Domain-specificity of Cd²⁺ and Zn²⁺ binding to rabbit liver metallothionein 2

Metal ion mobility in the formation of Cd₄-metallothionein α -fragment

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The yield of the α -fragment of rabbit liver metallothionein 2 was used to test the domain-specificity and mobility of Cd²⁺ and Zn²⁺ when bound to metallothionein. Increasing molar ratios of Cd²⁺ were added to either Zn₇-metallothionein or the metal-ion-free apo-metallothionein. The enzyme subtilisin was used to digest those parts of the peptide chain that were not bound to Cd²⁺. Analysis of the digestion products was carried out by separation by polyacrylamide-gel electrophoresis. The chelation agent EDTA was used as a competitive chelator. It was found that the presence of excess EDTA greatly enhances the formation of the Cd₄-metallothionein α -fragment, and catalyses the complete digestion of all other the metal-ion-containing peptides, so that even Cd₇-metallothionein, formed when 7 molar equivalents of Cd²⁺ are added to Zn₇-metallothionein, is digested to the α -fragment. These results suggest that the Cd²⁺ bound in the β -sites is very labile, much more labile than the kinetics of the off-reaction would suggest. The observation of significant amounts of α -fragment on the gels, even when the stoichiometry of the metal ions initially present in the protein should not have resulted in much concentration of Cd₄- α -fragment clusters, indicates that as the digestion proceeds the metal ions move to sites that form complete clusters and therefore selectively protect that part of the peptide chain from digestion. We also find that rabbit Cd₄-metallothionein 2 α -fragment stains near to the top of the gel, in complete contrast with the location of rat Cd₄-metallothionein 2 α -fragment. This difference in the mobilities suggests that the α -fragment prepared from rabbit metallothionein 2 is much less negatively charged than the analogous protein fragment prepared from rat liver metallothionein 2.

INTRODUCTION

The protein metallothionein (MT) contains 20 cysteine residues out of a total of 61 amino acid residues (Kägi & Vallee, 1961). MT can be readily isolated from the livers and kidneys of a wide variety of mammals, although rats and rabbits have provided much of the protein used in spectroscopic studies. A remarkably wide range of metal ions bind to MT, both *in vivo* (Piotrowski *et al.*, 1979) and *in vitro* (Nielsen *et al.*, 1985). However, it has been the two-domain structure adopted for Cd²⁺, Cu²⁺ and Zn²⁺ (and probably all other metal ions that bind to MT) that has attracted most attention in structural studies. MT has been well studied by a range of spectroscopic techniques (Kägi & Vallee, 1961; Otvos & Armitage, 1980; Neuhaus *et al.*, 1985; Furey *et al.*, 1986; Stillman *et al.*, 1987a; Stillman & Zelazowski, 1988). Definitive information on the geometry of the binding sites for Cd²⁺ and Zn²⁺ has been obtained from ¹¹³Cd n.m.r. (Otvos & Armitage, 1980), two-dimensional ¹H n.m.r. (Neuhaus *et al.*, 1985) and X-ray-diffraction studies (Furey *et al.*, 1986). The MT molecule exhibits the unique property of binding either 7 Cd²⁺ ions or 7 Zn²⁺ ions, or a mixture of 7 of these metal ions, in two domains, named α and β (Otvos & Armitage, 1980). Stability constants were reported first by Kägi, summarized by Nordberg &

Kojima (1979), and by Petering *et al.* (1987) as approx. 10¹¹ M for Zn²⁺ binding to MT and 10¹⁵ M for Cd²⁺ binding to MT, both at pH 7. Absorption, c.d., magnetic-c.d. and emission spectroscopic studies have provided considerable detail about the stoichiometry of the metal-ion replacement reactions that take place when Cd²⁺ (Stillman *et al.*, 1987b; Stillman & Zelazowski, 1988), Cu²⁺ (Winge & Miklossy, 1982; Nielsen *et al.*, 1985; Gasyna *et al.*, 1988) or Hg²⁺ (Stillman *et al.*, 1983; Cai & Stillman, 1988b) is added to apo-MT, Zn₇-MT or Cd,Zn-MT. Emission spectra for Cu-MT (Stillman *et al.*, 1985; Gasyna *et al.*, 1988) and Ag⁺ (Stillman *et al.*, 1988) and c.d. spectra of Cd²⁺ binding to Zn-MT and apo-MT (Stillman *et al.*, 1987b; Stillman & Zelazowski, 1988) have shown particular sensitivity towards cluster formation within MT. However, only very recently have studies concentrating on the initial site for binding of these metal ions appeared (Nettesheim *et al.*, 1985; Stillman *et al.*, 1987b; Stillman & Zelazowski, 1988).

In this present paper we examine how the differences between binding-site formation constants controls the yield of Cd₄- α -fragment made by adding Cd²⁺ to either apo-MT or Zn²⁺-containing protein, using the enzyme subtilisin to carry out selective digestion of the protein (Winge & Miklossy, 1982), and EDTA as a competitive chelator.

Abbreviation used: MT, metallothionein.

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MATERIALS AND METHODS

Zn₇-MT 2 was purified from the liver of a rabbit injected with ZnSO₄ at the rate of 20 mg of Zn/kg body wt. every second day over a 2-week period. The protein was purified as previously reported (Zelazowski *et al.*, 1980). Apo-MT 2 was prepared from rabbit Zn-MT 2 by gel filtration on Sephadex G-25 (Stillman *et al.*, 1987b). Protein concentrations were assayed by titration of the RSH groups with 5,5'-dithiobis-(2-nitrobenzoic acid) (Birchmeir & Christen, 1971) or by determination of the metal content by atomic absorption spectrometry. Polyacrylamide-gel electrophoresis was performed by using the methods of Jovin *et al.* (1964). Portions (100 µg, 1.4 nmol) of the protein were dissolved in 100 ml of Tris buffer and the pH was adjusted to between 7 and 8 with HCl. The solutions were saturated with Ar to prevent oxidation. A 77-fold excess of EDTA was added, together with subtilisin (Sigma Chemical Co.), which was added at an enzyme/protein ratio of 1:20 (w/w), and the solution was incubated at room temperature for 2 h. The products in the final solution were separated by polyacrylamide-gel electrophoresis. The final gels were immersed in a solution of Coomassie Blue R-250 (in an aqueous solution of ethanol, trichloroacetic acid, sulphosalicylic acid and acetic acid) overnight to fix and stain the protein. Then 5 mm sections of duplicate gels were digested with H₂O₂ overnight at 80 °C to solubilize the gel. After evaporation of the solvent, the solid was redissolved in HCl and applied to the atomic absorption spectrometer for analysis of metal content. The atomic-absorption-spectrometry values for the Cd content are plotted for each gel adjacent to the gel photographs.

RESULTS

Partial digestion by subtilisin of rabbit liver Zn₇-MT 2 and apo-MT 2 to which various amounts of Cd²⁺ had been added was used to investigate how the varied occupancy of the α- and β-domains by Cd²⁺ and Zn²⁺ affects the formation of a specific product, the Cd²⁺-saturated Cd₄-MT 2 α-fragment.

It is well known that the section of the peptide chain that is involved in Cd²⁺-thiolate cluster formation is afforded protection against digestion by subtilisin (Winge & Miklossy, 1982). The relative yield of Cd₄-αMT fragment compared with Zn₇-MT or fully saturated Cd₇-MT shows how the different cluster stabilities affect the mechanism of the digestion with subtilisin after the mixing of known stoichiometric ratios of Zn²⁺ and Cd²⁺ with the protein. The experiment probes the binding constants for filled domains within the protein, i.e. Cd₄-α-domain versus Cd₃-β-domain versus the mixed Cd,Zn-α-domain and Cd,Zn-β-domain.

Digestion of Zn₇-MT 2 partially metallated with Cd²⁺

Subtilisin was mixed with separate solutions of the Zn-MT protein to which different amounts of Cd²⁺ had been added. The solutions used were prepared as follows: portions of Cd²⁺, with increasing concentrations between 1 and 7 molar equivalents, in steps of 1 molar equivalent, together with excess EDTA, were mixed with separate samples of Zn₇-MT 2, and subtilisin was added. These solutions were incubated for 2 h at room temperature

and then applied to a polyacrylamide gel and the products were separated by electrophoresis.

Fig. 1(a) shows the stained gels obtained after electrophoresis of eight samples of Zn-MT 2. Lane 0 is the native Zn₇-MT, with no Cd²⁺ added. This gel provides a calibration of the mobilities for both Zn₇-MT and Cd₇-MT, because the Cd₇-MT is expected to behave identically with the Zn₇-MT as there are no differences in binding geometry (both tetrahedral) or overall charge. The development of the Cd₇-MT band at the same location in Fig. 3 confirms this. This calibration for rabbit liver Cd₄-αMT 2 fragment is provided in lanes 3–6. We can use these data because, under the conditions used for the solutions separated by the gels in Fig. 2, the only product expected is Cd₄-αMT fragment.

For lanes 1–7 in Fig. 1 the protein was treated as described above so that the number of the lane corresponds to the number of molar equivalents of Cd²⁺ added to the Zn-MT. Thus, before addition of subtilisin, the protein added to lane 1 could be written as Cd₁Zn₆-MT, lane 2 as Cd₂Zn₅-MT, up to lane 7, in which the initial Zn₇-MT was fully saturated with Cd²⁺ to give Cd₇-MT. From our spectral studies we know that at room temperature Cd²⁺ will be distributed across both domains up to Cd₆Zn₁-MT (Stillman *et al.*, 1987b; Stillman & Zelazowski, 1988). The solutions were applied to the gel at the top of the Figure. Native Zn₇-MT (in lane 0) moves to a position near the bottom (indicated by the black star), while the intact α-domain, Cd₄-αMT 2 fragment, remains at a position near the top of the gel (seen as the band in each of lanes 1–7, and indicated by a white star within a black circle). The gel patterns are different when compared with similar separations carried out for rat liver MT2. In those cases the α-fragment was detected nearer the middle of the gel (Winge & Miklossy, 1982; Zelazowski *et al.*, 1984).

Fig. 1(b) shows the distribution of Cd, measured by atomic absorption spectrometry, for 5 mm sections of each gel. The intensity of the band due to Cd₄-αMT fragment in Fig. 1(a) increases as the stoichiometry of the original protein changes from Cd₁Zn₆-MT to Cd₄Zn₃-MT, with little other proteinaceous material being discerned. In accord with the staining, the concentration of Cd also increases up to 5 molar equivalents of Cd²⁺ added. As expected, in the stoichiometric range of 5–7 molar equivalents of Cd, that is from Cd₄Zn₃-MT to Cd₇-MT (lanes 4–7), there is little increase in intensity of Cd₄-αMT fragment, which indicates that the maximum amount of the α-fragment made in this experiment is reached when between 4 and 5 molar equivalents of Cd²⁺ are added to the Zn-MT. Surprisingly, there is almost no indication of the presence of undigested Cd₇-MT, even when 7 molar equivalents of Cd²⁺ have been added. The Cd₇-MT would be observed at the same position as the Zn₇-MT (lane 0). Very slight staining is found in this region in lanes 6 and 7, which could represent a minimal concentration of Cd₇-MT; however, there is little Cd²⁺ associated with the protein at this point on the gel. The band near the centre of the gel (white star) has not been identified, but it might have been produced as a result of incomplete digestion of protein (Klauser *et al.*, 1983). The atomic-absorption-spectrometric analysis shows that a small amount of Cd²⁺ binds to this material.

Clearly, in the presence of EDTA, digestion by subtilisin results in the formation of α-fragment (left-hand side of Fig. 1a), together with progressively more un-

Fig. 1. Set of polyacrylamide gels photographed after electrophoresis of the MT fragments formed by subtilisin digestion of rabbit liver Zn₇-MT 2 in the presence of EDTA

(a) There are eight lanes. Cd²⁺ was added (lanes 1–7) to a solution of Zn₇-MT and EDTA (in approx. 77-fold excess), and subtilisin was then added. The lane markings indicate the number of molar equivalents of Cd²⁺ added to the Zn-MT. For lane 0 no Cd²⁺ was added. Cd₄- α MT fragment stains at the top, marked by a white star within a black circle; Cd₇-MT and Zn₇-MT stain at the bottom of the gel (marked by a black star). The white star marks material of unknown origin. Protein was added to the gel at the top of the photograph. (b) The seven panels show the Cd concentrations measured for 5 mm segments down each of the seven gels (lanes 1–7) in (a). The top of the gel appears on the left of the panel. The panel number coincides with the number of molar equivalents of Cd²⁺ added to the Zn-MT in the presence of EDTA, before digestion by subtilisin.

identified Cd-containing material that is indicated by the stain with above 3 molar equivalents of Cd²⁺. Although the Cd₃-S₉ cluster in the β -domain of the intact protein forms at some stage after 5 molar equivalents of Cd²⁺ added (Stillman *et al.*, 1987b), the β -domain is not protected from digestion in the presence of the EDTA. It is significant that under these experimental conditions almost no Cd₇-MT forms, even after the maximum amount of Cd₄- α MT fragment forms with 4–5 molar equivalents of Cd²⁺ added. As displayed in the stained gel and by the metal analyses, all protein in excess α -fragment is digested.

Digestion of apo-MT 2 partially metallated with Cd²⁺ in the presence of EDTA

When apo-MT 2 was used as a starting material (Fig. 2a) we found a considerable increase in protein-free Cd²⁺, which is located at the bottom of the gel, a decrease in α -fragment formation and, again, minimal undigested Cd₇-MT. In these experiments molar-equivalent portions of Cd²⁺ were added to seven different solutions of rabbit

liver apo-MT 2 at pH 2, and the pH was adjusted up to 8. Lane numbers indicate the number of molar equivalents of Cd²⁺ added to the apo-MT. Lane 8 has no Cd²⁺ added; a solution of apo-MT and subtilisin was added to the gel. Spectral studies (Stillman *et al.*, 1987b) show that Cd²⁺ added to apo-MT specifically fills the α -domain first to form Cd₄- α MT fragment, then the β -domain. Even though subtilisin was added to Cd₇-MT in lane 7, which from previous studies (Nielsen & Winge, 1983) would have been expected to be resistant to enzymic digestion, Fig. 2(a) shows only minor staining, this indicating the presence of only small amounts of undigested Cd₇-MT. Fig. 2(b) shows the Cd distribution along the gels displayed in Fig. 2(a). The pattern of the Cd distribution is quite different when compared with the Zn-MT gels. Most of the Cd in these gels is found at the bottom of the gel by the atomic-absorption-spectrometric analysis. Because there is no Coomassie Blue stain, we suggest that this Cd is in the form of the EDTA complex. The concentration of Cd in the α -fragment region up to the Cd₄-MT point is about the same as in

Fig. 2. Set of polyacrylamide gels photographed after electrophoresis of the MT fragments formed by subtilisin digestion of apo-MT 2 in the presence of EDTA (in 77-fold excess)

(a) There are eight lanes. Cd^{2+} was added (lanes 1–7) to solutions of apo-MT and EDTA, and subtilisin was then added. The lane markings indicate the number of molar equivalents of Cd^{2+} added to the apo-MT. For lane 8, no Cd^{2+} was added. The regions in which stained lines are seen are the same as for Fig. 1(a). (b) The seven panels show the Cd concentrations measured for 5 mm segments down each of the seven gels (lanes 1–7) in (a). The top of the gel appears on the left of the panel. The panel number coincides with the number of molar equivalents of Cd^{2+} added to the apo-MT in the presence of EDTA, before digestion by subtilisin.

Fig. 1. After this point, the concentration of Cd in the α -fragment region is constant.

Digestion of apo-MT 2 partially metallated with Cd^{2+} in the absence of EDTA

Cd^{2+} (1–7 molar equivalents) was added to portions of apo-MT at pH 2, then adjusted to pH 8 with Tris buffer, and subtilisin was mixed with the protein for 2 h. Clearly, there is a significant difference between the staining in Fig. 2(a) (apo-MT, with EDTA) and Fig. 3(a) (apo-MT, with no EDTA added). Unlike the Cd distribution observed for the other experiments, we now find (Fig. 3b) that Cd is distributed in two main areas of the gel. In Fig. 3(a) new staining appears in the Cd_7 -MT region of the gel (three-quarters of the way down, indicated by the black star) for all samples in which 4 or more molar equivalents of Cd^{2+} have been added to the apo-MT. At the same time as the Cd_7 -MT staining increases in intensity (from 5 to 6 to 7 molar equivalents of Cd^{2+}), staining the Cd_4 - α MT fragment region (the top of the gel, indicated by the white star within a black circle) decreases in intensity, until no α -fragment is detected

when 7 molar equivalents of Cd^{2+} have been added. There is a significant increase of Cd in the Cd_7 -MT region at the expense of Cd_4 - α MT fragment once more than 4 molar equivalents of Cd^{2+} have been added to the apo-MT. Little protein-free Cd^{2+} collects in the sections of the gel near the bottom. The atomic-absorption-spectrometry results show more clearly than the protein staining how both the Cd_4 - α MT fragment and Cd_7 -MT exist in the gel even with only 1 molar equivalent of Cd^{2+} added.

In a separate series of experiments, Zn^{2+} was added to apo-MT 2, and the digestions were carried out as described above. In the presence of EDTA, subtilisin completely digested the protein at all stoichiometric Zn/MT ratios. No bands were detected after staining. No Figure is shown here.

DISCUSSION

Native MT containing a mixture of both Cd and Zn that is isolated from rat and rabbit livers exhibits considerable domain-specificity (Otvos & Armitage,

Fig. 3. Set of polyacrylamide gels photographed after electrophoresis of the MT fragments formed by subtilisin digestion of apo-MT in the absence of EDTA

(a) There are seven lanes. Cd^{2+} was added to solutions of apo-MT in the absence of EDTA, and subtilisin was then added. The lane markings indicate the number of molar equivalents of Cd^{2+} added to the apo-MT. The α -fragment stains at the top and Zn₇-MT stains at the bottom of the gel. Protein was added to the gel at the top of the photograph. (b) The seven panels show the Cd concentrations measured for 5 mm segments down each of the seven gels (lanes 1–7) in (a). The top of the gel appears on the left of the panel. The panel number coincides with the number of molar equivalents of Cd^{2+} added to the apo-MT in the absence of EDTA, before digestion by subtilisin.

1980; Winge & Miklossy, 1982; Nielsen *et al.*, 1985; Furey *et al.*, 1986). The stoichiometric Cd/Zn ratio for native liver protein often approximates 5:2, with the Cd localized in the α -domain and the Zn localized in the β -domain (Otvos & Armitage, 1980; Nielsen *et al.*, 1985). Except for Cd^{2+} binding selectively to the α -domain in apo-MT (Winge & Miklossy, 1982; Nielsen & Winge, 1983; Stillman *et al.*, 1987b; Stillman & Zelazowski, 1988) and Cu^{2+} binding selectively to the β -domain (Nielsen *et al.*, 1985) in apo-MT, the domain-selectivity in the initial binding step for many metal ions has not yet been established conclusively. ^{113}Cd n.m.r. (Nettesheim *et al.*, 1985) and optical spectra recorded during Cd^{2+} binding to both apo-MT and Zn₇-MT (Stillman *et al.*, 1987b; Stillman & Zelazowski, 1988) have suggested that the domain specificity observed *in vitro* depends on whether the binding sites were occupied by Zn or whether the thiolate groups were protonated before binding the

Cd^{2+} (as in apo-MT). We have previously suggested (Stillman *et al.*, 1987b; Stillman & Zelazowski, 1988), using c.d. spectroscopy, that Cd^{2+} binds to Zn₇-MT in a distributed manner without significant cluster preference. Similar results were found in ^{113}Cd n.m.r. titrations (Nettesheim *et al.*, 1985).

At room temperature these distributed arrangements are relatively stable (Byrd & Winge, 1986). However, native Cd₅Zn₂-MT is found with Cd primarily bound in the α -domain and the Zn bound in the β -domain, an arrangement not readily achieved *in vitro* (Stillman & Zelazowski, 1988). The MT-bound Cd can rearrange into the domain-specific species if the temperature is raised (Stillman *et al.*, 1987b; Stillman & Zelazowski, 1988); the effect is especially pronounced for metal-ion-binding experiments carried out below 0 °C (Stillman & Zelazowski, 1988). In a separate study (M. J. Stillman, Z. Gasyna & A. J. Zelazowski, unpublished work), we

have found that Cd and Zn rearrange between the α - and β -domains following the mixing of stoichiometric amounts of Zn_7 -MT and Cd_7 -MT. This reaction occurs very much faster than expected for the low concentrations of free metal ion available with the high formation constants measured for Cd^{2+} and Zn^{2+} binding to MT (Kägi & Vallee, 1961). These results lead to questions concerning the mechanism of formation of the domain-specific native Cd_7Zn_7 -MT.

The experiments described in this paper compare the stabilities of the clusters of metal ions in the α - and β -domains. With the 10^4 -fold difference in stability constants for Cd^{2+} with respect to Zn^{2+} binding to MT (Nordberg & Kojima, 1979; Petering *et al.*, 1987), the Cd^{2+} readily displaces bound Zn. As digestion takes place, resistance (which translates into a marked decrease in the extent of digestion within a certain time period) is observed for those parts of the protein that contain metal ions. The experiments with subtilisin illustrate that the Cd and Zn atoms bound in MT are quite mobile. When the distributed model for Cd^{2+} binding to Zn_7 -MT operates, the product formed at room temperature following the addition of Cd^{2+} to Zn_7 -MT is a protein with Zn and Cd distributed between both domains up to the 5 molar equivalents of Cd level (Stillman & Zelazowski, 1988).

The essential feature in these experiments is that we can use subtilisin to digest those parts of the peptide that are not strongly bound to Cd^{2+} . EDTA will extract Zn from Zn-containing MT, for example the mixed-metal Cd_7Zn_7 -MT (Nicholson & Sadler, 1987), but not the Cd in such protein. Therefore we might predict that, when the distributed model operates following the addition of Cd^{2+} to Zn -MT, excess EDTA should selectively extract Zn from the protein, leaving a polypeptide chain clipped, or cross-linked, by regions of Cd-SR-Cd unit distributed across both domains. Such a structure should be resistant to proteolytic digestion in all regions where Cd^{2+} -thiolate binding is present. Therefore digesting such a partially occupied protein should lead to a chaotic mixture of MT fragments, because there would be no complete clusters units available to protect the whole of either the α - or the β -domain. This large number of partially digested fragments would produce many bands, or just a broad smudge, on polyacrylamide-gel electrophoresis.

The picture that we find is completely different from this predicted one. Under the experimental conditions used here with excess EDTA (Figs. 1a and 2a) we form, almost exclusively, Cd-containing α -fragment. There is only one additional, unidentified, band observed. Significantly, we cannot detect any staining due to Cd_7 -MT, even when 7 molar equivalents of Cd^{2+} are added to either Zn_7 -MT or apo-MT, as long as excess EDTA is used in the incubation solution.

The presence of the EDTA is clearly an important factor in determining this selectivity. When the experiments are carried out in the absence of EDTA, quite different products are formed. In particular, large amounts of Cd_7 -MT are formed and remain undigested, even when there are empty Cd^{2+} -binding sites in other MT molecules. For example, both Cd_4 - α MT fragment and Cd_7 -MT form when only 1 molar equivalent of Cd^{2+} has been added to the apo-MT. We illustrate the reactions that take place in Fig. 4. Because of the very great difference between the reactions taking place in the presence and in the absence of EDTA, we suggest that

Fig. 4. Schematic representation of the reaction sequence when Cd_7Zn_7 -MT is mixed with EDTA and then partially digested by subtilisin

In the first step, the EDTA removes Zn from the protein, leaving a mixture of RS-Cd-SR cross-linked units and unco-ordinated thiolate groups, RSH . In the second step EDTA acts as a carrier for Cd in the β -domain and catalyses the rearrangement of Cd from the β -domain to the α -domain. EDTA may then chelate the Cd remaining in the β -domain, leaving the β -domain peptide chain available for digestion.

EDTA not only competes for Zn bound to the protein but also acts as an intermediary in redistributing Cd from the β -domain to the α -domain once Zn has been removed to form Zn^{2+} -EDTA complexes. This is the last step before digestion. Thus the EDTA catalyses the rearrangement of metals to the thermodynamic rather than the kinetic, binding-site arrangement. This mechanism does not appear to operate in the absence of the subtilisin (Nicholson & Sadler, 1987). Clearly, the metal-ion-binding sites in the MT are not energetically equal. Formation of complete clusters, both Cd_4 and Cd_3 , appears to be more energetically favourable than formation of partially filled clusters.

A second scenario might be that EDTA chelates only the Zn, leaving a series of vacant binding sites in the α - and β -domains. Cd in the β -domain could then migrate to the α -domain without the use of the carrier. The experiments shown in Fig. 3 examine this effect because it has been proposed that Cd^{2+} binds selectively to the α -domain in titrations of apo-MT (Nettesheim *et al.*, 1985; Stillman *et al.*, 1987b). What we find in the absence of EDTA is that several different fragments are left over after the digestion. However, there are two main fragments with significant concentrations, and these appear

with mobilities the same as those of Cd_4 - α MT fragment and the undigested Cd_7 -MT. The lack of the single uniform product that is observed with EDTA strongly suggests that the Cd is not mobile enough within this time scale (2 h at room temperature) so that the subtilisin digests the kinetic product, which in the solution contains a distribution of the two most stable configurations, i.e. Cd_4 - α MT fragment and Cd_7 -MT, between 1 molar equivalent and 7 molar equivalents of Cd^{2+} added to the solution. This implies that for Cd to migrate completely from the β - to the α -domain at Cd/MT stoichiometric ratios less than 4:1 a carrier is necessary. In our case this is EDTA. The sequence of events that occur during these reactions is described in Fig. 4. The consequence of such a scenario is the following sequence of stability constants: $K_f(\text{Cd}_4\text{-}\alpha\text{-domain}) > K_f(\text{Cd}^{2+}\text{-EDTA}) > K_f(\text{Cd}_7\text{-MT}) \approx K_f(\text{Cd}_3\text{-}\beta\text{-domain})$.

A major consequence of this type of chemical equilibrium is that there can be an accumulation of Cd in one of the two clusters, e.g. Cd in the α -cluster may not be as labile as Cd in the β -cluster, which can lead to an observed binding preference *in vivo*. Rearrangement during chemical procedures carried out after the initial metal-ion-binding has occurred could result in the preferential binding for many metal ions that has been well documented by Nielsen and co-workers (Nielsen & Winge, 1983; Nielsen *et al.*, 1985).

In this present study we are testing the various models for metal-ion binding to MT against very simple experimental results. However, we find that the predicted outcome of the experiments is not observed. During the addition of Cd^{2+} to Zn_7 -MT, we would expect that when the Cd exceeded the capacity of the α -domain (4 molar equivalents) the Cd would accumulate in the β -domain, displacing the Zn (Winge & Miklossy, 1982; Nielsen *et al.*, 1985). From competitive binding studies with EDTA (Li *et al.*, 1980) and immobilized EDTA (Chelex-100) (Cai & Stillman, 1988a), we would expect that the Cd^{2+} would bind to the β -domain tighter than it would to the EDTA [Petering *et al.* (1987) report that the stability constant for Cd^{2+} binding to MT is 10^2 times greater than for Cd^{2+} binding to EDTA]. However, our data show that simply incubating Cd_7 -MT with EDTA and subtilisin for 2 h is sufficient to remove the 3 Cd atoms from the β -domain. This strongly suggests that the lability of the Cd in the β -domain may be similar to that of the Zn in the β -domain. It requires several days of incubation under these same conditions to pull out the Cd from the α -domain (Winge & Miklossy, 1982).

Finally, from these experiments we have observed that there is a significant difference in the relative mobilities of the Cd_4 - α MT 2 fragments from the rat and the rabbit. On electrophoresis α -fragment produced from rabbit MT 2 moves at a significantly slower pace compared with the rat Cd_4 - α MT 2 fragment. We find that the protein mobilities on these gels are very reproducible over several years. We have previously (Zelazowski *et al.*, 1984) reported gels showing the separation of the products of identical reactions carried out with rat liver protein. In this 1984 paper, the rat liver Cd_4 - α MT 2 fragment stains near our 'white star' symbol, in the middle of the gels in Figs. 1–3. This is in a completely different region from that where the rabbit liver Cd_4 - α MT 2 fragment is found. The difference in mobilities suggests that rabbit α -fragment is much less negatively charged than is the rat α -fragment. We have recently also found that rabbit MT

2 protein behaves quite differently in its reaction with Hg^{2+} when compared with the analogous protein from the rat. Whereas the Hg_{18} -MT species that is formed from rabbit liver MT 2 is characterized by a strong c.d. spectrum, which we have interpreted as arising from a particularly tightly wound peptide (Cai & Stillman, 1988b), we do not find the same type of c.d. spectrum for the Hg-containing rat liver MT 2. It is possible that both of these observations are related to significant differences in the metal-ion-binding capabilities of the rabbit MT 2.

What is the physiological significance of these results? We can speculate that metals (Cd in this case) also stabilize MT *in vivo*, making it resistant to enzyme proteolysis. Because the Cd in the β -domain is not bound tightly, exchange with Zn^{2+} could readily take place forming the Cd_4Zn_3 -MT species, the native protein with domain-specificity that is isolated from liver tissue. This model implies that the β -domain is able to bind metal ions much more freely than is the α -domain.

CONCLUSIONS

The formation of Cd_4 - α MT fragment has been used to determine the extent of digestion of MT by subtilisin. Digestion of the protein depends both on the metal loading and on the presence or the absence of a metal-transfer agent, a competitive chelator such as EDTA.

The experiments discussed above show that: (1) in the presence of EDTA, only Cd_4 - α MT fragment is formed following proteolytic digestion, with no evidence of Cd_7 -MT, even when 7 molar equivalents of Cd^{2+} have been added; (2) Cd located in the β -domain in MT is as labile as Zn in the β -domain, with respect to chelation by EDTA; (3) these results suggest that a metal-transfer agent could operate *in vivo* to catalyse the rearrangement of Cd bound to Zn-MT in a distributed manner, so that the domain-specific Cd_4Zn_3 -MT is formed in tissue and is isolated as the native protein; (4) rabbit liver Cd_4 -MT α -fragment is much less negatively charged than the analogous rat liver protein.

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