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Application of ^{113}Cd NMR to metallothioneins

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Abstract

Metallothioneins constitute a class of ubiquitously occurring low molecular mass proteins (6–7 kDa) possessing two cysteine thiolate-based metal clusters usually formed by the preferential binding of d^{10} metal ions such as Zn^{II} and Cd^{II}. The three-dimensional solution structure of mammalian proteins has been determined by two-dimensional NMR spectroscopy of $^{113}Cd_7$-metallothionein. The structure shows two protein domains encompassing the $M_3(CysS)_9$- and $M_4(CysS)_{11}$-cluster with each metal ion being tetrahedrally coordinated by thiolate ligands. The application of ^{113}Cd NMR proved to be indispensable in the structural studies of metallothioneins. Thus, both homonuclear ^{113}Cd decoupling studies and ^{113}Cd-^{113}Cd COSY of $^{113}Cd_7$-metallothionein established the existence of two metal-thiolate clusters in this protein. The identification of sequence specific cysteine-cadmium coordinative bonds came from heteronuclear ^{113}Cd-^{1}H COSY experiments. Independently, the ^{113}Cd NMR characterization of the intermediate metal-protein complexes, leading to the cluster structure in $^{113}Cd_7$- metallothionein, revealed a stepwise cluster formation process with the $Cd_4(CysS)_{11}$-cluster being formed first. The recent demonstration of a Karplus-like dependence between the heteronuclear $^3J(^{113}Cd, ^1H)$ coupling constants for the cysteine C^β protons and the H^β-C^β-S^γ-Cd dihedral angles should allow to derive the geometry of the Cd-(S-Cys) centers in various metallothioneins and related metalloproteins. A possible application of ^{113}Cd NMR to the study of metallothioneins in the environment is discussed.

Abbrevations used: MTs – metallothioneins

Introduction

Metallothioneins (MTs) is a collective name for a superfamily of ubiquitous low molecular mass (6–7 kDa) cysteine- and metal-rich proteins or polypeptides. They are characterized by metal-thiolate clusters formed by the coordination of d^{10} metal ions with cysteine thiolates. Despite a lapse of 40 years since its discovery (Margoshes & Vallee 1957; Kägi & Vallee 1960) and intensive investigations (reviewed by Kägi 1991; Templeton & Cherian 1991; Vašák & Kägi 1994; Poutney et al. 1995) the primary physiological role of MTs is still a topic of discussion. However, in view of several metallic constituents of this protein it is believed that MTs play an important role in heavy metal detoxification (Cd, Hg) and in metabolism and modulation of biological activity of essential metal ions (Zn, Cu). Moreover, in recent years an increasing body of evidence has accumulated suggesting that MTs play an important role in protecting the cells against the mutagenic effects of free radicals produced during oxidative stress and of some neoplastic drugs which are potent DNA alkylating agents (Kägi 1991; Templeton & Cherian 1991).

The best studied mammalian MTs (MT-1 and MT-2 isoforms) are composed of a single polypeptide chain of about 60 amino acids, 20 of which are cysteine and none of which are aromatic amino acids or histidine (Vašák & Kägi 1994; Pountney et al. 1995). Recently, two other mammalian isoforms (MT-3 and MT-4) have been discovered. Unlike MT-1 and MT-2, which are expressed in most organs, MT-3 expression

appears to be restricted to the brain and that of MT-4 to certain stratified squamous epithelia (Pountney et al. 1995 and references therein).

There are a variety of proteins and peptides that bear a functional and structural relationship to the well-characterized mammalian MTs. In the absence of a more meaningful system of nomenclature, the MTs have been divided into three arbitrary classes. Class I MTs constitute proteins with 20 cysteines closely related to mammalian MTs. Class II encompasses analogous proteins with locations of cysteines only distantly related to mammalian MTs. Class III are enzymatically synthesized peptides such as the poly-(γ-glutamylcysteinyl) glycines occurring in some plants and fungal species (Fowler et al. 1987).

The subject of this article is to highlight the application of ^{113}Cd NMR in the study of the cluster structure in MTs and in the determination of the three-dimensional structure of mammalian MTs by NMR.

Properties of cadmium as a structural probe

Since the demonstration of the utility of ^{113}Cd as a probe for the metal binding site in metalloproteins by Armitage et al. (1978) the ^{113}Cd NMR spectroscopy has received considerable attention. ^{113}Cd NMR spectroscopy has been used successfully to examine the coordination environment of a number of metalloproteins with Zn, Ca, Cu, Cd, Mn and Mg binding sites. In general, Cd-substituted metalloproteins retain, at least to some extent, their biological activity. In the case of MTs the natural occurrence of cadmium in the protein makes the use of ^{113}Cd NMR advantageous. Several reviews which include selected aspects of the ^{113}Cd NMR literature have appeared (Armitage & Boulanger 1983; Summers 1988).

Sensitivity. ^{113}Cd has a spin of 1/2, a natural abundance (N) of 12.26%, and a relative sensitivity (at 100% enrichment) of 1.09×10^{-2} relative to ^{1}H (Table 1). Thus the relative sensitivity is comparable to that of ^{13}C which is 1.59×10^{-2}. ^{111}Cd is another naturally occurring isotope suitable for NMR (I = 1/2, N = 12.75%). Since the relative sensitivity of ^{111}Cd with 9.54×10^{-3} is about 10% lower than that of ^{113}Cd, the majority of the NMR studies have been performed with the latter nucleus.

Chemical shifts and chemical exchange. ^{113}Cd has a demonstrated chemical shift range of over 900 ppm. The value of the chemical shift has been shown to depend on the nature, number, and geometric arrangement of the coordinating ligands. The chemical shift of ^{113}Cd resonances is usually reported relative to a standard (0.1 M aqueous $Cd(ClO_4)_2$). The greater chemical shift dispersion of ^{113}Cd in comparison to other common high-resolution nuclei, e.g., ^{1}H, ^{19}F, ^{31}P, ^{13}C, and ^{15}N, reflects the sensitivity of shielding of the ^{113}Cd nucleus to the local environment. The deshielding of the $^{113}Cd^{II}$ ion by complexing ligands was found to increase in the order of S>N>O. In past the chemical shift position of ^{113}Cd resonance has often be used to derive structural features of the metal binding site. However, recent studies indicate that ^{113}Cd NMR chemical shift information alone is inadequate for unambiguous determination of structural features or structural changes at metal binding sites of metalloproteins (Summers 1988). Due to the high dispersion of the ^{113}Cd chemical shifts, the position of the resonance is exceedingly sensitive to subtle differences in the coordination environment. This results in the excellent spectral resolution. This is exemplified by the ^{113}Cd NMR spectrum of MT in which multiple coordination sites of very similar structure are well resolved (Figure 1). However, in some unfavourable cases this very same property can lead to difficulties in resonance detection, owing to chemical exchange broadening. In this case both the chemical shift and the line widths of ^{113}Cd resonances can be affected. The extent of the effect depends on the rate of exchange properties relative to the chemical shift difference between the exchanging species. In the fast exchange regime, appreciable variations in the chemical shift can be obtained as the observed chemical shifts represent an average between those of the $^{113}Cd^{II}$ ion in two or more states. Under intermediate-exchange condition, problems can be more serious because of the broadening of the resonances over a fairly large range of exchange rates, which may prevent the observation of the resonances. However, in many instances expected resonance(s) became detectable upon variations of the pH, temperature or ionic strength conditions of the sample. These changes of conditions can favourably alter the time scale of the exchange process.

Coupling constants. The observation of scalar couplings in the ^{113}Cd NMR spectrum of proteins can provide unique information regarding the nature of the ligands and their mode of binding. In MTs the

Table 1. Nuclear properties of selected isotopes

Isotope (I = 1/2)	ν at 11.74 T (MHz)	Natural abundance (%)	Sensitivity Rel.[a]	Sensitivity Abs.[b]
^{1}H	500.0	99.98	1.00	1.00
^{13}C	125.7	1.11	1.6×10^{-2}	1.8×10^{-4}
^{15}N	50.7	0.37	1.0×10^{-3}	3.8×10^{-6}
^{113}Cd	110.9	12.26	1.09×10^{-2}	1.33×10^{-3}
^{111}Cd	106.0	12.75	9.54×10^{-3}	1.21×10^{-3}
^{112}Cd (I = 0)	–	24.07	NMR inactive	

[a] Relative sensitivity at constant field for equal number of nuclei.
[b] Product of relative sensitivity and natural abundance.

observation of ^{113}Cd–^{113}Cd two-bond couplings (^{2}J between 29–48 Hz) has led to the elucidation of the two-cluster structure. It may be noted that while at intermediate magnetic fields the homonuclear couplings are still relatively well resolved, at high magnetic fields, due to chemical shift anisotropy (CSA) of the ^{113}Cd nucleus, their lower resolution is observed. The observation of heteronuclear ^{113}Cd-^{1}H three-bond couplings (^{3}J between 1–70 Hz) to cysteines allowed their direct identification as the sole coordinating ligands in MTs and by using two-dimensional heteronuclear (^{113}Cd, ^{1}H) correlated spectroscopy (COSY) the determination of sequence specific ^{113}Cd-cysteine coordinative bonds (Wüthrich 1991). In general, the magnitude of three-bond coupling constants (^{3}J) for lighter nuclei (^{1}H, ^{1}H; ^{15}N, ^{1}H; ^{13}C, ^{1}H) depends on the dihedral angle and follows a Karplus-type of dependence. A similar correlation between the magnitude of heteronuclear (^{113}Cd, ^{1}H) three-bond coupling constants and the dihedral angles could be potentially useful in defining the geometry of the metal binding sites in ^{113}Cd-substituted MTs and other metalloproteins. Recently, using heteronuclear (^{113}Cd, ^{1}H) multiple quantum coherence (HMQC) experiments such a Karplus-like dependence has been established between the H^{β}-C^{β}-S^{γ}-Cd dihedral angles and the magnitude of the heteronuclear ^{3}J ($H^{a,b}$-^{113}Cd) coupling constants (Zerbe et al. 1994). This information will be of great use as an additional input parameter in three-dimensional protein structure analysis by NMR methods.

Practical considerations. The coordination sphere of the metal binding sites in metalloproteins always contains three or more ligands provided by the protein. In this case the ^{113}Cd resonance experiences heteronuclear (^{113}Cd, ^{1}H) couplings. Because of the negative value of ^{113}Cd magnetic moment, ^{113}Cd can experience nuclear Overhauser enhancements (NOE's), η + 1, which ranges from about 1 to -1.2 depending on the correlation time, τ_c. Where τ_c corresponds roughly to the interval between two successive reorientations or positional changes of the molecule. Thus NOE decreases as τ_c increases. Further, NOE also decreases with increasing magnetic field. Consequently, for cadmium substituted metalloproteins NOE values near zero are often observed resulting in sever reduction or even loss of resonance intensity. Such a situation exists at intermediate magnetic fields for metallothioneins. To overcome this problem the ^{113}Cd NMR spectrum is acquired by gating of the decoupler. Additional parameters which should be considered are the T_1 relaxation and the signal line width. The T_1 relaxation of ^{113}Cd sites in MTs is of the order of 1 s and the line widths are about 40 Hz (Nettesheim et al. 1985). Using a 1–2 mM sample contained in 5 mm NMR tube the one-dimensional ^{113}Cd NMR spectrum (96% ^{113}Cd enrichment) can be obtained in 5–10 h at the magnetic field of 11.7 T.

^{113}Cd metallothioneins

Protein and sample preparation. ^{113}Cd enriched samples should be used when studying the details of the cluster structure in MTs. However, since the Cd_7-MT form cannot be isolated from mammalian species this form is prepared by metal reconstitution. In the original study by Otvos & Armitage 1980, $^{113}Cd^{II}$ salts have been administered to rabbits and Zn_2,$^{113}Cd_5$-MT isolated. For five ^{113}Cd ions present in distinct sites five ^{113}Cd resonances would be expected. However, in the ^{113}Cd NMR spectrum of the isolated $^{113}Cd_5$,Zn_2-MT form at least 15 resolved ^{113}Cd resonances were observed (see Figure 1). This fea-

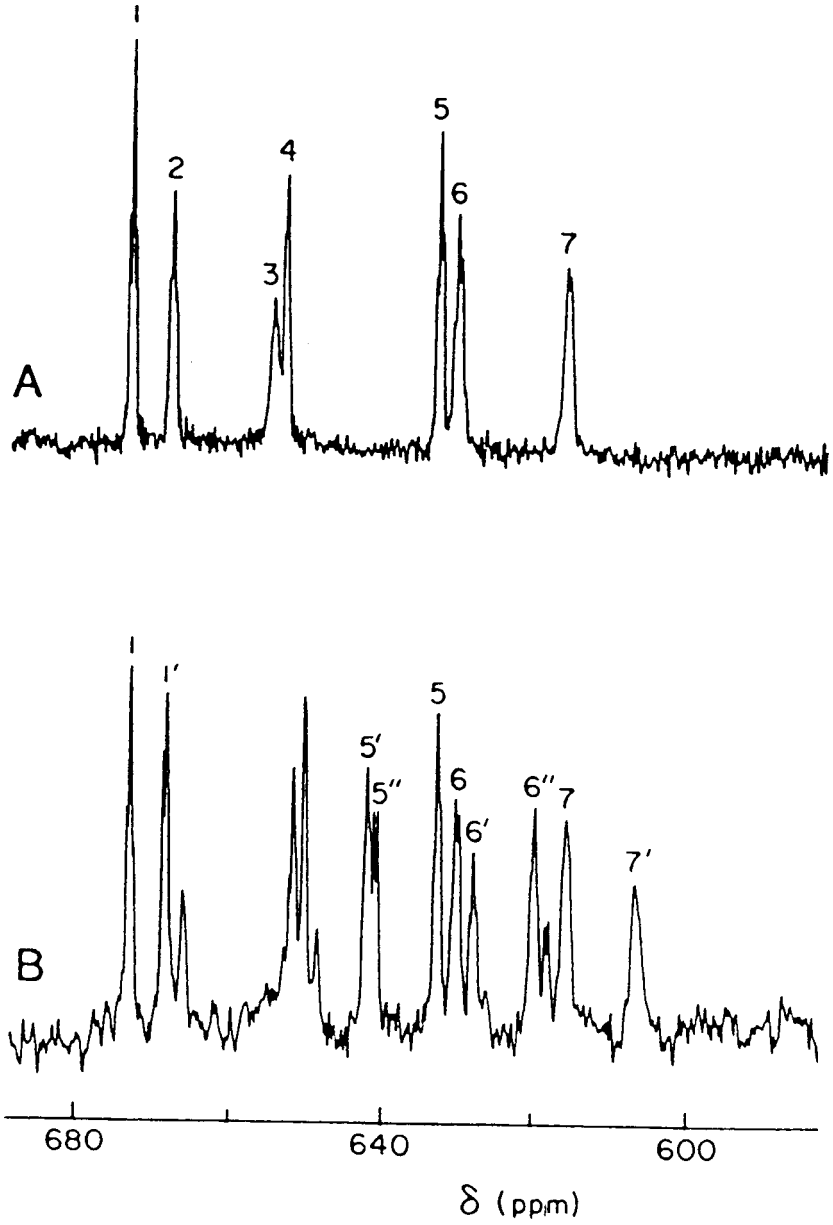

Figure 1. Comparison of one-dimensional ^{113}Cd NMR (^{1}H-decoupled) spectra at 25 °C of (A) reconstituted rat $^{113}Cd_7$-MT-2 and (B) isolated rat $^{113}Cd_5,Zn_2$-MT-2. The ^{113}Cd signals in (A) are numbered according to decreasing chemical shift. The extra signals in (B) indicate the presence of at least two additional, different molecular species (adapted from Vašák et al. 1987).

ture reflects a Cd-distribution among a number of mixed Zn,Cd-clusters in native Cd_5,Zn_2-MT making the structural studies virtually impossible. To generate the uniformly labeled $^{113}Cd_7$-MT, the remaining about two Zn^{II} ions in $^{113}Cd_5,Zn_2$-MT were subsequently substituted for $^{113}Cd^{II}$ by an exposure of the sample to the $^{113}Cd^{II}$ ions. The latter step is not required for invertebrate MTs as the protein possessing homometallic ^{113}Cd occupation of all six metal binding sites can be directly isolated, i.e., $^{113}Cd_6$-MT (Narula et al. 1995). However, the above approach requires a rather large quantity of the costly ^{113}Cd isotope. An alternative metal reconstitution method which requires the use of apoMT form and a small amount of $^{113}Cd^{II}$ has also been described. Briefly, the Zn^{II} and/or Cd^{II} ions present in isolated MTs are removed by a low

pH treatment. In the subsequent anaerobic step the required mol equivalents of $^{113}Cd^{II}$ are added to the solution of apo-MT and the pH adjusted to neutral (Vašák 1991). Protein samples for NMR are then concentrated by ultrafiltration and 10% of D_2O is added to provide the field-frequency lock.

For a number of MTs from different species only the gene sequences are known. To learn more about the structure of these MTs they are expressed in *E. coli* cell cultures as the Cd containing form. The assumption made in such a structural study is that the protein as isolated from *E. coli* represents a correctly folded native MT form. For NMR studies of these MTs a following strategy has been adopted. The protein is expressed as Cd-MT using natural abundance Cd salts. The natural abundance one-dimensional ^{113}Cd NMR spectrum is then obtained which provides the basis for the metal reconstitution. For the natural abundance spectrum an approximately 5–8 mM sample of the protein is required. The ^{113}Cd-MT form (96% ^{113}Cd enrichment) is then generated by the method of metal reconstitution as described above. The subsequently obtained one-dimensional ^{113}Cd NMR spectrum of reconstituted ^{113}Cd-MT must match that obtained using the natural ^{113}Cd abundance. With such a sample more advanced NMR experiments including ^{113}Cd-^{113}Cd COSY and (^{113}Cd, ^{1}H) HMQC can be performed.

Cluster structure in metallothioneins from ^{113}Cd NMR

The presence of two metal-thiolate clusters in mammalian $^{113}Cd_7$-MT has been established by one-dimensional homonuclear decoupling studies of this metalloform (Otvos & Armitage 1980). In this metal homogenous form the seven ^{113}Cd resonances for seven metal binding sites are present. By analysis of the two-bond ^{113}Cd-^{113}Cd spin coupling connectivities, responsible for the multiplet splitting patterns of each resonance, the presence of two separate clusters was deduced, i.e., the cyclohexane-like $M^{II}_3(Cys)_9$ cluster and the adamantane-like $M^{II}_4(Cys)_{11}$ cluster (Figure 6). It may be noted that in the X-ray structure of rat Zn_2,Cd_5-MT only an unique distribution of these ions in this metalloform crystallized (Robbins et al. 1991). Using two-dimensional NMR technique the same information regarding the cluster structure in MTs can be obtained much easier by homonuclear ^{113}Cd COSY experiments (Frey et al. 1985).

An example of the study of cluster structures in MT, where only the gene sequence is available, is that of sea urchin MT which has been expressed in *E. coli* as the Cd containing form (Wang et al. 1995). In Figure 2 the one-dimensional ^{113}Cd NMR spectra of sea urchin Cd_7-MT at natural abundance and at about 96% ^{113}Cd enrichment are shown. The close similarity between both spectra established the correctness of metal reconstitution. Subsequent ^{113}Cd-^{113}Cd COSY provided the information about the cluster organization in this protein (Figure 3). This information has been derived from the cross-peak pattern. Thus, the three cross peaks associated with ^{113}Cd resonances IV, V, VI, and VII and two of them with resonances I, II and III suggested the presence of a two cluster structure. In the one-dimensional ^{113}Cd NMR spectrum the resonances V, VI show almost identical chemical shift positions which precluded the details of this Cd_4-cluster structure to be obtained from ^{113}Cd-^{113}Cd COSY. The fact that the cluster topology of Cd_4-cluster is similar to that found in mammalian MTs came from the analysis of the two-bond ^{113}Cd-coupling pattern of the resonance IV obtained by selective decoupling of the overlapping resonances V and VI, i.e., original quartet collapsed into a doublet. From these studies the presence of two metal-thiolate clusters of three- and four-metal ions have been concluded. However, although the same type of clusters exists also in mammalian Cd_7-MTs, in sea urchin Cd_7-MT they are interchanged in their location along the polypeptide chain.

Cluster dynamics

In order to function as efficient metalloregulatory agents, MTs should be able to acquire and release metal ions rapidly. Indeed, the metal-sulfur coordinative bonds of MT clusters have been found to be highly labile by a variety of spectroscopic techniques. Direct evidence for metal fluxionality in Cd_7-MT was furnished by ^{113}Cd NMR saturation transfer experiments which established the presence of inter- and/or intramolecular metal exchange within cluster B (β-cluster) with a half-life in the order of 0.5 s (Nettesheim et al. 1985). The absence of such NMR-detectable processes within cluster A (α-cluster) is apparently responsible for the usually observed about 20% increased signal intensity of the four ^{113}Cd resonances of this cluster (see Figure 4A). The confirmation of similar processes taking place within the

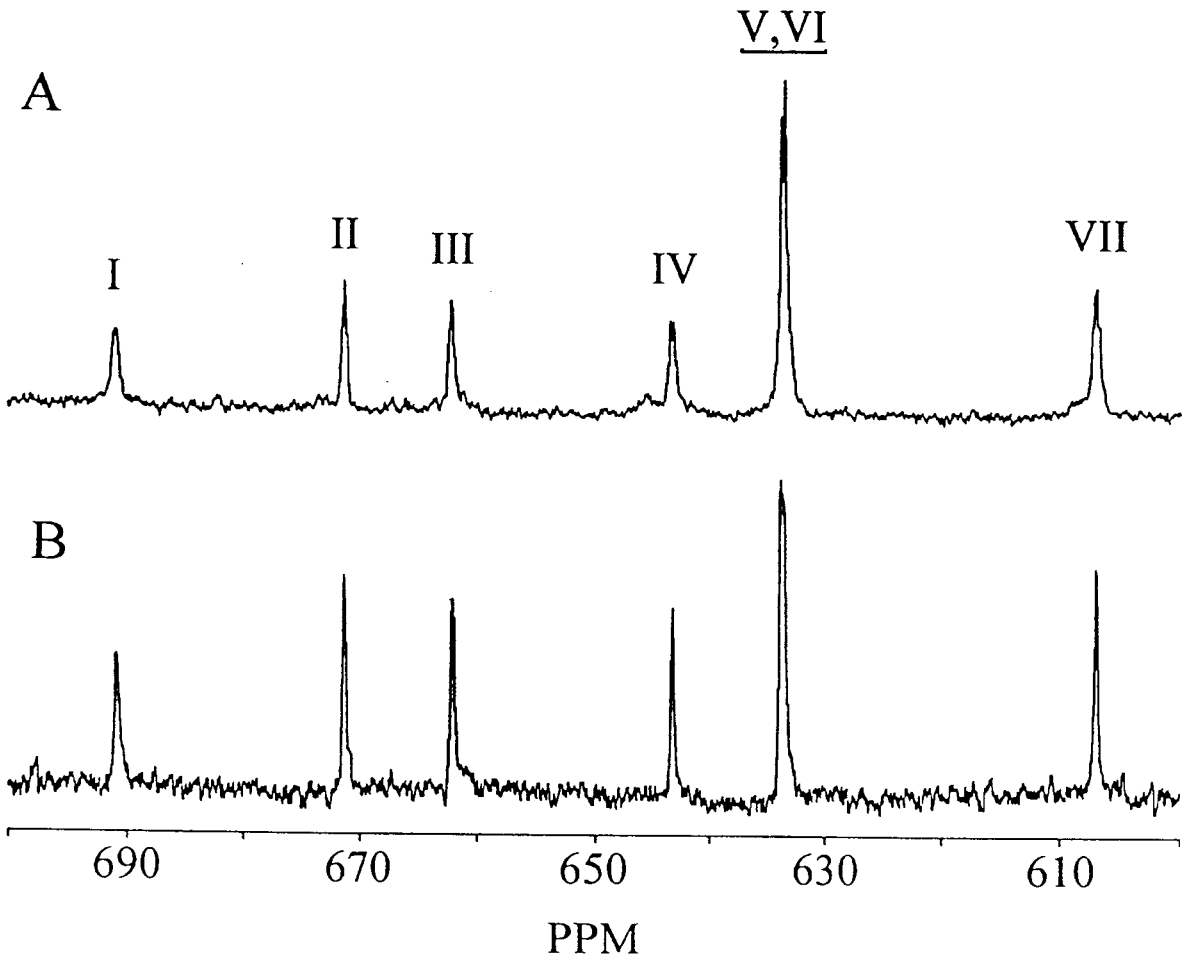

Figure 2. Comparison of one-dimensional ^{113}Cd NMR (^{1}H-decoupled) spectra at 27 °C of (A) reconstituted sea urchin $^{113}Cd_7$-MT (3.7 mm) and (B) isolated recombinant sea urchin Cd_7-MT at natural abundance (5.7 mm). The ^{113}Cd signals in (A) are numbered according to decreasing chemical shift (from Wang et al. 1995).

α-cluster of Cd_7-MT, but with a half-life of about 16 min, was afforded by metal exchange studies using the radioactive ^{109}Cd isotope (Kägi 1991). Such dynamic fluctuations in MT clusters are thought to be a manifestation of the non-rigid nature of the overall protein structure as supported by NMR ^{1}H-^{2}H amide exchange studies (Messerle et al. 1990). The mechanism underlying the fluxionality of the MT structure and its extent is currently unknown. It should be noted, however, that very large structural fluctuations have also been observed in ^{1}H NMR studies of adamantane-like cages with the general formula $[M_4(SPh)_{10}]^{2-}$ (M = Cd^{II}, Zn^{II}, Co^{II}, and Fe^{II}) (Hagen et al. 1982). These results were interpreted in terms of a nonrigid cluster model in which the metal-thiolate bonds are temporarily broken and reformed giving rise to a number of interchanging cluster substates of comparable stability and structure. It is likely that similar types of dynamic processes take place in MT. Overall, these data reaffirm that notwithstanding the high thermodynamic stability of M^{II}_7-MT complexes they are kinetically very labile, i.e. that the thiolate ligands undergo both metallation and demetallation rapidly. Interestingly, a metal exchange rate with a half-life of minutes also occurs in the binuclear Zn_2Cys_6 cluster of a GAL4 protein involved in the stabilization of the DNA binding domain of this transcription factor as well as in zinc containing DNA binding 'zinc-finger proteins'. By contrast, in zinc enzymes, e.g., alkaline phosphatase, carboxypeptidase, the exchange half-lifes are in the order of hours and days. Thus, the actual exchange rates of metal ions of protein complexes are

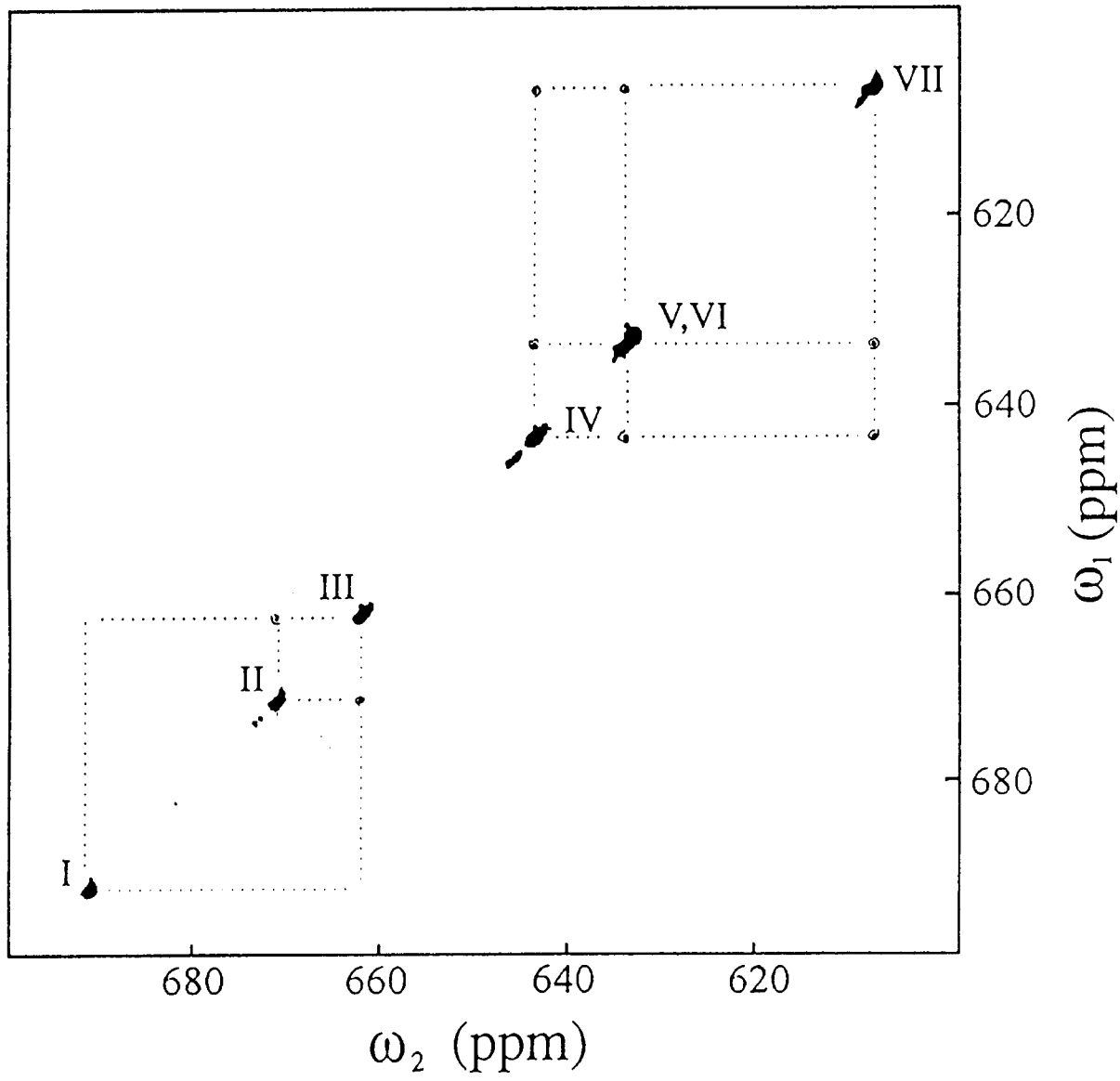

Figure 3. Two-dimensional homonuclear ^{113}Cd COSY spectrum at 27 °C of reconstituted sea urchin $^{113}Cd_7$-MT (5.7 mm) (from Wang et al. 1995).

not an intrinsic feature of bonding properties of the metal but are determined by the energies and kinetics of protein folding (structure flexibility).

To date, structural and biological studies of mammalian MTs have focused mainly on the monomeric form of this protein containing 7 equivalents of divalent metal ions. However, *in vitro* studies have shown that exposure of Cd_7-MT from rabbit liver to free Cd^{II} ions in phosphate buffer yields non-oxidized dimers which contain additionally 2 less strongly bound Cd^{II} ions and 1 inorganic phosphate per monomeric protein unit (Palumaa et al. 1992). The structural features of generated cluster forms have been examined by ^{113}Cd NMR. However, although the resonances of the unperturbed four-metal cluster were observed, the remaining ^{113}Cd resonances of the dimer remained undetected due to an unknown metal-exchange process(es).

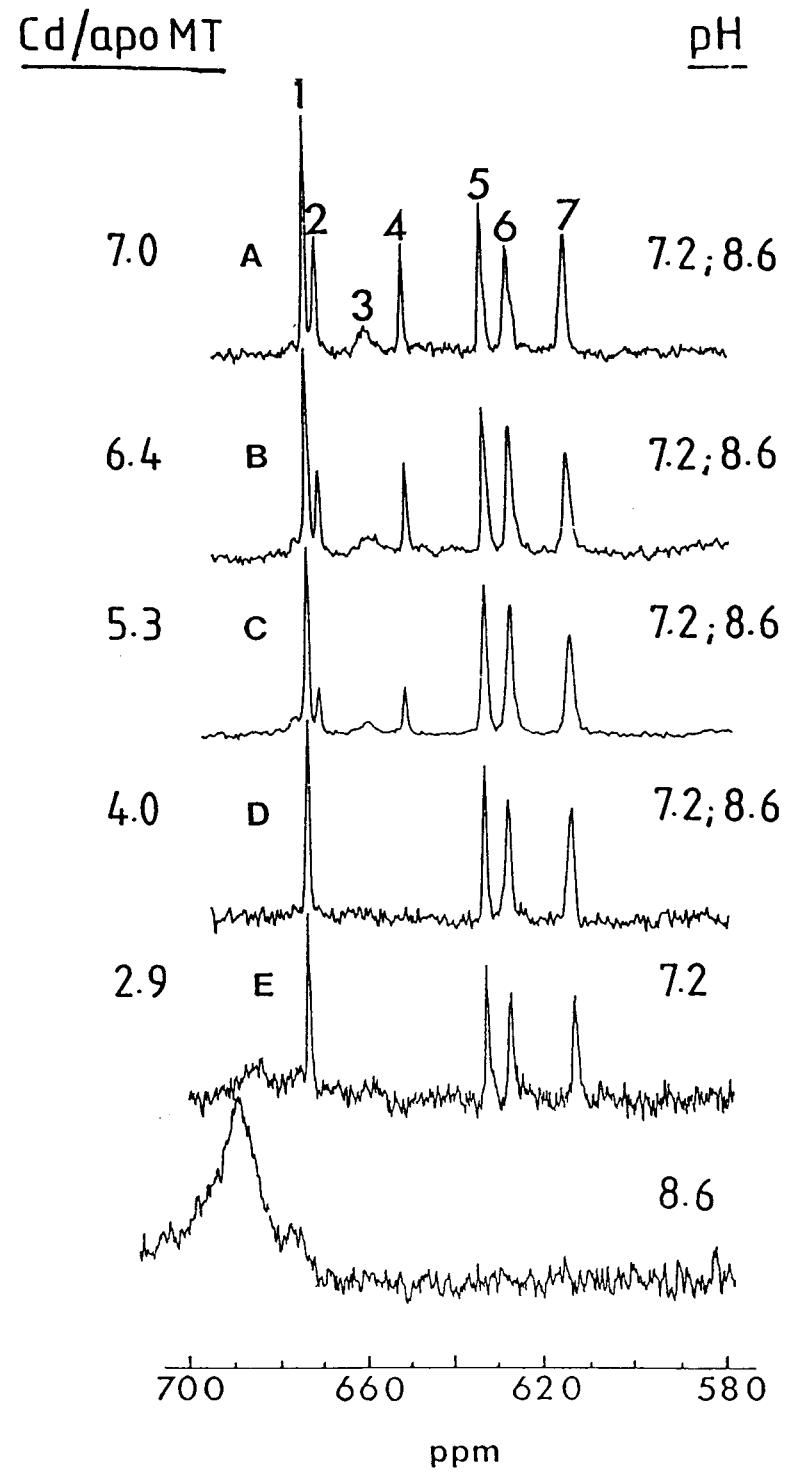

Figure 4. One-dimensional ^{113}Cd NMR (^{1}H-decoupled) spectra of ^{113}Cd-MT-2 from rabbit liver as a function of ^{113}Cd to apoMT-2 ratio obtained at both 7.2 and 8.6 pH values. The spectra A-D were identical at both pH values. The ^{113}Cd signals in (A) are numbered according to the metal positions given in Figure 6 (adapted from Good et al. 1988).

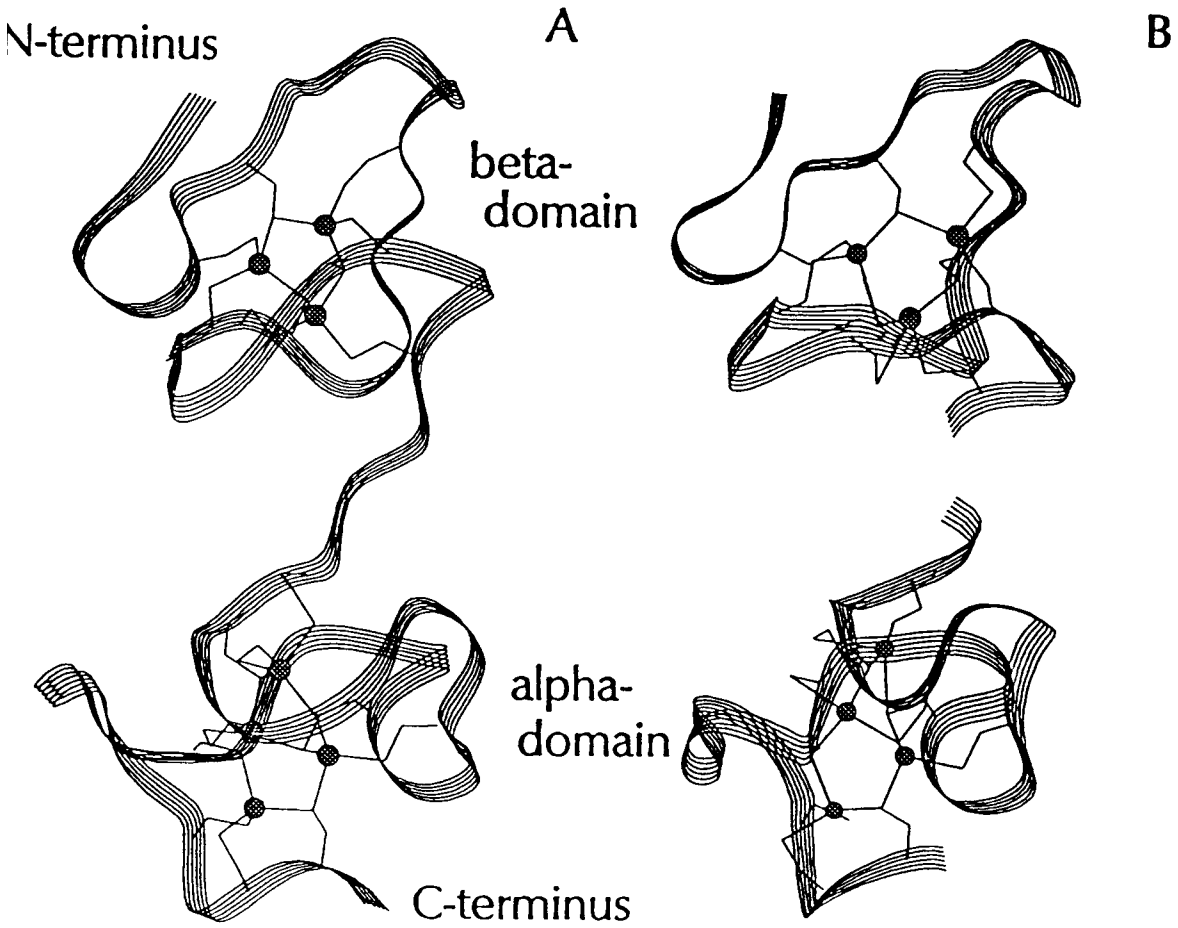

Figure 5. Ribbon drawing of the rat MT three-dimensional structure as determined (A) by X-ray crystallography (Robbins et al. 1991) and (B) by NMR (Schulze et al. 1988) in aqueous solution. Metals are shown as shaded spheres connected to the protein backbone by cysteine thiolate ligands. (Adapted from entries made to Brookhaven Data Bank).

Cluster Formation

The process of cluster formation has been studied for Cd^{II} and Co^{II} MT derivatives (Good et al. 1988; Bertini et al. 1989). In both instances the formation of each cluster appears to proceed in a cooperative manner at neutral pH, whereas, under alkaline conditions (pH 8.6), isolated sites are first formed with up to four metal equivalents after which cluster formation sets in. Thus in the ^{113}Cd titration studies of rabbit apoMT-2 at pH 7.2 and at low metal occupancy (2.9 and 4 ^{113}Cd equivalents) only the four-metal cluster (α-cluster) resonances 1, 5, 6 and 7 were observed (Figure 4, spectra D and E). Further addition of $^{113}Cd^{II}$ ions generated all the resonances of the three-metal cluster (resonances 2, 3 and 4) in succession. In contrast, similar studies at pH 8.6 and with less than four ^{113}Cd equiv (2.9 equiv) gave rise to a broad NMR signal centred at 688 ppm (Figure 4 bottom). This feature is characteristic of a metal exchange between clustered and mononuclear metal centers. When at this pH value exactly four ^{113}Cd equivalents were bound to apoMT the ^{113}Cd NMR spectrum changed to the characteristic spectrum of the α-cluster and further addition of $^{113}Cd^{II}$ ions again led to the successive generation of the resonances of the β-cluster (Figure 4, spectra D–A). These results demonstrated that the formation of metal-thiolate clusters in MT is sequential and cooperative at physiological pH with the C-terminal four-metal cluster being formed first.

This pH-dependent difference in the pathway of cluster formation can be accounted for by the com-

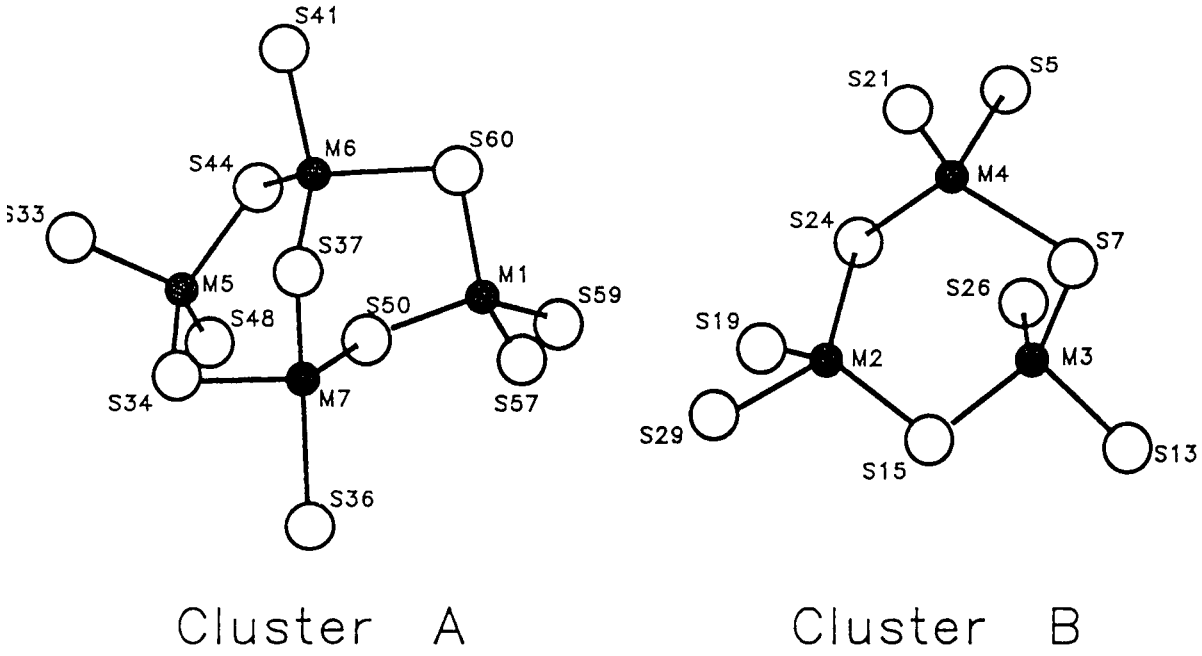

Figure 6. Schematic drawing of the two metal-thiolate clusters in mammalian Cd_7-MT and Zn_2,Cd_5-MT. M denotes the respective divalent metal ions with M2 and M3 in cluster B of Zn_2,Cd_5-MT being occupied by Zn^{II}. S_γ atoms are labeled by the Cys residue number. (Adapted from entries made to Brookhaven Data Bank).

petition between metal ions and protons for the thiolate sulfurs of the cysteine residues. Thus, at neutral pH the binding of substoichiometric amount of metal ions is accompanied by the displacement of cysteine thiol protons. In this case the formation of clusters with bridging cysteines at low metal-to-protein ratios will require fewer protons to be displaced per metal bound than if each metal ion were coordinated by four separate terminal thiolates (Good et al. 1988).

Three-dimensional structure of mammalian Cd_7-metallothioneins

The characteristic feature of all MTs is the occurrence of Cys-Xaa-Cys tripeptide sequences, where Xaa stands for an amino acid residue other than Cys. In all mammalian forms the 20 Cys residues are highly conserved and involved in the binding of seven divalent metal ions (Vašák & Kägi 1994).

The three-dimensional structures of mammalian MT were determined in aqueous solution by two-dimensional NMR spectroscopy using the reconstituted Cd_7-MT from rabbit (Arseniev et al. 1988), rat (Schultze et al. 1988) and human (Messerle et al. 1990) liver, and in crystals by X-ray diffraction of native Zn_2,Cd_5-MT (Robbins et al. 1991) from cadmium overloaded rat liver at 2.0 Å resolution. So far attempts to crystallize metal-homogeneous Cd_7- and Zn_7-MT have been unsuccessful. In the NMR studies the homonuclear ^{1}H COSY of Cd_7-MT with either the NMR inactive ^{112}Cd isotope (I = 0) or NMR active ^{113}Cd (I = 1/2) allowed the identification of all 20 cysteines on the basis of heteronuclear ^{113}Cd-^{1}H couplings. Subsequent heteronuclear ^{113}Cd-^{1}H COSY experiments yielded the sequence specific cysteine-cadmium coordinative bonds. The latter information provided crucial constraints as to the polypeptide fold around the metal clusters in MTs.

Both the NMR structure of Cd_7-MT in solution and the 2.0 Å resolution X-ray structure of Zn_2,Cd_5-MT determined for the same molecular species reveal identical metal-thiolate cluster structures and closely comparable global polypeptide folds (Figure 5) (Braun et al. 1992). The crystal packing reveals intimate association of MT molecules as dimers interdigitated about a 2-fold axis containing trapped ions of crystallization (modelled as two phosphates and two sodium ions). The monomeric species, in both solution and crystal structures, has a dumbbell-like shape with uniformly sized and almost spherical *C*-terminal α-domain and *N*-terminal β-domain each with a diameter of 15–20

Å and containing at their centers the respective four and three metal cluster (Figure 6).

The cluster geometries can best be described as distorted chair (3-metal cluster) and adamantane-like (4-metal cluster) polyhedra with tetrahedral metal centers inaccessible to the solvent. The polypeptide backbone wraps around both clusters forming two large helical turns. In the *N*-terminal alpha-domain the spiral of the peptide fold is left-handed and in the *C*-terminal beta-domain it is right-handed. The two protein domains are connected by a flexible hinge region formed by the conserved Lys-Lys segment (residues 30 and 31) in the middle of the polypeptide chain. The discontinuity in the NMR structure between these two residues is due to the lack of NOE-based information in the NMR data as to the mutual orientation of the two domains (Figure 5B). Consequently, the NMR structure calculations were performed separately for the alpha-domain and the beta-domain. The secondary structure appears to be constrained by metal coordination. In both NMR and X-ray structures the local conformation in seven Cys-Xaa-Cys segments constitute an unusual type of secondary structure, the half turn, so far unique to MTs. Additional secondary structure elements include two 3_{10} helical segments (residues 41–47 and 57–61). Closely similar three-dimensional structures have also been found in solution NMR structures of mammalian MTs from human (Messerle et al. 1990) and rabbit liver (Arseniev et al. 1988).

^{113}Cd NMR in environmental pollution

In the field of environmental pollution the study of toxic metals, in particular Cd, has received a great deal of attention. Apart from its acute and chronic toxicity, long recognized as important in relation to the industrial production and use of this metal, the cumulative properties of the Cd^{II} ion in living organisms provide a special problem. Many studies have been devoted to the involvement of MTs in intracellular sequestration of Cd. It has been shown that MT provides cells with a mechanism to attenuate, at least temporarily, the toxicity of Cd (Templeton & Cherian 1991). Due to the Cd accumulation in MT this protein has been used as a biomarker of Cd toxicity. However, so far ^{113}Cd NMR has not been used to monitor this protein in the environment. Major problems with such a study are the low natural abundance and sensitivity of the ^{113}Cd isotope (see Table 1). However, the observation of the ^{113}Cd resonances of MT is conceivable under conditions where large protein amounts (5–10 mM concentrations) are available and/or wide-bore NMR magnets are employed.

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