groups increases in a nonlinear fashion. Thus, after the first 4 equiv of Cd ions is bound, protection by the metal against modification by the reagents becomes much more pronounced. In the samples with the highest Cd content, i.e., Cd_5–MT and Cd_7–MT, only a minor fraction of the Cys undergoes reaction within the duration of the experiment.

In order to study the effect of Cd binding on the reactivity of the individual Cys or of groups of Cys located in a given segment of the polypeptide chain, samples of MT with varying

[2] Addition of Cd^{2+} to apo-MT at pH ≥5 results in quantitative incorporation of the metal into the protein (Kägi & Vallee, 1961). At the conditions employed in this work (pH 8.6), the average apparent association constant of the complex of Cd^{2+} with the thiolate metal binding sites is estimated to be of the order of 10^{20} M^{-1} (Vašák & Kägi, 1983).

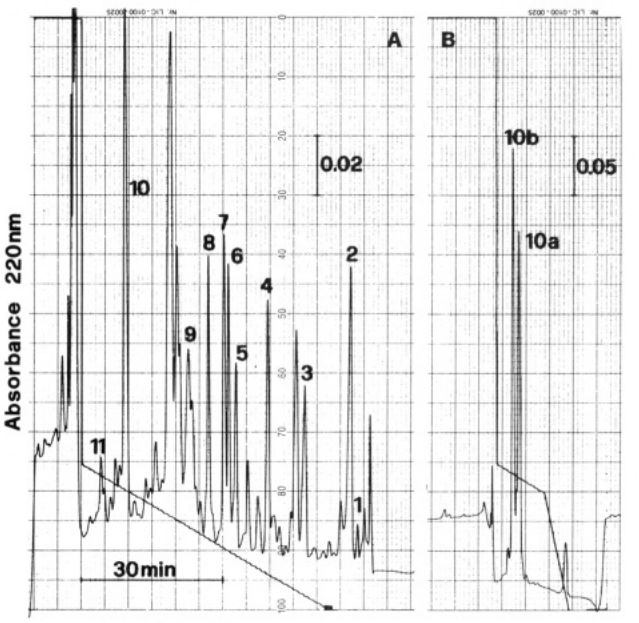

FIGURE 2: (A) HPLC chromatogram of tryptic digest of 50 nmol of MT S-alkylated by iodoacetamide. Buffer A was 0.1% TFA; buffer B was 0.1% TFA/60% acetonitrile. Flow rate 1 mL/min. (B) Rechromatography of tryptic peptides 10a and 10b at the same conditions.

Table I: Tryptic Peptides of Rabbit Liver MT 2 Modified with Iodoacetamide Separated by Reverse-Phase HPLC on an RP-18 Column (Figure 2)

HPLC peak no.	residue no. in sequence	ranking no. of Cys in sequence	amino acid sequence of peptide[a]
1	21–22	6	CK
2	23–25	7	ACK
	52–56		GASDK[b]
3	26–30	8, 9	CTSCK[c]
4	57–61	18–20	CSCCA[c]
5	26–30	8, 9	CTSCK
6	57–61	18–20	CSCCA
7	1–5	1	MDPNC
8	6–15	2–5	SCAADG(SCTC)
9	32–43	10–14	SCCSCCPAGCTK
10a	44–51	15–17	CAQGCICK
10b	1–15	1–4	MDPNCSCAADG(SCTC)
11	1–20	1–5	MDPNCSCAADG(SCTCATSCK)

[a] The probable sequence of the subform of rabbit liver MT 2 compatible with these peptides is N-Ac-M^1DPNC/SCAAD^{10}G(SCTC/ATSCK^{20})/CK/ACK/CTSCK^{30}/K/SCCSCCPAG^{40}CTK/CAQGCIC^{50}K/GASDK/CSCC^{60}A. (/) Indicates points of tryptic cleavage. Taken from the partial amino acid sequence of Kimura et al. (1979) and from unpublished results from P. Hunziker, A. Kern, P. Kaur, and J. H. R. Kägi; parantheses indicate assumed order of the amino acids in the sequence. [b] This peptide coelutes with peptide 23–25. [c] See text.

Cd-to-protein ratios were subjected to differential modification with [^{14}C]iodoacetamide and cold iodoacetamide (see Materials and Methods). After completion of the modification reaction, the samples were digested with trypsin. The resulting peptides were separated by reverse-phase chromatography (HPLC). A representative peptide map is shown in Figure 2. The peptides were identified with known segments of the sequence on the basis of their amino acid composition (Table I). Except for Lys-31, all tryptic fragments were recovered. In addition, there was partial cleavage after Cys-5 and Cys-15. Two peptides (residues 26–30 and 57–61) eluted with two distinct peaks (peaks 3 and 5 and peaks 4 and 6, respectively), implying the occurrence of unidentified chemical heterogeneity.

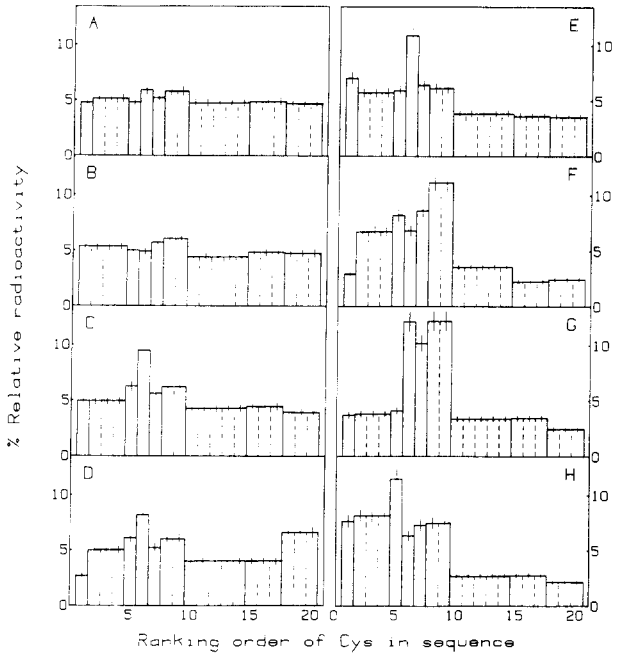

FIGURE 3: Effect of Cd binding on differential alkylation of Cys in MT by [^{14}C]iodoacetamide (for conditions of alkylation, see Materials and Methods). The bars indicate the radioactivity of the individual CAM-Cys along the polypeptide chain (numbered from 1 to 20) in percent of total radioactivity incorporated (=relative specific radioactivity). The length of the vertical lines indicates the fractional standard deviation (see Materials and Methods). In tryptic peptides containing two or more CAM-Cys (indicated by broken-line subdivisions), the same average radioactivity is assigned to each individual CAM-Cys. The Cd content (mol of Cd/mol of MT) of the samples was as follows: (A) none, (B) 1, (C) 2, (D) 3, (E) 4, (F) 5, (G) 6, and (H) 7.

The extent of differential labeling of peptides with [^{14}C]-iodoacetamide was assessed by comparing their specific radioactivity. In peptides containing several Cys, only an average value for the specific radioactivity of each Cys could be determined.

The reactivity profiles reflected by the variation in specific radioactivity of the modified Cys along the polypeptide chain for samples of different Cd-to-protein ratios are shown in Figure 3. The specific radioactivity values of the CAM-Cys are given in percent of the specific radioactivity of the modified protein and thus represent the relative distribution of the radioactivity incorporated into the particular sample. The data show that in the absence of bound metal the radioactivity is distributed about equally over all CAM-Cys (Figure 3A), indicating that in the apo form all Cys are equally reactive toward iodoacetamide. Binding of 1 equiv of Cd ion to apo-MT induces no appreciable alteration in this distribution (Figure 3B). However, some differences in relative specific radioactivity appear when more equivalents of metal are added. Thus, already at 2 equiv of Cd ions bound, a profile emerges that could be caused by a slight relative increase in the specific radioactivity of the CAM-Cys between residues 20 and 30 (Figure 3C). At 3 equiv of Cd incorporated, the average specific radioactivity of the CAM-Cys located between residues 33 and 51 is distinctly lower than that of the remaining CAM-Cys (Figure 3D). In Cd_4–MT, this relative reduction extends over the entire COOH-terminal half of the chain (Figure 3E). Beginning with Cd_5–MT, there is also a lowering of the specific radioactivity in the CAM-Cys at the NH_2-terminal part (Figure 3F). This relative reduction in the extent of labeling in these regions is compensated by a marked increase in relative specific radioactivity of the CAM-Cys between residues 20 and 30, denoting a greater relative reactivity of the Cys located in this segment. This inequality in labeling is still more pronounced in Cd_6–MT, where the relative specific radioactivity of these residues is about 3–4 times higher than that of the CAM-Cys in the flanking COOH- and NH_2-terminal portions (Figure 3G). These differences are diminished again, when the last equivalent of Cd is bound, i.e., when the protein is fully reconstituted to Cd_7–MT. The resulting pattern shows a characteristic two-level profile (Figure 3H) made up of the NH_2-terminal half with CAM-Cys having an average relative specific radioactivity of about 8% and a COOH-terminal half containing CAM-Cys with an average relative specific radioactivity of about 2.5%. It should be noted that the break between the two portions coincides exactly with the boundary of the metal–thiolate cluster domains in the protein (Winge & Miklossy, 1982).

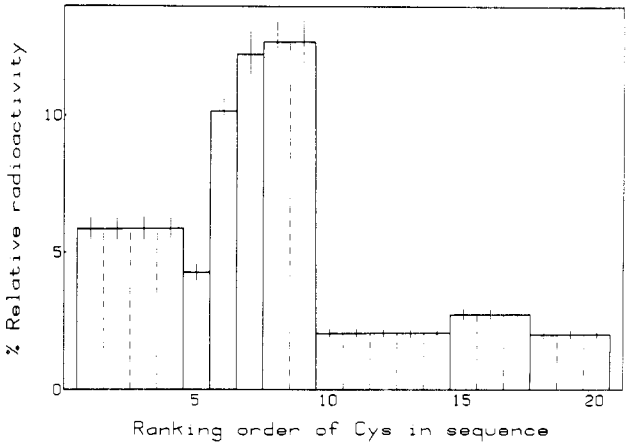

FIGURE 4: Effect of partial metal removal on differential alkylation of Cys in MT by [^{14}C]iodoacetamide. Cd_7–MT was preincubated for 10 min with 1 mM EDTA, in 25 mM phosphate buffer at pH 7.5. At these conditions, only 6 mol of Cd/mol of MT is bound to the protein. Differential alkylation and preparation of the tryptic digest was carried out as described under Materials and Methods. As in Figure 3, the bars indicate the relative radioactivity of the individual CAM-Cys in percent of the total radioactivity incorporated (=relative specific radioactivity). The length of the vertical lines indicates the fractional standard deviation (see Materials and Methods).

These changes in the relative specific radioactivity profiles are reproduced also by selective removal of Cd from Cd_7–MT. This is illustrated by the effect of incubation with 1 mM EDTA at about pH 7.5 (Figure 4). Under these conditions, EDTA was found to remove preferentially 1 equiv of Cd from the protein, thereby generating Cd_6–MT (Nicholson et al., 1986). The similiarity of the profiles of Figure 4 and Figure 3G supports this view and reemphasizes that in the Cd-deficient derivative the Cys located between residues 20 and 30 are reacting preferentially with iodoacetamide.

Discussion

The present results document explicitly the strong influence of metal binding on the chemical reactivity of the mercapto groups of Cys with alkylating and oxidizing reagents and demonstrate the suitability of such chemical modification studies for the elucidation of the topochemical and thermodynamic aspects of metal complex and cluster formation in this system. The occurrence of large reactivity differences between metal-containing and metal-free forms of MT have been recognized already in early studies. Thus, native MT containing the full complement of metal is remarkably stable toward air oxidation while apo-MT is readily transformed to the disulfide from (Weser et al., 1973). Analogous differences between metal-containing and metal-free forms have been

noted in the reactivity of MT toward NEM (Kägi & Vallee, 1961) and DTNB (Bühler, 1974; Li et al., 1981).

Under the conditions employed in this study, the mercapto groups of Cys occur in four chemical forms, i.e., RSH, RS^-, RSCd, and $RSCd_2$, the latter two constituting thiolate ligands coordinated singly and doubly to Cd, respectively. Of these forms, RS^- is by far the strongest nucleophile and, hence, the most reactive species. At pH 8.6, about 25% of the Cys side chains of apo-MT are present in this form (Vašák & Kägi, 1983). Due to the Lewis acid character of the metal ion, this nucleophilicity is diminished on binding of Cd. Thus, judging from the difference in initial rate, alkylation of Cd_7–MT is 8 times slower than that of apo-MT (Figure 1). Hence, it is concluded that occupation of the thiolate ligands by the metal is the major factor reducing their reactivity and that, as a corollary, rate measurements and comparative labeling studies can yield information on the relative stability of the metal complexes in this system (vide infra).

The disproportionately large difference in reactivity with iodoacetamide and DTNB observed between Cd_4– and Cd_5–MT (Figure 1) is most readily explained by the significant emergence of Cd–thiolate clusters. A similar sharp transition from separate mononuclear Cd–thiolate complexes formed at low metal-to-protein ratio to oligonuclear Cd–thiolate clusters at a higher ratio was recently observed in spectrophotometric and spectropolarimetric titrations of apo-MT with Cd (H. Willner, M. Vašák, and J. H. R. Kägi, unpublished results). The essential chemical change in the transition from mononuclear tetrathiolate metal complexes to clustered tetrathiolate structures is the involvement of some of the thiolate ligands in the coordination of two vicinal Cd ions to yield thiolate bridges, i.e., $RSCd_2$. This entails a further reduction in the nucleophilic character of such sulfur ligands and may thus contribute to the loss of reactivity of Cys in MT toward the modifying reagents, which develops with increasing metal occupation. Another factor reducing the reactivity is probably the loss of intramolecular motional freedom associated with cluster formation, which leads to a reduced accessibility of Cys to the modifying reagents.

The differential chemical modification data obtained in this study provide topographic information for resolving the pathway of metal complexation and cluster formation in this system. The same procedure has been applied successfully by Kaplan et al. (1971) to the assessment of the environment of amino groups at protein surfaces and by Bosshard (1979) for mapping contact surfaces in supramolecular structures. The method has the advantage over conventional chemical modification in that the initial labeling step employs only a relatively small quantity of radioactive reagent, reducing the incidence of multiple modifications of the same molecule and thus avoiding complications in the kinetics of the reaction. The subsequent modification of the remaining groups by a large excess ensures the chemical homogeneity of the product.

The choice of the alkylating agent iodoacetamide was prompted by its high selectivity for mercapto groups at the conditions used and by the fact that its derivative with apo-MT introduces no electric charge and is water-soluble and susceptible to digestion by trypsin. The same advantages have recently led to its use in the sequence determination of the two charge-separable forms of bovine MT (Münger et al., 1985). The number of peptides obtained by tryptic digestion and their amino acid compositions (Table I) are consistent with the partial sequence data of rabbit MT 2 reported by Kimura et al. (1979). Since most peptides contain several CAM-Cys and since no effort was made for further fragmentation, the specific radioactivity values obtained constitute but an average value. Thus, the resolution of the present analysis is limited by the length of the peptides examined.

The nearly constant specific radioactivity of all CAM-Cys upon modification of apo-MT indicates that the uncomplexed Cys do not differ measurably in reactivity and, hence, in their exposure to the alkylating reagent (Figure 3A). This is in agreement with the earlier evidence from ^1H NMR measurements that upon metal removal all peptide hydrogens are equally exchangeable with the aqueous solvent, indicating the absence of a fixed secondary and tertiary structure (Vašák et al., 1980). The same featureless specific radioactivity profile results when CD_1–MT and, to a lesser degree, Cd_2–MT are exposed to [^{14}C]iodoacetamide, suggesting that the first metal is bound at random to the polypeptide chain (Figure 3B,C). However, differences in the modification pattern begin to emerge when more Cd is bound. The significantly lower degree of labeling noted in the Cys-rich segment stretching from residue 33 to residue 50 of Cd_3–MT and Cd_4–MT suggests a tendency toward a preferential occupation of this portion of the chain (Figure 3C,E). In all likelihood, this change in profile reflects the inception of the Cd_4 thiolate cluster (cluster A; Otvos & Armitage, 1981) by a cooperative nucleation event. The extension of the protection to the three last Cys in the chain when more Cd is added signals the completion of cluster A (Figure 3E). The subsequent changes in profile on proceeding to Cd_5–MT, Cd_6–MT, and Cd_7–MT can be correlated with the additional stabilization of this cluster and the emergent formation of the NH_2-terminal Cd_3 thiolate cluster (cluster B). The pronounced protection from alkylation of the most NH_2-terminally located Cys makes it likely that in the formation of cluster B these residues are complexed first by the added metal while the Cys of the intermediate segment (residues 20–30) remain unoccupied (Figure 3F,G), thereby manifesting their distinctly lower affinity for the metal. These residues lose their preferential reactivity only after the seventh equivalent of Cd is also added (Figure 3H).

The weaker binding of Cd to the seventh site is also supported by the effect of EDTA on the reactivity profile (Figure 4). That EDTA removes selectively some Cd from Cd_7–MT has been noticed by several authors (Nicholson et al., 1986; Nielson & Winge, 1983). The latter authors have also adduced evidence that the Cd is lost from a labile site in cluster B. The close similarity of the modification profile obtained after exposure of Cd_7–MT to 0.1 M EDTA (Figure 4) to the profiles observed for reconstituted Cd_5– and Cd_6–MT (Figure 3F,G) supports this localization and identifies the peptide segment from residue 20 to residue 30 as forming part of the metal-binding site depleted by this chelating agent. The reasons for the reduced affinity of Cd for this site are not immediately obvious. It could arise from steric constraints or alternatively from an unfavorable charge distribution since five of the eight positive charges of MT are located in this region of the sequence. It is also conceivable that completion of cluster B necessitates an energy-requiring rearrangement of the metal–thiolate bonds within the cluster that goes at the expense of the free energy of binding of the last Cd.

Both the order in which clusters A and B emerge upon the addition of Cd to apo-MT and the greater degree of protection afforded to the Cys of cluster A reaffirm the suggestion of Nielson and Winge (1983) that this cluster is appreciably more stable than cluster B. Within the limits of the resolving power of the method, the present data even allow some semiquantitative assessment of their difference in stability. Thus, on the reasonable assumption that the ratio of the average relative

specific radioactivity of the CAM-Cys of the two clusters of Cd_7–MT (Figure 3H) reflects the ratio of the average relative availability of their thiolate ligands for reaction with the alkylating reagent, it follows from the about 3-fold difference in labeling that the average difference in free energy of binding per Cd–thiolate bond is about 0.7 kcal/mol. On account of the overall stoichiometry of nearly three Cys per Cd bound, this figure allows for a difference in free energy of Cd binding in the two clusters of about 2.1 kcal/mol of Cd, equivalent to a 30-fold difference in binding constant. It is interesting that two distinct sets of binding sites for Cd with an about 10–50-fold difference in binding constant have also been deduced from spectrophotometric and potentiometric pH titration studies of Cd_7–MT (Vašák & Kägi, 1983; Gilg, 1985). The correspondence with the figure derived from reactivity measurements supports the view that these two sets of binding sites relate to clusters A and B, respectively.

On the basis of their putative identification of only a single intermediate in Cd repletion of MT, Nielson and Winge (1983) suggested that in vitro renaturing of apo-MT with Cd proceeds by a compulsory two-step process in which the first 4 equiv of Cd is bound in the COOH-terminal domain (cluster A) and the last 3 equiv in the NH_2-terminal domain (cluster B). For each domain, Cd binding was proposed to be strictly cooperative, thereby excluding intermediates other than the Cd_4–MT species in which cluster A is occupied selectively. This is in marked contrast to the data presented in this paper. While the development of the reactivity profiles (Figure 3) with Cd repletion provides support for some cooperative metal binding, it is obvious that at the conditions used in this study the complete reconstitution of Cd_7–MT is a multistep process involving both stochastic and ordered events. The emergence of the more stable cluster A is clearly preceded by the formation of complexes of Cd with groups of Cys selected at random along the polypeptide chain. Cooperativity appears as an ordering principle only after the options for the formation of separate metal–tetrathiolate complexes are exhausted. In contradiction to the claims of Nielson and Winge (1983), there is also no clear evidence for a cooperative process in the formation of cluster B. Quite to the contrary, the distinctly lower affinity of the last equivalent of Cd for cluster B (vide supra) signifies that the completion of this cluster is anticooperative.

That fully cooperative two-step scheme of cluster formation of Nielson and Winge (1983) is not followed is also confirmed by spectrophotometric and spectropolarimetric titration of apo-MT with increments of Cd (H. Willner, M. Vašák, and J. H. R. Kägi, unpublished results). As was previously shown for the binding of Co(II) to apo-MT (Vašák & Kägi, 1981), the first few equivalents of Cd are bound to the polypeptide chain by forming mononuclear tetrathiolate complexes. Only after addition of more than 3 equiv of Cd is significant cluster formation observed.

Thus, the implication emerging from these data is that the generation of the Cd–thiolate cluster structures in MT is a probabilistic process modulated by the constraints and the chemical forces effective in the system. In line with this reasoning, the pathways followed will depend both on the metal species and on the choice of the experimental conditions. Nielson and Winge (1984) have suggested that in rat liver MT Cu prefers binding to cluster B rather than to cluster A. We are currently investigating by differential chemical modification whether for Zn and Cu the pathway of cluster formation differs from that for Cd. Only when such data are available will it be meaningful to speculate on the functional significance of the two clusters in cellular metal homeostasis of these two essential trace metals.

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