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Preparation and ^{113}Cd NMR Studies of Homogeneous Reconstituted Metallothionein: Reaffirmation of the Two-Cluster Arrangement of Metals†

James D. Otvos,* Helen R. Engeseth, and Suzanne Wehrli

Department of Chemistry, University of Wisconsin—Milwaukee, Milwaukee, Wisconsin 53201

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ABSTRACT: ^{113}Cd NMR analysis of rabbit liver metallothionein 2 reconstituted with ^{113}Cd at all seven binding sites has previously indicated that the metals are arranged in two metal–thiolate clusters [Otvos, J. D., & Armitage, I. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7094–7098]. Spectra of the protein always contained more than seven resonances, however, suggesting the samples were in some way heterogeneous. Results of a recent study of ^{113}Cd metallothionein reconstituted in a different manner but also giving spectra with more than seven resonances have been interpreted as arguing against the two-cluster model of metal binding and in favor of a model in which structural flexibility of the protein allows many configurational substates of the cluster(s) to coexist [Vasak, M., Hawkes, G. E., Nicholson, J. K., & Sadler, P. J. (1985) *Biochemistry 24*, 740–747]. Data are presented here that indicate that dimers and larger oligomers of metallothionein formed as byproducts of metal reconstitution are the likely source of at least some of the ^{113}Cd resonances attributed by these workers to configurational substrates. Removal of the contaminating oligomers by gel filtration yields a verifiably homogeneous protein whose ^{113}Cd spectrum consists of seven resonances of comparable intensity. Unambiguous confirmation of the existence and structures of the two previously proposed metal–thiolate clusters was obtained by two-dimensional chemical shift correlation spectroscopy and spectral simulation of the ^{113}Cd–^{113}Cd splitting patterns of the individual resonances.

Metallothioneins (MTs)[1] comprise a unique family of low molecular weight, cysteine-rich metal binding proteins thought to be centrally involved in resistance to heavy-metal toxicity and the homeostasis of zinc and copper (Nordberg & Kojima, 1979; Webb & Cain, 1982; Brady, 1982). In keeping with these postulated functions, MT has the capacity to bind an unusually large number of metals (e.g., 7 mol of Cd or Zn/mol of protein), and its synthesis is closely regulated by many of the metals with which it interacts (Nordberg & Kojima, 1979; Anderson et al., 1979; Bremner & Young, 1976).

A detailed knowledge of the mode(s) of binding of different metals to MT is generally considered to be a prerequisite for advancing significantly beyond our current incomplete understanding of the physiological functions of the protein. Since an X-ray crystal structure is still unavailable, it has been necessary to rely entirely on chemical and spectroscopic evidence, of which a great deal has been accumulated (Nordberg & Kojima, 1979; Otvos & Armitage, 1982; Vasak & Kägi, 1983). Chemical analyses of mammalian MTs have indicated the existence of two major isoprotein forms (MT-1 and MT-2) that each consist of 61 amino acid residues, 20 of which are cysteines occupying strictly conserved positions in the sequence (Kägi et al., 1980). The thiolate sulfur atoms of these cysteine residues all participate in coordination of the seven bound Cd and/or Zn ions (Kägi & Vallee, 1961). Most spectroscopic studies using, among others, techniques such as electronic absorption, CD, MCD, EPR, and EXAFS have been able to provide only a general picture of the coordination environments

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[1] Abbreviations: MT, metallothionein; Cd_7-MT and Zn_7-MT, metallothionein in which all seven binding sites are occupied by Cd or Zn, respectively; Cd,Zn-MT, metallothionein whose seven binding sites are occupied by a mixture of Cd and Zn; MT-1 and MT-2, the two major isoprotein forms of metallothionein; CD, circular dichroism; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; HPLC, high-pressure liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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of the metals in MT because of their limited abilities to resolve spectral contributions from the individual metals. Nonetheless, they have firmly established that every metal in the protein has a tetrathiolate coordination sphere of roughly tetrahedral geometry (Vasak & Kägi, 1983).

By far the most detailed description of the mode of metal binding to MT has been furnished by ^{113}Cd NMR (Otvos & Armitage, 1982). Cd chemical shifts are very sensitive to subtle differences in coordination environment, and as a result, separate resonances are resolved from every Cd ion in the protein. The earliest spectra of ^{113}Cd-enriched rabbit liver MT produced by ^{113}Cd induction exhibited resonances that were split into multiplets by ^{113}Cd–^{113}Cd scalar coupling, which provided the first evidence that metals are linked to one another in the protein by bridging thiolate ligands (Otvos & Armitage, 1979). Further analysis of these spectra by selective ^{113}Cd decoupling to establish the number and nature of the metal–thiolate clusters was hindered by a property of all mammalian MTs induced by Cd administration, namely, that the isolated protein is heterogeneous in its metal composition, containing in addition to Cd approximately 2 equiv of Zn distributed nonuniformly among the seven binding sites (Otvos & Armitage, 1982). These Zn ions not only interrupt the spin coupling networks that would otherwise exist if every site were occupied by a spin $^1/_2$ ^{113}Cd ion but also perturb the chemical shifts of Cd ions in neighboring sites, leading to spectra containing many more than the seven resonances expected for a homogeneous Cd_7-MT. To overcome these complications, the native Zn was replaced with ^{113}Cd in vitro prior to protein purification by adding excess $^{113}CdCl_2$ to the homogenates of livers from ^{113}Cd-exposed rabbits (Otvos & Armitage, 1980). Spectra of the resultant ^{113}Cd-MT were considerably simpler than those of ^{113}Cd,Zn-MT, but contrary to expectation still contained more than seven resonances. The conclusion was that the protein, despite now being metal homogeneous, was in some other respect heterogeneous, possibly as the result of partial metal ion deficiency or the presence of two or more protein species with limited microheterogeneity of sequence (Otvos & Armitage, 1980; Boulanger & Armitage, 1982). Although the extra resonances complicated the analysis of ^{113}Cd decoupling experiments, it was nevertheless possible to conclude that the protein contains two separate metal–thiolate clusters containing four and three metals, respectively (Otvos & Armitage, 1980). Subsequent studies of human liver MT (Boulanger & Armitage, 1982) and calf liver MT (Briggs & Armitage, 1982) have confirmed this arrangement, and it appears that the cysteine ligands to the metals in each cluster occupy contiguous positions in the primary structure (Boulanger et al., 1982; Winge & Miklossy, 1982).

Recently, ^{113}Cd NMR studies have been reported of rabbit liver $^{113}Cd_7$-MT-2 prepared by two new reconstitution methods (Vasak et al., 1985). The protein produced by both methods was analyzed by HPLC to consist of a single isoprotein and contained the full complement of seven metals, yet failed to give spectra composed of seven ^{113}Cd resonances of comparable intensity. Instead, more than seven signals were observed, many with subintegral intensities, and *all* of these were claimed on the basis of two-dimensional *J*-resolved spectra to be composed of envelopes of several (three to seven) overlapping resonances. The conclusion drawn from these data was that metallothionein does not possess a rigid structure containing seven metals bound in two distinct clusters but rather has considerable structural flexibility that permits the existence of multiple configurational substates whose relative populations are controlled by conditions such as temperature and ionic strength. If true, the authors suggest that this more complex picture of metal binding to MT would need to be taken into account when assessing the relevance of any future X-ray crystallographic structure of what might only be a minor configurational substate of the protein in solution.

In this paper, we present evidence that dimers and larger oligomers of MT are formed as byproducts of the production of Cd_7-MT by in vitro reconstitution. These species are shown to be responsible for at least some of the extra ^{113}Cd resonances that were attributed by Vasak et al. (1985) to configurational substates of the protein. Careful chromatographic purification of reconstituted $^{113}Cd_7$-MT-2 gives a monomeric protein whose ^{113}Cd NMR spectrum consists of seven resonances of comparable intensity. Unequivocal confirmation of the existence and structures of the previously proposed four- and three-metal clusters is provided by spectral simulation of the complex multiplet splittings of the resonances and by two-dimensional ^{113}Cd chemical shift correlation spectroscopy.

Materials and Methods

Protein Preparations. Zn_7-MT-2 was isolated from the livers of rabbits which had been subjected to daily injections of 0.15 M $ZnSO_4$ for 6 days to give a total dose of 0.32 mmol of Zn per kilogram body weight. The animals were sacrificed 6 h after the final injection and the livers stored at −40 °C. Protein was purified as previously described (Otvos & Armitage, 1980) except that a 5–125 mM gradient of Tris-HCl, pH 8.6, was used in the DEAE-cellulose chromatography step to separate MT-2 from MT-1. $^{113}Cd_7$-MT-2 was prepared by direct displacement of Zn^{2+} from pure Zn_7-MT-2 by addition of a stoichiometric amount of $^{113}CdCl_2$ (95 atom % from Prochem Isotopes). Several steps were taken to minimize the time-dependent oligomerization of the protein that we have found takes place when MT is allowed to incubate in the presence of excess Zn^{2+} or Cd^{2+}. First, instead of adding the entire complement of $^{113}Cd^{2+}$ at once, several sequential additions of 1–2 molar equiv each were made from a 0.5 M stock solution of $^{113}CdCl_2$ to a solution of Zn-MT-2 (0.5–2 mM) in 50 mM Tris-HCl, pH 8. After each Cd addition, Chelex 100 resin (Bio-Rad Corp.) was immediately added to remove the displaced Zn, followed by its removal (within 1 min) by filtration. The resultant protein was concentrated for NMR by Amicon ultrafiltration using a UM-2 membrane and analyzed for metal composition by atomic absorption spectroscopy. Protein concentration was determined by the absorbance at 220 nm in 0.01 M HCl using a molar absorptivity of 47 300 M^{-1} cm^{-1} (Bühler & Kägi, 1979). All protein designated as Cd_7-MT in these studies was analyzed as having a metal to protein ratio of 7 ± 0.5.

The extent of any protein oligomerization produced during the preparation of Cd_7-MT was assessed on an analytical scale by gel filtration HPLC or Sephadex G-75 chromatography. When necessary, monomeric protein was separated from dimers and higher oligomers on a preparative scale by applying 2 mL of protein solution to a 95 × 1.5 cm column of Sephadex G-75 in 10 mM Tris-HCl, pH 8, and eluting with the same buffer at 25 °C with a flow rate not exceeding 0.5 mL/min.

NMR Methods. ^{113}Cd NMR spectra were recorded at 55.5 MHz on a Bruker WM-250 spectrometer using continuous broad-band proton decoupling. Protein samples of about 1.8 mL were contained in 10-mm tubes in 50 mM Tris-HCl, pH 8, buffer containing 10% D_2O to provide the field-frequency lock. Spectra were accumulated by using a 70° pulse width and a 1-s pulse repetition rate. Since the T_1 values of the seven resonances of Cd_7-MT-2 are not equal, ranging from 0.22 s for resonance VII to 1.27 s for resonance VI (Nettesheim et

al., 1985), spectra acquired under these conditions were not "fully relaxed". Chemical shifts are reported in parts per million downfield from the ^{113}Cd resonance of 0.1 M $Cd(ClO_4)_2$. Multiplet structures of selected ^{113}Cd resonances were simulated by using the Bruker PANIC subroutine. ^{113}Cd homonuclear chemical shift correlated (COSY) spectra were acquired by using a $90°-\tau-60°$ pulse sequence with proton decoupling only during acquisition. The evolution period τ was varied from 0 to 29 ms in 128 0.227-ms steps to give a spectral width in the second dimension of 4400 Hz. A total of 1704 transients were accumulated for each value of τ.

Results and Discussion

Homogeneous MT containing only Cd in its seven binding sites is a species that offers many advantages over mixed-metal MTs in studies of the protein's chemistry and structure (Otvos & Armitage, 1980; Vasak & Kägi, 1983). However, Cd_7-MT is not produced in mammals by Cd induction and must therefore be prepared by some type of reconstitution procedure. Two general methods have been used. In one, the apoprotein is produced by removal of the native metals by acid treatment, and Cd is then introduced anaerobically as the pH is readjusted to neutrality (Nielson & Winge, 1983; Vasak et al., 1985). In the other, the Zn in native Cd,Zn-MT is stoichiometrically replaced by Cd added either to the purified protein (Vasak et al., 1985) or to the crude protein in liver homogenates (Otvos & Armitage, 1980). Although both methods produce MT containing close to the expected 7 molar equiv of Cd and 20 equiv of thiol, it is important to prove whether the metals in the reconstituted protein occupy sites identical with, not just similar to, those in native MT. The best spectroscopic method available to address this question is ^{113}Cd NMR, both because Cd chemical shifts are sensitive to very minor differences in coordination environment and because spectra of native ^{113}Cd,Zn-MT are available for comparison with spectra of reconstituted protein (Otvos & Armitage, 1982). ^{113}Cd spectra of Cd_7-MT generated by the above procedures have not, in fact, been found to be identical, which indicates that not all methods of reconstitution are equally "accurate". Moreover, there is a question whether any of the methods give accurate reconstitution since all of the reported spectra contain more than the seven resonances expected of a protein containing seven discrete metal binding sites[2] (Otvos & Armitage, 1980; Vasak et al., 1985). A very different interpretation of these data, advanced by Vasak et al. (1985), is that the extra ^{113}Cd resonances are indicative not of imperfectly reconstituted protein but of binding sites that are flexible in nature, thereby allowing multiple configurational substates of comparable energy to coexist.

In the course of recent studies of the metal exchange chemistry of Zn-MT (Nettesheim et al., 1985), we discovered that protein oligomers can form during Cd reconstitution if the protein is allowed to incubate in the presence of excess Cd^{2+} or Zn^{2+}. The nature of the reaction responsible for oligomer formation is not yet known, though its partial reversibility by thiol reagents suggests an oxidative mechanism. The rate of oligomerization is dependent on the concentrations of protein and free metal ion. At millimolar concentrations of both, high molecular weight insoluble species form within an hour and precipitate from solution. If Chelex resin is used to rapidly remove excess metal from the protein solution (see Materials and Methods), oligomer formation can be kept to a minimum.

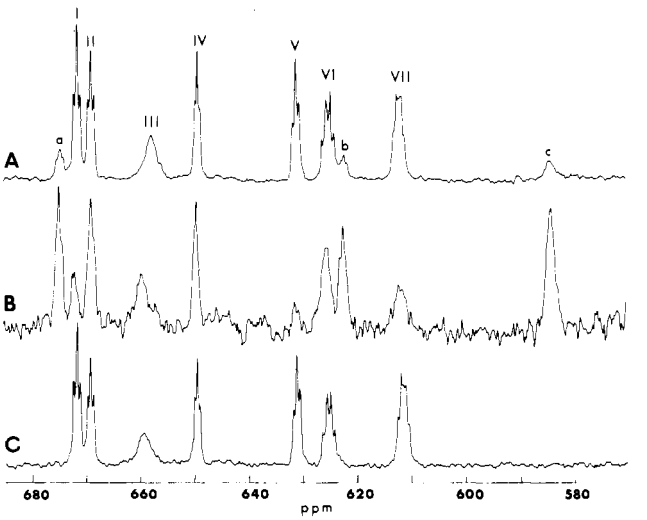

FIGURE 1: (A) ^{113}Cd NMR spectrum at 26 °C of 8 mM $^{113}Cd_7$-MT-2. The spectra in (B) and (C) are of the pooled and concentrated fractions 45–60 (dimer) and 61–75 (monomer), respectively, from the gel filtration chromatographic separation of this protein shown in Figure 2.

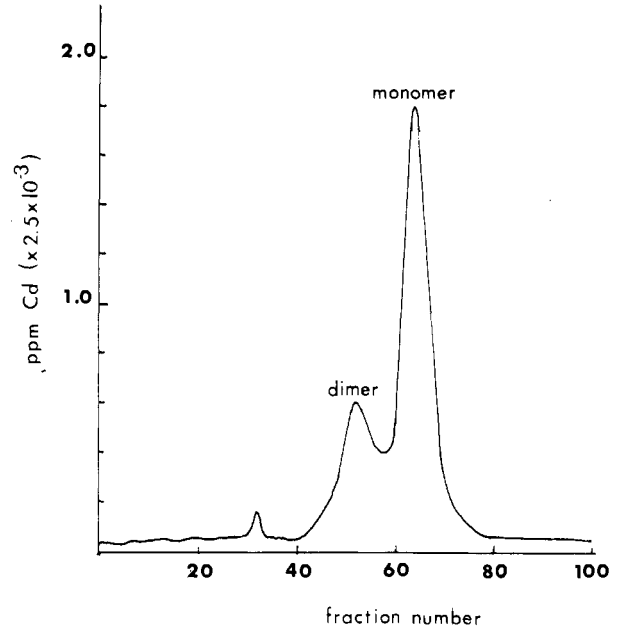

FIGURE 2: Sephadex G-75 (95 × 1.5 cm) chromatographic profile of the $^{113}Cd_7$-MT-2 sample whose ^{113}Cd spectrum appears in Figure 1A. 1.4-mL fractions were collected.

The spectra in Figure 1 provide evidence that oligomers of Cd_7-MT exhibit ^{113}Cd NMR properties that are distinguishable from those of authentic monomeric protein and are the likely origin of the extra resonances that Vasak et al. (1985) attributed to configurational substates of MT. Shown in Figure 1A is a spectrum that is typical of those obtained of $^{113}Cd_7$-MT-2 produced by in vitro reconstitution of Zn_7-MT-2. In addition to the seven prominent resonances labeled I–VII, there appear three smaller peaks labeled a, b, and c at 673.9, 622.5, and 584.0 ppm, respectively. Minor resonances at the same positions as peaks a and b were also observed by Vasak et al. (1985) in spectra of Cd_7-MT-2 reconstituted from apothionein or native Cd,Zn-MT-2.[3] The heterogeneous

[2] The one exception is a spectrum of rabbit liver ^{113}Cd-MT-2 prepared by acid reconstitution which contains only seven resonances, though of unequal intensity (Delgarno & Armitage, 1984).

[3] The spectral region examined by Vasak et al. (1985) did not extend far enough upfield to allow detection of a resonance at the position of peak c.

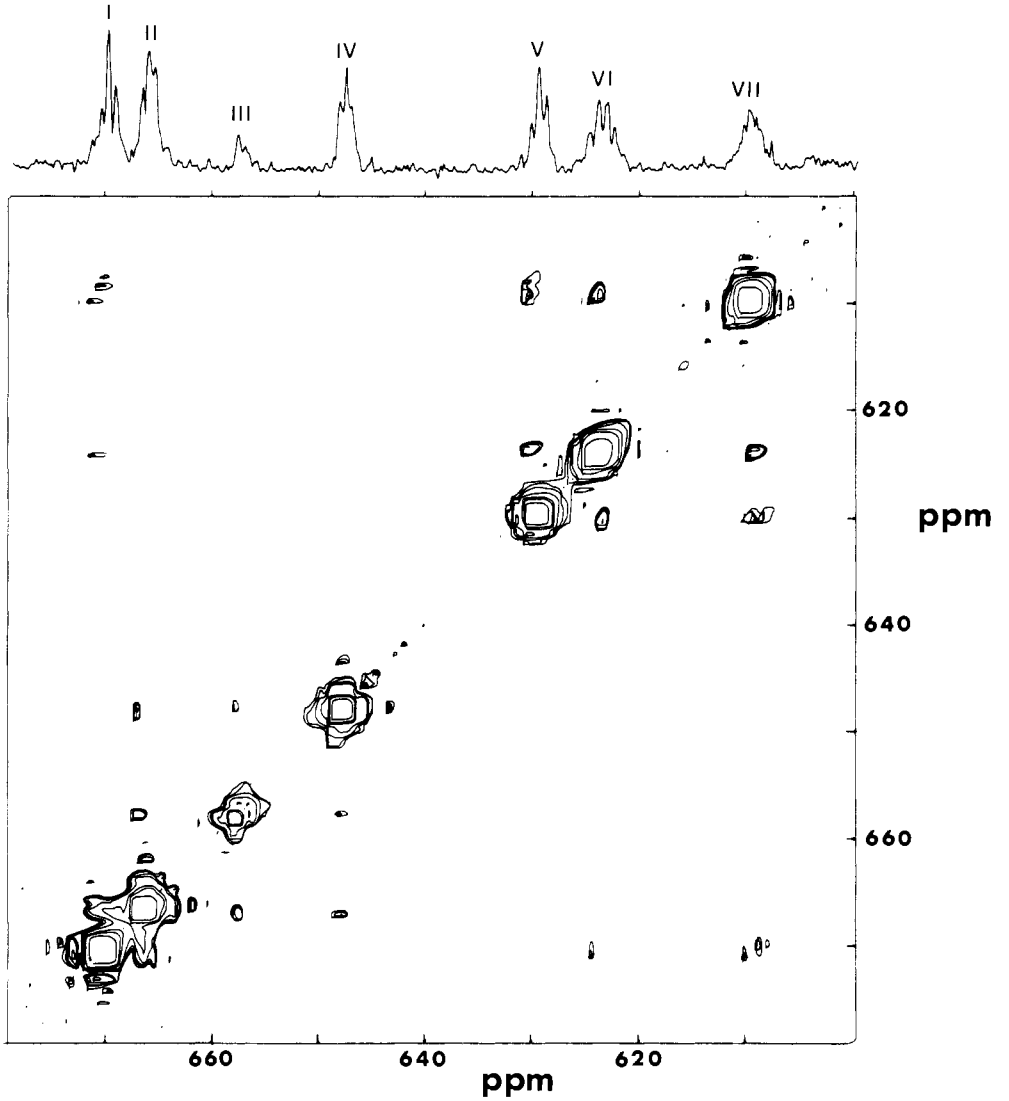

FIGURE 3: ^{113}Cd chemical shift correlation (COSY) spectrum of 4.5 mM monomeric $^{113}Cd_7$-MT-2 at 35 °C obtained as described under Materials and Methods. The spectrum at the top is the absolute value mode projection onto one axis of the two-dimensional data set.

nature of the sample giving the spectrum in Figure 1A is clearly demonstrated by its gel chromatographic profile in Figure 2. In addition to a small amount of protein polymer eluted in the void volume (fractions 30–33) there is a significant amount of protein (ca. 20%) which elutes at a volume characteristic of material with approximately twice the molecular weight of monomeric MT.

^{113}Cd spectra of the pooled and concentrated fractions corresponding to the dimeric and monomeric species are clearly different, as shown in spectra B and C, respectively, of Figure 1. The monomer spectrum completely lacks the three minor resonances and contains only the seven signals previously attributed to ^{113}Cd in the three-metal cluster (peaks II–IV) and four-metal cluster (peaks I and V–VII) (Otvos & Armitage, 1980). Peaks a, b, and c are easily assigned to the dimer since three prominent resonances at the same chemical shifts appear in the spectrum in Figure 1B. Three other signals of comparable intensity in the dimer spectrum appear at the positions of resonances II–IV in the monomer spectrum, which identifies them as arising from Cd at three-metal cluster sites whose environments are apparently indistinguishable in the two species. The smaller resonances in Figure 1B at 671.8, 631.0, and 611.7 ppm have shifts that are identical with resonances I, V, and VII of the monomer and originate from the residual amount of this species present in the dimer sample resulting from their incomplete separation on the Sephadex G-75 column. The intensity of the remaining peak in Figure 1B at 625.2 ppm is too great to be accounted for by the amount of contaminating monomer in the sample and is therefore attributed to superimposed peaks from monomer (resonance VI) and dimer.

A detailed understanding of the structural basis of MT oligomerization must await more extensive analysis of spectra of the dimer and larger oligomers that are more highly purified. It is clear, however, that such species are created as byproducts of several different methods of metal reconstitution and can greatly complicate structural interpretation of ^{113}Cd NMR spectra if their presence is unrecognized. In this regard, we note that large polymers of $^{113}Cd_7$-MT (M_r >100000) have been found to give spectra exhibiting three-metal cluster resonances whose intensities are greatly attenuated (for unknown reasons) compared to those of the four-metal cluster. This observation should be taken into account when assessing any spectrum containing resonances of subintegral intensity in terms of relative binding site occupancy or structural heterogeneity of the metal clusters.

With the availability for the first time of verifiably homogeneous, fully reconstituted $^{113}Cd_7$-MT comes the opportunity to unequivocally confirm the organization of the seven metal binding sites by an analysis of the through-bond spin coupling

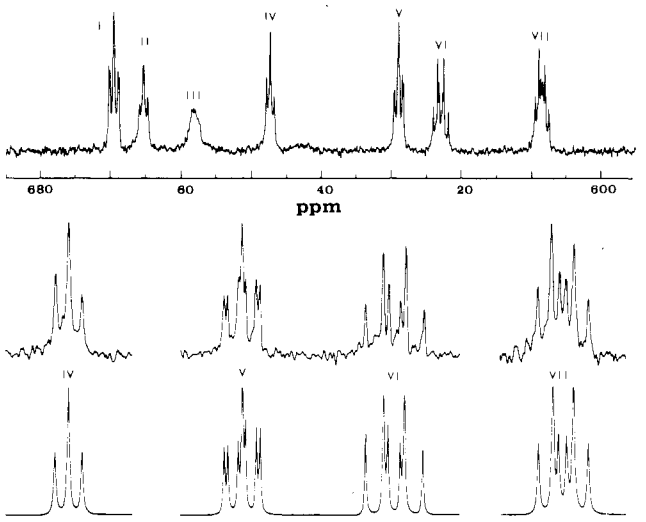

FIGURE 4: (Top) ^{113}Cd NMR spectrum at 44 °C of 7 mM monomeric $^{113}Cd_7$-MT-2. (Middle) Expanded plots of resonances IV–VII from the top spectrum after multiplication of the free induction decay with a Gaussian function to enhance resolution. (Bottom) Spectral simulations of the multiplet structures of resonances IV–VII using the spin coupling constants and coupling connectivities shown in Figure 5A.

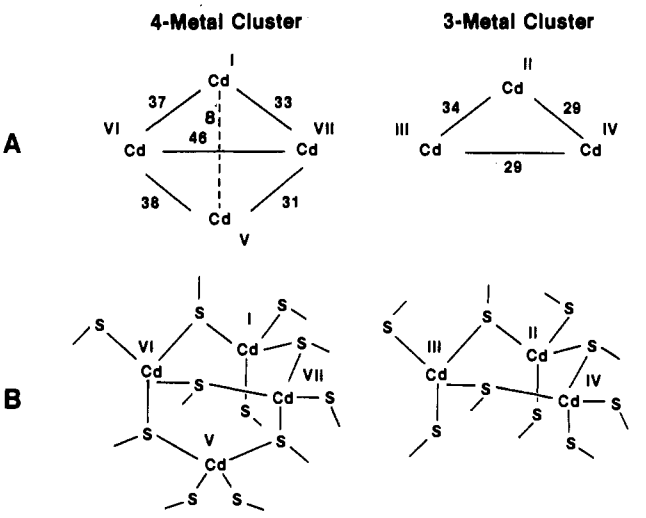

FIGURE 5: (A) Schematic representations of the metal clusters in Cd_7-MT-2 showing the ^{113}Cd–^{113}Cd two-bond (solid lines) and four-bond (dashed line) scalar coupling interactions between metals. The roman numeral beside each Cd refers to the corresponding resonance in the spectra in Figures 1, 3, and 4, and the numbers on each line are the observed ^{113}Cd–^{113}Cd coupling constants. (B) Postulated structures for the metal–thiolate clusters based on previous ^{113}Cd NMR analyses (Otvos & Armitage, 1980) and confirmed by the data in Figures 3 and 4.

connectivities between ^{113}Cd ions. This is most simply accomplished by homonuclear chemical shift correlation spectroscopy (COSY) as shown in Figure 3. The group of cross-peaks between resonances II, III, and IV clearly confirms their previous assignment (Otvos & Armitage, 1980) to occupants of an independent three-metal cluster in which every Cd ion is linked to the other two by a bridging thiolate ligand (Figure 5B). Similarly, the cross-peaks between the other resonances validate the structure of the four-metal cluster represented in Figure 5B. It is noted in particular that no cross-peak is seen between resonances I and V, indicating the absence of the two-bond interaction between these metals that is predicted by the adamantane-like model of the cluster proposed by Vasak & Kägi (1983).

The strongest argument presented to support the contention that metallothionein does not contain two discrete metal–thiolate clusters but instead can accommodate a large number of configurational substates owing to its structural flexibility was the purported composite nature of all ^{113}Cd resonances in the spectrum of rabbit liver $^{113}Cd_7$-MT-2 (Vasak et al., 1985). This conclusion was based on the authors' observation of very poorly resolved multiplet structures and two-dimensional, *J*-resolved spectra that gave more than the anticipated number of peaks. As shown in Figure 4, we experience no such difficulty resolving the complex, first-order ^{113}Cd–^{113}Cd splitting patterns of every resonance[4] and are able to perfectly match their multiplet structures by a spectral simulation calculation using the coupling constants and spin connectivities presented in Figure 5A. No stronger proof than this can be offered to corroborate the existence of discrete four- and three-metal clusters in MT since any other model would fail to give the observed agreement between experimental and theoretical spectra. It must therefore be concluded that the complex nature of the spectra obtained by Vasak and coworkers was due not to an underlying structural complexity of the metal binding sites but rather to undetected heterogeneity in their protein preparations.

[4] The one exception is resonance III, whose much greater line width is due to chemical exchange broadening (Nettesheim et al., 1985).

Registry No. ^{113}Cd, 14336-66-4.

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Calcium-Induced Increase in the Radius of Gyration and Maximum Dimension of Calmodulin Measured by Small-Angle X-ray Scattering†

B. A. Seaton,*,‡,§ J. F. Head,‖ D. M. Engelman,§ and F. M. Richards§

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511, and Department of Physiology, Boston University School of Medicine, Boston, Massachusetts 02118

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ABSTRACT: We have used solution small-angle X-ray scattering to characterize bovine brain calmodulin in the presence and absence of calcium. In the presence of calcium, calmodulin exists in solution as an elongated molecule with a radius of gyration of 21.5 Å and a maximum vector length of approximately 62 Å. These values are consistent with the dimensions recently determined for the crystal form of rat testis calmodulin. In the absence of calcium, the calmodulin molecule is shorter, the radius of gyration decreases to 20.6 Å, and the maximum vector length decreases to approximately 58 Å. This change in dimensions is consistent with an overall contraction of the protein through movement of the two lobes closer to each other upon removal of calcium from calmodulin.

Small-angle X-ray scattering (SAXS)[1] from macromolecules is used to measure shape-related parameters of individual molecules in free solution. As a low-resolution technique, solution scattering is insensitive to fine structural features but can accurately provide details related to size and shape. For biological macromolecules such as proteins, where interactions with substrates or regulators can change the shape, X-ray scattering provides a useful means of measuring such effects [e.g., see McDonald et al. (1979) and Pickover et al. (1979)]. If the crystal structure of a protein is known, this technique can also help determine whether the molecule exists in solution in a similar conformation to that found in the crystal. In the present study, we have used SAXS to characterize bovine brain calmodulin in solution. Calmodulin is a ubiquitous, multifunctional intracellular calcium receptor protein that belongs to a family of homologous, low molecular weight proteins which includes troponin C, parvalbumin, S-100 protein, and vitamin D dependent intestinal calcium binding protein. A three-dimensional structure has been recently determined, by X-ray crystallography, for calmodulin in which all four calcium binding sites are occupied by calcium (Babu et al., 1985). This structure shows a long, dumbbell-shaped molecule in which the calcium binding sites are arranged in two pairs, well separated by a single, long central α helix. In overall appearance, the tertiary structure is similar to that observed in the crystal structure of troponin C (Sundaralingam et al., 1985; Herzberg & James, 1985).

As with many other members of this family of proteins, calmodulin undergoes a conformational change when it binds calcium [for a review, see Klee & Vanaman (1982)]. Its α-helix content increases, and a new hydrophobic surface appears which has been implicated in the calcium-dependent interactions of calmodulin with its target proteins, peptides, and phenothiazine drugs. In addition, proteolytic fragmentation, chemical modification, and electrophoretic migration patterns change considerably when calmodulin binds calcium. Some of the structural changes are probably localized near the sites of calcium binding and may be confined to each lobe of the molecule. The magnitude of many of these changes, involving rearrangements of parts of the polypeptide chain or of individual groups of atoms, would preclude SAXS detection. However, as noted by Babu et al. (1985) and Sundaralingam & Rao (1985), the additional possibility exists, in both calmodulin and troponin C, that movement of the two lobes relative to each other occurs on binding calcium. SAXS has proven extremely useful in detecting changes on this level.

Here we describe studies using small-angle X-ray scattering to measure the size and shape parameters of calmodulin in solution and to determine how the binding of calcium to the molecule changes these parameters.

Experimental Procedures

Materials. Calmodulin was prepared from bovine brain by the method of Masure et al. (1984). Calmodulin-dependent phosphodiesterase was prepared from bovine brain as described by Head et al. (1979). Hen egg-white lysozyme (grade I), EGTA, and MOPS were obtained from Sigma.

Sample Preparation. Calmodulin with calcium was prepared by dialyzing protein for at least 24 h at 4 °C against 5 mM $CaCl_2$, 100 mM KCl, and 50 mM MOPS–KOH, pH 7.4, using washed and rinsed Spectrapor 1 dialysis tubing (molecular weight cutoff = 8000).

Calmodulin free of calcium was prepared by exhaustively dialyzing protein against 300–500 volumes of 5 mM EGTA,

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* Author to whom correspondence should be addressed.
‡ Present address: Department of Chemistry, Harvard University, Cambridge, MA 02138.
§ Yale University.
‖ Boston University School of Medicine.

[1] Abbreviations: SAXS, small-angle X-ray scattering; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate.

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