Characterization and Sequential Localization of the Metal Clusters in Sea Urchin Metallothionein†

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ABSTRACT: The mode of metal binding in sea urchin metallothionein (MT) was explored by electronic absorption, chiroptical, NMR, and mass spectroscopic methods. Recombinant sea urchin MT containing 7 equiv of the natural mixture of Cd isotopes was stripped of the metal by exposure to low pH and reconstituted with ^{113}Cd (>95% enriched). Comparison of the electronic spectroscopic and chiroptical features and the ^{113}Cd NMR spectra of the reconstituted material with those of the native recombinant material indicated that the reconstituted material had regained the native conformation. The shoulder at 250 nm in the electronic absorption spectrum, the biphasic circular dichroism profile centered at 250 nm, and the chemical shift positions (605−695 ppm) of the seven ^{113}Cd NMR resonances all strongly suggested that sea urchin MT like all other well characterized MTs contains clusters made up of tetrahedral Cd−thiolate units. The ^{113}Cd chemical shift correlation spectrum of the reconstituted protein proved the existence of such metal clusters and allowed the unambiguous assignment of some of the metal connectivities. Homonuclear decoupling experiments in which Cd resonances were selectively saturated indicated moreover a partitioning of the metal complement into two separate clusters containing three and four Cd ions. The same proposition was supported by the selective reduction of three ^{113}Cd resonances upon partial metal depletion following exposure of the protein to EDTA. Thus, the three-metal cluster is energetically less stable than the four-metal cluster. That the two clusters are separate entities was also demonstrated by the isolation of a protein fragment containing the four-metal cluster resulting from partial proteolysis of sea urchin MT by subtilisin in the presence of EDTA. Amino acid sequence and electrospray mass spectroscopic analysis identified this fragment as the N-terminal portion of the whole protein. This is in marked contrast to the known mammalian forms where the more stable four-metal cluster is associated with the C-terminal domain. One can conclude therefore that the sea urchin MT contains the same type of metal-thiolate clusters as those found in mammalian MTs, but that they are interchanged in their location along the polypeptide chain.

Metallothioneins (MT)[1] [see Roesijadi (1993) and Kägi (1993) for reviews] are widely found low molecular weight cysteine rich proteins distinguished by an exceptionally high content of d^{10} metal ions (e.g., Zn^{2+}, Cd^{2+}, and Cu^{1+}). They have traditionally been thought to be involved in cellular detoxification of metals, but more central functions such as an involvement in the regulation of Zn-dependent processes in gene expression (Zeng *et al.*, 1991a,b) and in development (Nemer *et al.*, 1984; De *et al.*, 1991) have also been suggested.

Compositional and spectroscopic analysis of mammalian MTs have established that Zn and Cd can replace each other isomorphously and that these metal ions are tetrahedrally coordinated to the cysteine sulfurs (Vašák & Kägi, 1983). Moreover, ^{113}Cd NMR spectroscopy has revealed that in mammalian and crustacean MTs the metal complexes are joined by bridging thiolates to form two separate clusters (Otvos & Armitage, 1980; Otvos *et al.*, 1982). Two-dimensional NMR and X-ray crystallographic studies have shown that rat MT-2 contains a Me_3Cys_9 and a Me_4Cys_{11} cluster located in the interior of the essentially globular N-terminal and C-terminal domains of the molecule, respectively (Schultze *et al.*, 1988; Robbins *et al.*, 1991). Crab MT, a class I MT [i.e., an MT showing sequence homology to mammalian MTs; see Kägi (1993) for review], contains, in an analogous fashion, two Me_3Cys_9 clusters (Otvos *et al.*, 1982; Narula *et al.*, 1993; Zhu *et al.*, 1994).

The MTs from sea urchin *Strongylocentrotus purpuratus*, exhibit little overall sequence homology with mammalian MTs and hence have been tentatively classified as class II MTs [i.e., MTs showing little sequence homology to mammalian MTs; see Kägi (1993) for review]. In terms of gene organization, however, the sea urchin MTs show great similarity to mammalian MTs with both a tripartite arrangement of coding exons and multiple metal regulatory elements

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[1] Abbreviations: apoMT, apometallothionein; CD, circular dichroism; Cd_7-MT, MT containing 7 Cd ions; ^{113}Cd NMR, nuclear magnetic resonance of ^{113}Cd nuclei; COSY, two-dimensional correlated NMR spectroscopy; 1D, one dimensional; 2D, two dimensional; EDTA, ethylenediaminetetraacetic acid; ES-MS, electrospray mass spectrometry; HPLC, high-pressure liquid chromatography; MCD, magnetic circular dichroism; Me, metal ion; NMR, nuclear magnetic resonance; MT, metallothionein; MTA, sea urchin MT isoform A; RP-HPLC, reversed-phase high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; UV, ultraviolet electronic absorption.

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(Bai *et al.*, 1993; Nemer *et al.*, 1985). The sea urchin MTs are also inducible by zinc, although with unequal thresholds (Wilkinson & Nemer, 1987), and additionally exhibit isoform specific temporal and spatial expression during development (Nemer *et al.*, 1991).

Intrigued by the conservation of gene organization but surprising lack of amino acid sequence homology between the sea urchin MT and vertebrate MTs, we expressed heterologously the protein encoding regions of the sea urchin MTA gene (Wang *et al.*, 1994) to investigate *inter alia* whether the differences seen at the primary structure level are reflected by differences in their spatial structure. It is expected that structural investigations will aid in identification of those structural elements which are important in the formation of metal thiolate clusters and their still elusive biological functions.

The initial characterization of the purified recombinant sea urchin MT indicated that it binds seven Cd ions per protein molecule. The position of the ^{113}Cd resonances (between 615 and 695 ppm) in the natural abundance ^{113}Cd NMR spectrum strongly suggested that the sea urchin MT possesses clusters composed of tetrahedrally coordinated cadmium thiolate units. From its large Stokes radius it was concluded that like the mammalian forms this protein has an elongated molecular shape. The two-step cadmium dissociation noticeable on acidification of the recombinant protein was tentatively explained by the existence of two distinct cadmium thiolate clusters of appreciably different stability (Wang *et al.*, 1994).

In this paper we describe the further structural characterization of the sea urchin MT, after reconstitution with ^{113}Cd, using 2D ^{113}Cd NMR to identify the cluster topology present in this protein. We have also investigated which of the clusters is energetically more stable by ^{113}Cd NMR analysis of EDTA treated sea urchin MT. Moreover, we have determined the position of the four-metal cluster by partial digestion of the whole protein in the presence of EDTA and subsequent isolation of the peptides by RP-HPLC.

MATERIALS AND METHODS

Reagents and Materials. Sephadex G-75 and G-25 were purchased from Pharmacia. DEAE Bio-Gel A and Chelex 100 was bought from Bio-Rad. Reverse-phase RP-300 HPLC columns were obtained from Brownlee Laboratories. Reverse-phase C_{18} microbore cartridges and reagents employed for protein sequencing analysis were purchased from Applied Biosystems. Water used for the above systems was Millipore Q purified, otherwise glass distilled water was used. Protease type VIII (Subtilisin Carlsberg) from *Bacillus licheniformis* otherwise called subtilisin (EC 3.4.21.62) was obtained from Sigma (P-5380). ^{113}Cd (>95% isotope purity) enriched was obtained from Harwell, England. 2′,2′-Dithiodipyridine was bought from Fluka. All buffers were made metal free by passing them through a Chelex 100 column. The pH of all Tris buffers was determined at 25 °C.

Protein Preparations. Recombinant sea urchin MTA was expressed in *Escherichia coli* 1B 392 Lon Δ1 grown in Luria Broth supplemented by the addition of 0.3 mM $CdCl_2$ to stabilize the newly synthesized apoprotein (Kille *et al.*, 1990; Wang *et al.*, 1994) and was purified by ethanol precipitation and Sephadex G-75 and DEAE Bio-Gel A column chromatography (Wang *et al.*, 1994). The apoprotein was prepared from the nascent recombinant protein by repeated concentration by ultra filtration, using a membrane with molecular weight cut off of 1000 Da, followed by dilution with 0.01 N HCl. The total dilution factor of the original buffer and Cd^{2+} was at least 16. Care was taken not to allow the protein concentration to exceed 0.5 mg mL^{-1} during the concentration steps. Reconstitution of the apoprotein with ^{113}Cd was performed using a slight modification of the method of Vašák (1991). Apoprotein and 7 equiv of $^{113}CdCl_2$ in 10 mM HCl were rendered oxygen-free by gently purging with a stream of argon for 30 min. This solution was then rapidly titrated with 1 M Tris base (oxygen free) to pH 8.6. The thus titrated solution was then incubated with Chelex 100 (approximately 1 mL of Chelex 100 slurry per milligram of protein) which had been pre-equilibrated to pH 8.6 with Tris-HCl buffer and stirred continuously over night at 4 °C. The Chelex was then removed by filtration, and the sample was applied to a Sephadex G-50 column (1.5 × 100 cm) in 10 mM Tris-HCl, pH 8.0, containing 10 mM NaCl. The metal to protein ratio was then checked.

All metal determinations were carried out by atomic absorption spectrometry using a VIDEO 12 aa/ae spectrophotometer.

Protein concentrations were derived either from the mercapto group content, determined with the 2′,2′-dithiodipyridine method at pH 4.0 (Pedersen & Jacobsen, 1980), or from measurement of protein absorption at 220 and 250 nm for the apoMT[1] and holoMT, respectively. For thiopyridinol a molar extinction coefficient of 7000 M^{-1} cm^{-1} at 342 nm, valid in the concentration range of 0.5−2.0 mM, was used (Wang *et al.*, 1994). The molar extinction coefficients of the protein were 4.59 × 10^4 M^{-1} cm^{-1} at 220 nm for apoMT and 10.19 × 10^4 M^{-1} cm^{-1} at 250 nm for the Cd-containing form (Wang *et al.*, 1994).

Spectroscopy. Electronic absorption measurements were made using a Cary 3 UV−visible spectrophotometer. A Jasco spectropolarimeter (model J-500) interfaced with an IBM PS/2 computer was used for CD[1] measurements. A magnetic field of 1.5 T was used for MCD[1] studies. Molar absorbancy, ϵ, molecular ellipticity, $[\theta]$, and magnetic molecular ellipticity, $[\theta_M]$, have units of M^{-1} cm^{-1}, deg cm^2 $dmol^{-1}$, and deg cm^2 $dmol^{-1}$ T^{-1}, respectively. The CD and MCD spectra were accumulated four times and recorded at the rate of 20 nm min^{-1} with a time constant of 2 s.

For ^{113}Cd NMR studies the following samples were dissolved in 0.5 mL of 50 mM NaCl and 20 mM Tris-HCl, pH 8.6, containing 99.8% D_2O: (a) 11.4 mg of lyophilized nascent recombinant sea urchin MT (Cd_7-MT) for one-dimensional studies; (b) 7.4 mg of lyophilized reconstituted sea urchin MT ($^{113}Cd_7$-MT) for one-dimensional studies; (c) 11.4 mg of lyophilized reconstituted sea urchin MT ($^{113}Cd_7$-MT) for two-dimensional studies. A fourth sample (d) was prepared as follows: 15 mL of 0.052 mM reconstituted sea urchin MT was incubated at 25 °C for 2.5 h with a 15-fold molar excess of EDTA (over Cd concentration) in Tris-HCl, pH 7.0. The sea urchin MT was then concentrated by ultrafiltration, using a membrane with molecular weight cut off of 1000 Da, and washed several times with 62.5 mM NaCl and 25 mM Tris-HCl, pH 7.0. After the final concentration step the volume of the protein solution was 400 μL. To this protein solution 100 μL of 99.8% D_2O was

added to give a final protein concentration of 1.58 mM in 50 mM NaCl and 20 mM Tris-HCl, pH 7.0, containing 20% D_2O. The sample was then sealed in a 5 mm NMR tube under argon. All the above procedures for sample d were carried out in an argon-purged glove box with degassed solutions.

In order to avoid signal decrease due to negative NOEs ^{113}Cd NMR spectra were recorded with inverse-gated proton decoupling on a Bruker AMX600 spectrometer operating at a ^{113}Cd frequency of 133.2 MHz. Chemical shifts are reported in parts per million with respect to the ^{113}Cd resonance of 0.1 M $Cd(ClO_4)_2$ in D_2O. For all samples approximately 32 800 transients were accumulated at 27 °C. Spectra were recorded with a recycle delay of 2 s using a 60° excitation pulse and broad-band proton-decoupling using WALTZ modulation during acquisition. For sensitivity enhancement an exponential function with a line broadening of 55 Hz was applied to the FID prior to Fourier transformation. ^{113}Cd homonuclear chemical shift correlated (COSY[1]) spectra were acquired by using the standard COSY sequence in phase-sensitive mode with proton decoupling during acquisition. The evolution period t_1 was varied in 206 increments from 0 to 7.7 ms covering a spectral width in the indirect (^{113}Cd) dimension of 13.3 kHz. A total of 256 transients were accumulated for each value of t_1.

Subtilisin Digests. Recombinant sea urchin MT was digested with the protease subtilisin under the following conditions: 0.86 mg of MT was incubated at 37 °C for 1.5 h with a 77-fold excess (over the Cd concentration) of EDTA in Tris-HCl buffer, pH 8.0, with a protein/enzyme ratio of 1:20 (w/w). The total reaction volume was 1 mL. The resulting protein fragments were then separated and analyzed by LC-MS according to the method of Hess *et al.* (1994). The fragments were applied to an RP-300 (C_8; 1 × 250 mm) column, and sample separation was achieved using a 25 mM ammonium acetate buffer (pH 6) system with an acetonitrile gradient. After separation the sample was then split using a low dead volume splitter T, and approximately 10% of the eluant was directed on-line into the electrospray of the mass spectrometer (SCIEX, API III) for mass determinations of the holoprotein fragments. The mass range between 800 and 2400 Da was scanned with a dwell time of 2 ms and a step size of 0.5 Da. The remaining 90% of the HPLC eluant was collected for mass determinations of the apoprotein fragment and amino acid and N-terminal sequence analyses. Dissociation of the metal from the protein for mass determinations of the apoprotein fragment was achieved by reducing the pH with 2% formic acid (1:1 v/v). This sample and reconstituted sea urchin MT were injected directly into the ion spray mass spectrometer.

Protein samples used for amino acid analysis were hydrolyzed *in vacuo* in 6 N HCl for 22 h at 110 °C and then analyzed using a Model 420A derivatizer (Applied Biosystems Inc.) equipped with an online HPLC system (Model 120) for detection of the phenylthiocarbamoyl derivatives according to the manufacturer's instructions. Cysteine content was determined on separate aliquots of the protein using 2′,2′-dithiodipyridine to determine the mercapto group concentration.

Amino acid sequence analysis was carried out on Applied Biosystems Inc. Model 477A with an online HPLC system (Model 120A) for separation of phenylthiohydantoin derivatives according to the manufacturer's instructions.

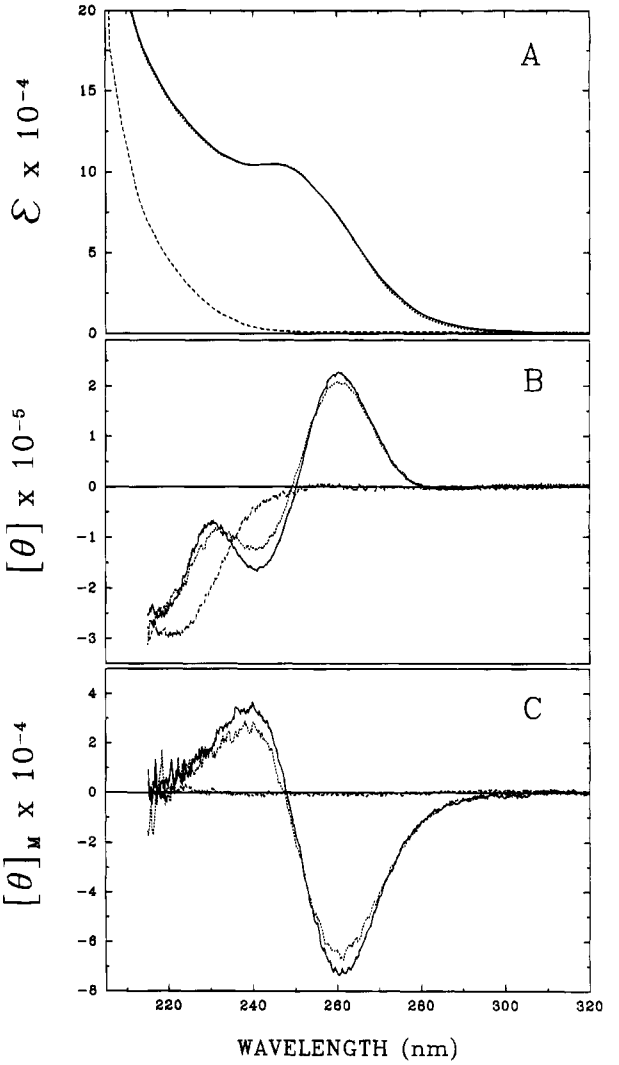

FIGURE 1: Comparison of the (A) electronic absorption, (B) circular dichroism, and (C) magnetic circular dichroism spectra of native recombinant Cd_7-MT (solid line), reconstituted $^{113}Cd_7$-MT (dotted line), and apo (dashed line) sea urchin MT. The holoMT samples had concentrations of 0.047 mM (native recombinant Cd_7-MT) and 0.025 mM (reconstituted Cd_7-MT) in 20 mM Tris-HCl, pH 8.6, containing 50 mM NaCl and were measured in 0.1 and 0.2 cm path length cuvettes, respectively, while the apoMT sample had a concentration of 0.046 mM and was measured in a cuvette of path length 0.2 cm as described in Materials and Methods.

RESULTS

Comparison of Native Recombinant and ^{113}Cd-Reconstituted Sea Urchin MT. A comparison of the electronic absorption (UV), circular dichroism (CD), and magnetic circular dichroism (MCD) spectra obtained from native recombinant sea urchin Cd_7-MT and the ^{113}Cd reconstituted form ($^{113}Cd_7$-MT) is shown in Figure 1. The features of these spectra correspond to those previously observed in mammalian Cd_7-MTs and thus reflect composition, coordination geometry, and symmetry properties of Cd-thiolate clusters (Vašák *et al.*, 1981; Willner *et al.*, 1987). The essential agreement of the corresponding spectra suggests identity of these structures in the two preparations. As with mammalian MTs the distinctive features of the holoMT from sea urchin spectra are lost upon acidification due to the release of the metal.

The native and reconstituted forms were also examined by ^{113}Cd NMR spectroscopy. Figure 2 shows the cor-

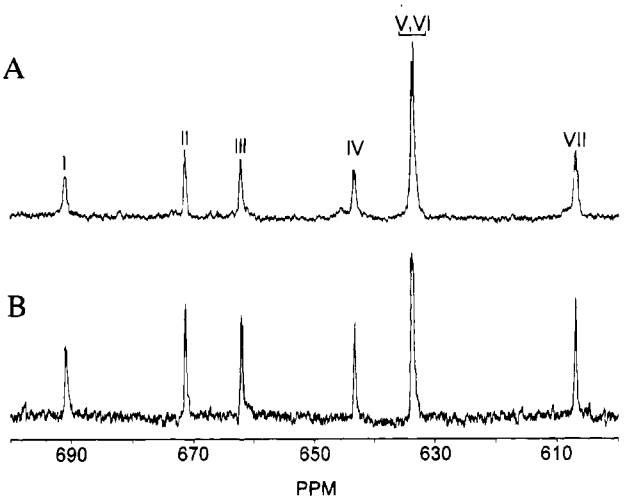

FIGURE 2: Comparison of one-dimensional ^{113}Cd NMR (^{1}H broad-band decoupled) spectra of (A) 3.7 mM solution of ^{113}Cd reconstituted sea urchin MT and (B) 5.7 mM solution of native recombinant sea urchin MT. Both samples were dissolved in 50 mM NaCl and 20 mM Tris-HCl, pH 8.6, containing 99.8% D_2O and were recorded at 27 °C as described in Materials and Methods. The resulting resonances have been numbered, with roman numerals, according to decreasing chemical shifts.

respondence between the natural abundance ^{113}Cd NMR spectrum of the native recombinant protein and the ^{113}Cd NMR spectrum of the ^{113}Cd (>95% enriched) reconstituted form. Both spectra display five distinct and two overlapping ^{113}Cd resonances numbered I−VII in the order of decreasing chemical shifts and correspondingly assignable to different environments of the Cd nuclei designated 1−7 in sea urchin Cd_7-MT. The chemical shifts of all resonances in the two ^{113}Cd NMR spectra are superimposable indicating indistinguishable chemical environments of the seven metal sites in the two forms. The only difference between the two spectra is the splitting observable in most resonances of the ^{113}Cd-reconstituted form arising from ^{113}Cd−^{113}Cd coupling. Inspection of expanded spectra (not shown) disclose that resonances I, II, III, and VII of Figure 2A are triplets and that resonance IV is a quartet thus establishing that in sea urchin MT, as in other well characterized MTs, the metal ions are linked in oligonuclear complexes or clusters. Because of signal overlap the multiplets of resonances V and VI cannot be distinguished.

Organization of the Cd Thiolate Clusters. Some of the coupled ^{113}Cd resonances are readily identified in the ^{113}Cd chemical shift correlation (COSY) spectrum of the reconstituted MT (Figure 3). The figure shows four clear cross peaks. A fifth rather weak cross peak connecting the resonances I and III was seen in the ω_2 cross section but could not be seen when plotting the spectrum on a "noise-free" level. The selective association of three of these cross peaks with resonances IV, V, VI, and VII and of two of them with resonances I, II, and III implied that the metal ions are partitioned into two distinct linkage groups thus strongly suggesting the existence of topologically separate clusters. The existence of two separate linkage groups composed of Cd1, Cd2, Cd3 and Cd4, Cd5, Cd6, Cd7, respectively, was confirmed by selective decoupling of resonances.[2] Thus, as shown in Figure 4, saturation of the overlapping resonances V and VI leads to a collapse of the quartet structure of resonance IV into a doublet. This means

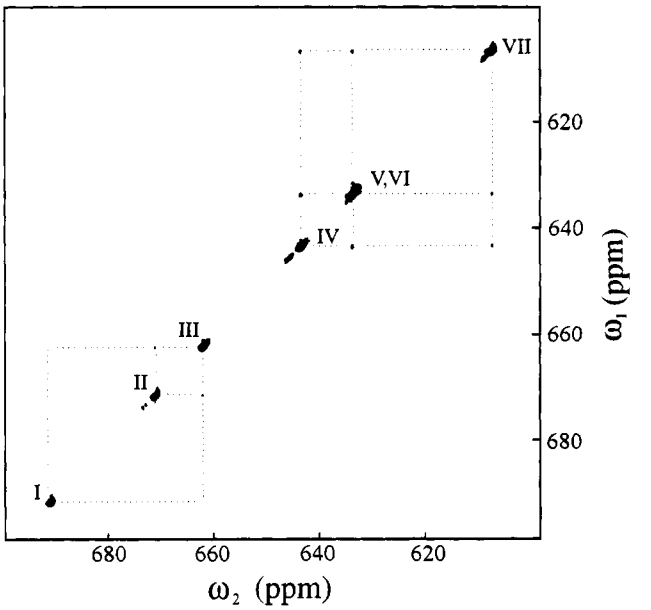

FIGURE 3: Two-dimensional homonuclear (^{113}Cd−^{113}Cd)COSY spectrum of a 5.7 mM solution of reconstituted sea urchin MT was recorded in 50 mM NaCl and 20 mM Tris-HCl, pH 8.6, containing 99.8% D_2O at 27 °C as described in Materials and Methods.

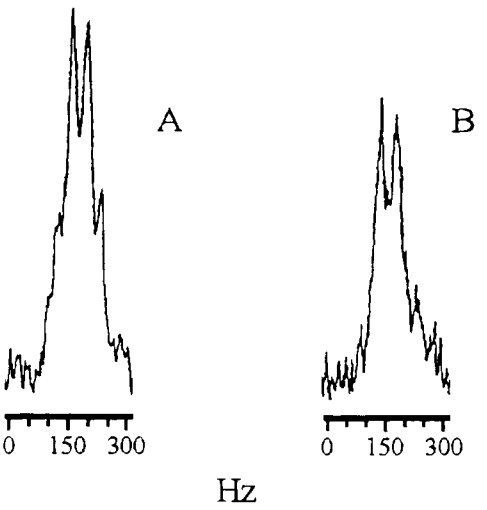

FIGURE 4: Comparison of the coupling pattern of the reconstituted sea urchin MT ^{113}Cd resonance IV (A) without and (B) with the selective decoupling of the overlapping resonances V and VI. The same sample as described in Figure 3 was used, and measuring conditions were as described in Materials and Methods.

that Cd5 and Cd6 must both be coupled to Cd4. This information cannot be extracted from the 2D spectrum due to the overlap of resonance V and VI. At the same time the triplet structure of resonance VII (not shown) was reduced to lower multiplicity while no effects were observed on the triplet features of resonances I, II, and III.

Relative Stability of the Cd Clusters. The exposure of 0.052 mM sea urchin $^{113}Cd_7$-MT to 5.5 mM EDTA at pH 7.0 for 2.5 h resulted in the loss of 1.5 equiv of Cd from the protein. The one-dimensional ^{113}Cd NMR spectrum of this sample after reconcentration is shown in Figure 5 and indicates that the three resonances I, II, and III are massively

[2] Unambiguous interpretation of homodecoupling experiments requires that all Cd−Cd J couplings give rise to well resolved multiplets with recognizable line splittings.

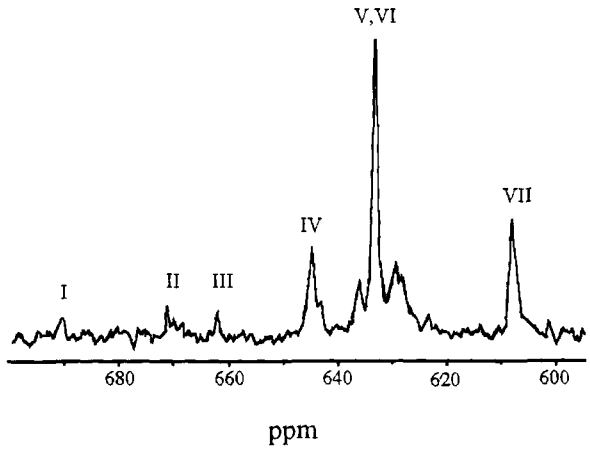

FIGURE 5: One-dimensional ^{113}Cd NMR spectrum of a 1.58 mM solution of reconstituted sea urchin MT which had been treated with EDTA resulting in the removal of 1.5 equiv of Cd. Under the conditions chosen for the sample preparation (see Materials and Methods) EDTA removes the metal in a biphasic reaction with a fast step completed within 5 h and a slow step proceeding over several days. By removing EDTA from the incubation mixture after 2.5 h both processes are arrested. The sample buffer and the NMR recording conditions were as described in the legend to Figure 3. The roman numerals refer to the corresponding resonances in Figure 2A.

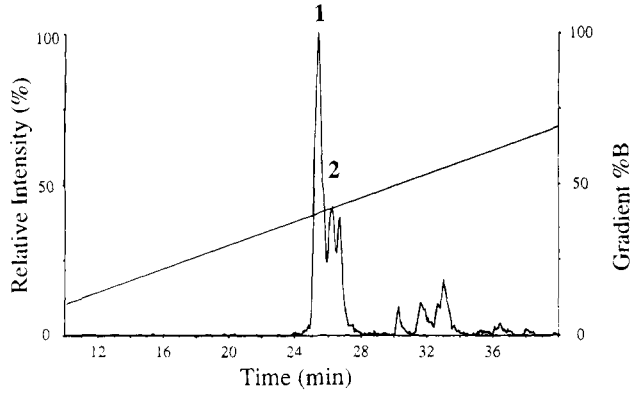

FIGURE 6: Separation of protein fragments generated from a subtilisin digest of EDTA treated sea urchin MT. EDTA treated recombinant sea urchin MT was digested with subtilisin and subsequently purified as described in Materials and Methods using a RP-HPLC system at pH 6 with an ammonium acetate buffer system and an acetonitrile gradient as shown. The eluant from the HPLC system was directly applied to an electrospray mass spectrometer and the total ion current of all ions between 800 and 2400 Da in this eluant is plotted as a function of time. The two major peaks are labeled 1 and 2.

and equally diminished relative to resonances IV, V, VI, and VII. Further changes are some additional resonances adjacent to resonances V and VI.

Localization of the Cd Clusters. To localize the metal thiolate clusters in the chain of sea urchin MT, holoprotein was incubated in the presence of 9.4 mM EDTA and subjected to limited proteolysis by subtilisin (for conditions see Materials and Methods). The fragments were separated by RP-HPLC at pH 6 and analyzed on-line by ES-MS. The total ion current of all masses between 800 and 2400 Da is shown in Figure 6. The mass of peak 1 is shown in Figure 7A. The signals of the mass to charge ratio at 1420 and 2130 represent the $[M + 3H]^{3+}$ and the $[M + 2H]^{2+}$ ions, respectively, of the peptide with a mass of 4258.3 Da. The molecular mass of the material corresponding to the minor

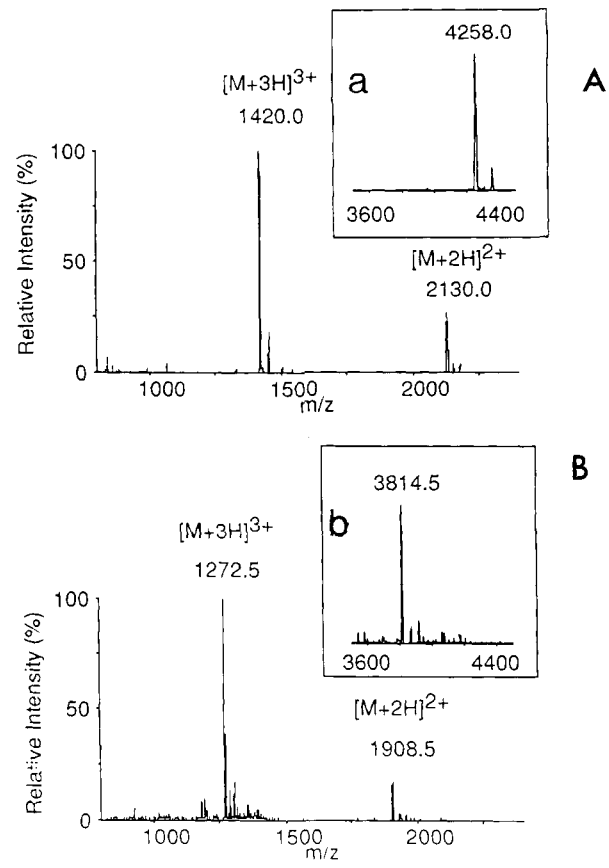

FIGURE 7: Electrospray mass spectra of (A) holo sea urchin MT fragment (represented by peak 1 in Figure 6) and (B) the apo fragment (generated by acidification from the material represented by peak 1 in Figure 6). The peaks representing the doubly and triply charged ions are indicated on both spectra. The inserts represent the calculated molecular masses generated from spectra A and B.

product (peak 2, Figure 6) has a net mass of 4388.0 Da. After acidification to dissociate Cd ions, the net molecular mass of the material represented by peak 1 was found to be reduced to 3813.4 Da indicating the loss of 4 Cd ions (Figure 7B). Thus as with Cd-reconstituted rabbit MT-2 (Yu *et al.*, 1993) the measurement by ES-MS of the digestion products allowed facile assessment of both the size and metal content of the undigested portion.

Five cycles of N-terminal sequence analysis of the material represented by peak 1 revealed a peptide with the N-terminal sequence PDVKC, thus identifying it as the N-terminal portion of the sea urchin MT. On the basis of mass data and the known sequence of sea urchin MT, this fragment is calculated to have a total length of 37 residues and corresponds to the underlined portion of the sea urchin MT amino acid sequence in Figure 8. The amino acid composition data also supported this conclusion (data not shown). The molecular mass of the material represented by peak 2 is approximately 130 Da more than that of the material emerging in peak 1. As this difference matches the mass of a methionine residue, we suggest that peak 2 represents the same portion of the sea urchin MT sequence as the peak 1 material but with a residual N-terminal initiator methionine residue. Thus, although previous studies had indicated that the predominant recombinant product had lost this methionine (Wang *et al.*, 1994), it appears that some protein preparations contain a significant proportion of material with this residue.

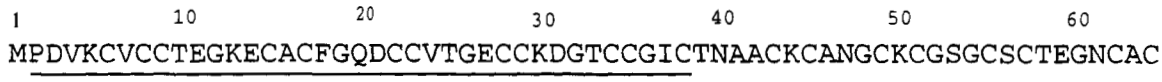

FIGURE 8: Amino acid sequence of sea urchin MTA (Nemer *et al.*, 1985). The underlined portion represents the undigested part of the molecule after incubation with subtilisin in the presence of EDTA (see Materials and Methods).

DISCUSSION

Comparison of the Native Recombinant and ^{113}Cd Reconstituted Sea Urchin MT. The successful elucidation of the organization of Cd thiolate clusters and the spatial solution structure of several Cd-containing MTs by NMR spectroscopic methods has been largely due to the favorable nuclear spin properties of Cd isotopes, such as ^{111}Cd and ^{113}Cd ($I = {}^1/_2$), which allows one to observe topologically interpretable homonuclear Cd−Cd and heteronuclear Cd−H interactions. For this reason for all such studies derivatives containing such isotopes must be prepared by isostructural substitution either by direct exchange methods (Otvos & Armitage, 1980; Otvos *et al.*, 1985) or by reconstitution of the holoprotein from the apo form with the desired isotope (Nielson & Winge, 1983; Vašák *et al.*, 1985; Otvos *et al.*, 1989). That both methods are equally successful has been shown both by comparing the 1D ^{113}Cd NMR spectra of ^{113}Cd$_7$-MT and by comparing the 2D ^{1}H NMR spectra of material prepared by reconstitution via the apoprotein or by direct exchange from native (Cd,Zn)-MT-2 of rabbit liver (Vašák *et al.*, 1987). The results revealed identical spectral patterns indicating identical metal-thiolate coordination and metal cluster architecture. In the same study it was also shown that ^{113}Cd resonances of *in vitro* reconstituted rat liver ^{113}Cd$_7$-MT coincide in chemical shift position with ^{113}Cd resonances of *in vivo* produced ^{113}Cd-containing MT-2 denoting identity of the mode of metal binding in the two forms. This is a conclusion also vindicated by the subsequent comparison of the solution structure of ^{113}Cd reconstituted rat liver MT-2 (Schultze *et al.*, 1988) with the crystal structure of native rat liver MT-2 (Robbins *et al.*, 1991). Accordingly, in the present study the ^{113}Cd derivative of sea urchin MT was prepared by *in vitro* reconstitution of the apoprotein generated by dissociation and removal of the natively bound metal at low pH, by adding the appropriate amount of ^{113}CdCl$_2$ and readjusting the pH to neutrality under anaerobic conditions (Vašák, 1991). The reestablishment of the native conformation was judged by the restitution of the chemical shift ^{113}Cd NMR pattern of the native recombinant form and the almost complete regeneration of the original CD profile. The latter method was used in preference to NMR measurements owing to the ease of analysis and its need of only small amounts of protein.

In contrast to the mammalian MT, the replacement of natural abundance Cd by ^{113}Cd in recombinant sea urchin MT by this method proved more difficult. Initial reconstitution experiments of the apoMT with ^{113}Cd following the above protocol yielded a product with the desired stoichiometry of 7 equiv of Cd per molecule and the anticipated UV absorption spectrum but it failed to restore the highly characteristic CD profile and the ^{113}Cd NMR spectrum of the native sea urchin Cd$_7$-MT (data not shown). As documented in Figure 1 the original CD spectrum was, however, largely regained when the reconstituted holoprotein sample was incubated and stirred overnight with the metal chelating resin, Chelex 100, at 4 °C before measuring. This sample displayed also all expected ^{113}Cd NMR features indicating that the coordination geometry of all seven Cd ions and hence their entire microenvironment was restored under these conditions of reconstitution. We attribute the failure to refold correctly in the absence of Chelex to a relative lack of residual structure determining features in the apo form of this protein and presume that the Chelex helps to mediate metal exchange from incorrectly folded structures thereby allowing rearrangement of the holoprotein to the thermodynamically most stable native conformation.

Organization of the Cd Clusters. While the ^{113}Cd NMR spectra of the native and reconstituted sea urchin MT display exactly the same resonance positions, they differ in that the resonances in the spectrum of the ^{113}Cd reconstituted sample are split into multiplets brought about by homonuclear ^{113}Cd−^{113}Cd couplings. Their splitting and the existence of cross peaks in the ^{113}Cd−^{113}Cd COSY spectrum (Figure 3) provides the most direct evidence for the arrangement of the metal in this protein in oligonuclear complexes held together by bridging thiolate ligands, an inference drawn earlier also from the chemical shift positions of the ^{113}Cd resonances (Coleman, 1993; Wang *et al.*, 1994). Independent evidence for cadmium thiolate clusters comes also from the electronic spectroscopic features of sea urchin Cd$_7$-MT. The UV absorption spectrum is dominated by the contributions from Cd−thiolate coordination while that of apoMT is almost featureless. The absorption shoulder near 250 nm of the holoMT corresponds to the incompletely resolved lowest energy band of the broad Cd−thiolate absorption envelope superimposed upon the plain spectrum of the apoprotein. Its position is close to that reported for Cd-containing mammalian MTs and complexes of Cd with 2-mercaptoethanol (Kägi & Vallee, 1961) and is thought to be indicative of tetrahedral coordination of Cd to four thiolate ligands (Vašák *et al.*, 1981). The multiphasic CD profile with positive and negative ellipticity lobes centered about an inflection point near 250 nm (Figure 1B) is also typical of all Cd-MTs thus far characterized (Bühler & Kägi, 1979; Willner *et al.*, 1987; Stillman *et al.*, 1987). These chiroptical features have been shown by coupled oscillator calculations to arise from excitonic coupling of strong dissymmetrically oriented transition dipole moments located at neighboring bridging sulfur ligands within the Cd−thiolate clusters (Willner *et al.*, 1992). Thus, the CD spectrum indicates the presence of such cluster structures in sea urchin Cd$_7$-MT, and, because of its explicit origin in the chiral arrangement of the coupled chromophores, it is exceedingly sensitive to structural changes in the microenviroment of the metal (see above). The MCD spectrum showing a strong biphasic profile (positive Faraday *A* term) associated with the lowest energy Cd−thiolate band is also a characteristic feature of the Cd-MTs (Law & Stillman, 1981; Vašák & Kägi, 1983; Willner *et al.*, 1992) and is thought to be a reflection of the high electronic symmetry of the degenerate excited state of the chromophores in the cluster (Carson *et al.*, 1981).

The most direct evidence for the existence and structure of two independent tetrahedral Cd−thiolate clusters in the sea urchin MT comes from a combination of the information

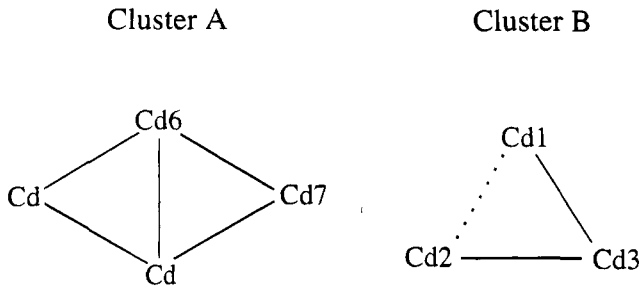

FIGURE 9: Schematic representation of the metal connectivities in the metal-thiolate clusters in sea urchin MT. Four-metal cluster of N-terminal domain (cluster A). Three-metal cluster of C-terminal domain (cluster B). The dotted connectivity in cluster B is inferential.

derived from the $^{113}Cd-^{113}Cd$ COSY spectrum (Figure 3) and the 1D ^{113}Cd NMR spectrum recorded with and without decoupling of selected resonances (Figure 4). Thus, the selective association of three cross peaks in the ^{113}Cd COSY spectrum with resonances IV, V, VI, and VII and two of them with resonances I, II, and III implies that the metal ions are partitioned into two groups. The key feature of the well-resolved 1D ^{113}Cd NMR spectrum is the fact that resonance IV is a quartet suggesting that Cd4 is coupled to three other ^{113}Cd ions. Upon saturation of the unresolved resonances V and VI, the quartet of resonance IV collapses to a doublet (and not a triplet) consistent with residual coupling to only one ^{113}Cd nucleus, i.e., Cd7, a conclusion derivable from the $^{113}Cd-^{113}Cd$ COSY spectrum. During the same experiment, the triplet of resonance VII collapses to a doublet thus revealing that Cd7 is coupled to either Cd5 or Cd6 but not to both. Thus, from the homodecoupling experiments and the observed cross peaks between resonances in the $^{113}Cd-^{113}Cd$ COSY spectrum, we conclude tentatively that the four-metal cluster has a bicyclic structure with the four metal ions interconnected by five bridging thiolate ligands forming a bicyclo[3.3.1]nonane-like system which was also found in mammalian forms (Otvos & Armitage, 1980) and in which two of the metal ions are linked to three other metals through bridging thiolate ligands and in which two of the metal ions have only two metal neighbors (Figure 9). From the connectivities between Cd1 and Cd3, and between Cd2 and Cd3, and the knowledge that the resonances I, II, and III are triplets we also infer tentatively that the three-metal cluster has a cyclic structure with the three metal ions interconnected by three bridging thiolate ligands forming a cyclohexane-like ring (Figure 9) as shown for the three-metal cluster of mammalian (Otvos & Armitage, 1980) and crustacean (Otvos *et al.*, 1982) MTs. Thus, we suggest on the basis of these inferences and the 7 Cd to 20 Cys stoichiometry (Wang *et al.*, 1994) that sea urchin MT like the mammalian proteins contains both a monocyclic Cd_3Cys_9 and a bicyclic Cd_4Cys_{11} cluster. The proposition that the MTs from sea urchin and mammals contain the same type of Cd–thiolate clusters is in accordance with the strong similarities of the proteins in overall structure and reactivity. It allows us to expect that sea urchin MT too is composed of two separate metal cluster enclosing protein domains and may thus account for its elongated shape deduced from Stokes radius measurements (Wang *et al.*, 1994).

Relative Stability of the Cd Clusters. The effectiveness of the chelating agent EDTA to remove Cd selectively from the three-metal cluster (Figure 5) while not affecting appreciably the four-metal cluster suggests that the former is kinetically and presumably also thermodynamically less stable. The latter inference is consistent with our earlier estimate, made from spectrophotometric pH-titration experiments, that at pH 7 the apparent average binding constant for Cd in the three-metal cluster of sea urchin MT is about 300 times lower than for the four-metal cluster (Wang *et al.*, 1994.) The unequal sensitivity to EDTA is another indication that the two clusters are independent of each other. The fact that with the removal of Cd by EDTA the resonances I, II, and III are reduced equally is strong experimental evidence that the three-metal cluster at pH 7 is cooperatively stabilized and that its emergence is an all-or-none process as has previously been reported for mammalian MT at neutral pH (Nielson & Winge, 1983; Dalgarno & Armitage, 1984; Good *et al.*, 1988). The spectral changes, noticeable in the chemical shift range of the four-metal cluster of the partially metal-depleted sample, could be the result of a perturbation of the coordination geometry in this cluster by unoccupied cysteine residues of the vacant three-metal site or might arise from transient complexes formed under conditions of lowered metal-to-thiolate ratios (Good *et al.*, 1988).

Localization of the Cd–Thiolate Clusters. The partial digestion studies carried out with the unspecific protease subtilisin on partially metal depleted sea urchin MT provide yet another indication that the two metal thiolate clusters are separate entities and suggest that the molecule is partitioned into distinct and separately arranged domains. By the same limited proteolysis method it has been previously shown that the mammalian MTs are composed of two domains, with the N-terminal half of the polypeptide chain accommodating the less stable three-metal cluster and the C-terminal half with the more stable four-metal cluster (Winge & Miklossy, 1982). As with these proteins incubation of EDTA-exposed sea urchin MT with subtilisin resulted in the selective digestion of the polypeptide portion of the metal-depleted three-metal domain yielding a protease-resistant polypeptide fragment containing four Cd ions (Figures 6 and 7). However, in contrast to the mammalian four-metal domain, which comprises the C-terminal half of the polypeptide chain, the undigested four-metal domain of the sea urchin MT contains the N-terminal portion, i.e., residues 2–38. Thus, one must conclude that in echinodermal and mammalian MT the arrangement of the stable four-metal cluster and the more labile three-metal cluster is inverted (Figure 9). Interestingly, this result is consistent with the proposal made by Nemer *et al.* (1985), who on the basis of sequence comparison with vertebrate forms suggested that in mammals and in the sea urchin the two halves of the polypeptide chain are reversed in their relative locations (Harlow *et al.*, 1989). These workers have also shown that the structural gene of sea urchin MTA like the vertebrate MT genes are made up of three exons and have suggested that two of them (exons 2 and 3) have evolved from an earlier gene duplication and that based on resemblances in the Cys arrangement the positions of the two exons are reversed in these two animal classes. From this inversion and from the overall spacing of the Cys in the chains, it appears in fact that the vertebrate and echinodermal proteins are quasi mirror images of one another, an impression supported by the present data.

In conclusion, we have reconstituted recombinant sea urchin Cd_7-MT with ^{113}Cd (>95% enriched) and have shown by electronic absorption, chiroptical, and ^{113}Cd NMR spectroscopic measurements that in this protein, like in mammalian MT, the metal ions are bound in oligonuclear complexes (i.e., clusters) made up of tetrahedral Cd−thiolate units. By partial metal depletion and limited proteolysis studies, we have furthermore established that the seven metal ions are partitioned in a less stable three-metal and a more stable four-metal cluster. From a combination of ^{113}Cd−^{113}Cd COSY measurements and decoupling studies, it appears moreover that sea urchin MT like the mammalian proteins possesses a monocyclic Cd_3Cys_9 and bicyclic Cd_4Cys_{11} cluster. However, in contrast to the mammalian forms in the sea urchin protein, their location along the polypeptide chain is reversed.

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