^{113}Cd NMR Studies of Reconstituted Seven-Cadmium Metallothionein: Evidence for Structural Flexibility†

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ABSTRACT: A reproducible method for the reconstitution of rabbit liver metallothionein (MT) containing seven cadmium atoms per mole of protein is described. This protein was studied in detail by ^{113}Cd NMR at 88-, 55-, and 44-MHz frequencies, including the effects of pH, temperature, and ionic strength on the spectra. Our results differ significantly from previous reports of ^{113}Cd NMR on similar samples. Thus, the spectra of both chromatographically distinguishable isoforms MT_1 and MT_2 were not identical, and neither could be interpreted in terms of a unique static model with the seven Cd ions forming two independent clusters of four and three Cd ions. Large differential shifts of ^{113}Cd resonances were observed with changes in temperature over the range 277–320 K and ionic strength (0.02–0.5 M). At low temperature a slow structural change (half-life of several minutes) was detected. The structure was more rigid at high ionic strength. The frequency dependence and two-dimensional *J*-resolved spectra revealed that ^{113}Cd resonances were composed of several overlapping peaks, complicating the interpretation of fine structure in one-dimensional spectra. A new flexible model of the Cd cluster in metallothionein is proposed. This model incorporates dynamic thiolate exchange reactions and involves several configurational substates of the protein. The possible relationship of such flexibility to the function of metallothionein is discussed.

Metallothionein (MT)[1] is an intriguing, low molecular weight (M_r 6000–7000), metal- and sulfur-rich protein that appears to play a fundamental role in the metabolism of the essential metals zinc and copper, toxic metals such as cadmium and mercury, and drugs containing gold (Nordberg & Kojima, 1979; Kojima & Kägi, 1978; Mogilnicka & Webb, 1981; Ryden & Deutsch, 1978). Knowledge of the mode of binding of metal ions to this protein is of considerable interest and is essential to any interpretation of its functions. Suggestions for these have included metal detoxification and storage (Kojima & Kägi, 1978) and the insertion of metals into apoproteins (Udom & Brady, 1980).

Metallothionein was first isolated from equine renal cortex by Margoshes & Vallee (1957) nearly 30 years ago. Since then, similar proteins have been isolated from kidney, liver, and intestines of a variety of animal species (Nordberg & Kojima, 1979), from fungi (Prinz & Weser, 1975; Lerch, 1980), from plants (Rauser & Curvetto, 1980), and recently also from metal-resistant bacteria (Higham et al., 1983, 1984). The best characterized mammalian forms to date contain a single polypeptide chain with 20 cysteinyl residues among a total of 61 amino acids and seven cadmium and/or zinc ions per mole of protein (Kojima et al., 1976). Another characteristic feature of metallothionein is the apparent absence of aromatic amino acids. Mammalian metallothioneins contain three Cys-X-Y-Cys sequences (where X and Y represent amino acids other than Cys) and seven Cys-X-Cys sequences (Kojima et al., 1976). The former are known to be involved in metal binding sites in other proteins, e.g., iron in ferredoxins (Yasunobu & Tanaka, 1973) and zinc in the structural site of horse liver alcohol dehydrogenase (Eklund et al., 1974), whereas the abundance of the latter appears to be unique to metallothionein. All 20 cysteinyl residues participate in metal binding via mercaptide linkages (Kägi & Vallee, 1961; Weser et al., 1973).

One of the most informative probes of metallothionein metal binding sites is ^{113}Cd NMR[1] (Otvos & Armitage, 1982). On

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[1] Abbreviations: CD, circular dichroism; COSY, chemical shift correlated spectroscopy; CSA, chemical shift anisotropy; 1D, one-dimensional; 2D, two-dimensional; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; HPLC, high-performance liquid chromatography; MCD, magnetic circular dichroism; MES, 2-(*N*-morpholino)ethanesulfonic acid; MT_1 and MT_2, two chromatographically distinguishable isoforms of metallothionein; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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this basis we were able to propose a model for Cd,Zn-MT in which metal was bound in near-tetrahedral sites with extensive thiolate bridging between metals (Sadler et al., 1978). Such bridging was subsequently verified by Otvos & Armitage (1980) by homonuclear decoupling experiments. Their decoupling connectivities for MT's in which Zn was replaced by ^{113}Cd in vitro suggested the presence of two distinct cadmium thiolate clusters containing three and four cadmium ions.

Independent support for metal cluster formation and the presence of primary and secondary tetrahedral metal binding sites was provided by EPR[1] and magnetic susceptibility measurements on Co(II)-MT (Vašák & Kägi, 1981). Perturbed angular correlation γ-ray spectroscopy using a short-lived isotope of ^{111}Cd recently also revealed the existence of two populations of metal sites with differently distorted tetrahedral environments (Vašák & Bauer, 1982), and EXAFS[1] studies have confirmed that Zn is coordinated to four sulfurs in a zinc metallothionein (Garner et al., 1982).

The protein samples used previously in ^{113}Cd NMR studies of ^{113}Cd-reconstituted rabbit liver metallothionein have been reported to have possible heterogeneity of primary structure (isoproteins) and deficiency of metal content (Otvos & Armitage, 1980). In an attempt to avoid most of these problems we have devised a more reproducible method of preparing fully reconstituted ^{113}Cd rabbit liver metallothionein from highly purified protein and have studied it in detail by ^{113}Cd NMR. Our results differ significantly in certain respects from those previously obtained. It is suggested that metallothionein should not be viewed as a rigid structure containing seven unique binding sites and that considerable structural flexibility is present that allows the existence of several configurational substates of comparable energy. This may bear an important relationship to its function.

Experimental Procedures

Chemicals. All inorganic chemicals were of reagent grade or better. For removal of traces of metal contaminants, buffer solutions were extracted with dithizone in CCl_4 or passed over a column of Chelex 100 (Bio-Rad Corp.).

Protein Isolation. Rabbit liver MT_2[1] was isolated from rabbits injected subcutaneously 15 times with a solution of cadmium chloride at a dose of 1 mg of Cd/kg of body weight in 2–3-day intervals (Kimura et al., 1979) and purified by the procedure adapted from Kägi et al. (1974). In a few preparations additional ion-exchange chromatography (Bio-Gel CM, Bio-Rad) was employed to maximize the homogeneity of the samples (M. Vašák, unpublished data). The purity of each preparation was examined by amino acid analysis (Durrum D-500) and atomic absorption spectrometry (Instrumentation Laboratory Model IL157), respectively.

Thionein, the metal-free protein, was prepared by dialyzing MT against three changes of 0.1 M HCl. The molecular weight cutoff of the dialysis tubing employed was 3500 (Spectrapor Co.). Protein concentration was determined spectrophotometrically (Perkin-Elmer 554) by measuring the absorbance of thionein at 220 nm in 0.1 M HCl and using an extinction coefficient of 7.9 mg cm^{-1} mL^{-1} (Bühler & Kägi, 1979).

Preparation of Cd_7-MT. Thionein at 0.5 mg mL^{-1} in 0.1 M HCl was mixed with an aqueous solution of $^{113}CdCl_2$ (^{113}CdO, 96% enriched from Oak Ridge, dissolved in HCl). To ensure full occupation of the Cd binding sites, about 7–7.5 molar equiv was added. Subsequently in an N_2-purged glovebag the pH of the thionein solution at 0.1 mM was adjusted stepwise to the desired value: pH 6.4 with 0.5 M MES buffer (Sigma) or pH 8.6 with Trizma base (Sigma, purified by Chelex treatment). The mixture and all other solutions used in the preparation of Cd-MT were degassed on a vacuum line prior to use. The excess Cd was removed during the subsequent dialysis or by addition of a small amount of Chelex 100. Samples for NMR were concentrated to about 1.2 mL on an Amicon ultrafiltration apparatus using a UM-2 membrane. The final concentrates were about 8 mM. The level of D_2O was raised to about 20% in the final stages to provide NMR lock.

Throughout this paper the term ^{113}Cd-MT is used to refer to protein containing 96% of the isotope ^{113}Cd.

To prepare Cd_7-MT with a natural abundance level of ^{113}Cd (12.3%), a sample of native Zn_2Cd_5-MT_2 (0.1 mM) in 50 mM Tris-HCl buffer, pH 8.6, was incubated with a 20-fold molar excess of $CdCl_2$ for about 30 min. The excess Cd was removed with Chelex 100 resin, following which the sample of protein was concentrated to about 1.2 mL (10 mM Cd_7-MT_2) on an Amicon ultrafiltration apparatus. The buffer for this sample was then changed to 50 mM phosphate, pH 7, by dialysis.

The protein-to-metal ratios were determined by atomic absorption and UV spectrophotometry at 220 nm (see above). Gel filtration (Sephadex G-50) of the fully occupied Cd_7-MT at pH 6.4 (50 mM MES) and at pH 8.6 (50 mM Tris-HCl) as well as of the standard Zn,Cd-MT at pH 8.6 revealed the same monomeric behavior.

NMR. The spectrometers used were Bruker WM200 (Biomedical NMR Centre, Mill Hill), Bruker WM250 (University of London Intercollegiate Research Service, King's College), and Bruker WH400 (University of London Intercollegiate Research Service, Queen Mary College) with ^{113}Cd frequencies of 44.4, 55.5, and 88.8 MHz, respectively. About 1.2–2 mL of sample in 10-mm outer diameter tubes was used. Chemical shifts are reported in parts per million downfield from the ^{113}Cd resonance of 0.1 M $Cd(ClO_4)_2$. All solutions were clear and colorless and were purged with N_2. Spectra were accumulated with a spectral resolution of at least 5 Hz by using 60°–90° pulses. Broad-band ^1H decoupling was gated off prior to each pulse to eliminate possible unfavorable NOE's. A relaxation delay of 2.4 s was usually introduced so that the total pulse repetition interval (acquisition time and delay) was about 3 s. Such spectra are thought to be fully relaxed. This was confirmed for a few samples by using longer delays and a 180°–τ–90° (inversion–recovery) pulse sequence, which suggested that the T_1's were <1 s at 298 K.

A standard Bruker program, consisting of repetitions of the Hahn spin-echo sequence 90°–τ–180°–τ–collect with variations of τ so as to cover the whole of the coupling constant dimension, was used for the 2D[1] J-resolved experiment, except that broad-band ^1H decoupling was applied on the final echo, i.e., at the time of acquisition.

Attempts to obtain 2D shift-correlated (COSY)[1] spectra at 88 MHz employed the sequence 90°–τ–45°–collect repeated 96 times, with ^1H decoupling only at the time of acquisition, with 512 values chosen so as to cover a sweep width of 7042 Hz in the second dimension. For detailed references to 2D experiments, see Bax (1982).

Results

^{113}Cd NMR is an attractive probe for studying the structures and assembly of the metal cluster(s) of MT. Cadmium-only proteins would appear to be ideal for the complete mapping of all binding sites, but such species have never been found in mammals. The MT's produced in response to administration of Cd salts usually contained at most five Cd ions together with two Zn ions per mole of protein. Previous workers (Otvos & Armitage, 1980) have treated rabbits with

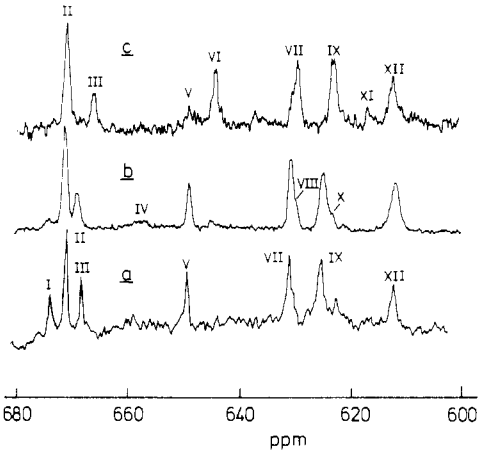

FIGURE 1: 88-MHz proton-decoupled ^{113}Cd NMR spectra recorded at 298 K of (a) Cd_7-MT_2 (natural abundance ^{113}Cd) in phosphate buffer, pH 7 (58 000 pulses, 1.5-s total delay, 25-Hz line broadening), (b) $^{113}Cd_7$-MT_2 in 50 mM Tris-HCl buffer, pH 8.6 (18 200 pulses, 3-s total delay, 10-Hz line broadening), and (c) $^{113}Cd_7$-MT_1 in 50 mM Tris-HCl buffer, pH 8.6 (1040 pulses, 3-s total delay, 10-Hz line broadening).

^{113}Cd salts and have subsequently obtained ^{113}Cd-replaced rabbit liver MT by in vitro treatment of the supernatants of liver homogenates with excess $^{113}CdCl_2$. They have also applied the same treatment to other mammalian MT's (Boulanger & Armitage, 1982). In our method the (natural abundance) Cd and Zn ions from highly purified, native (Cd-induced) MT's were first removed by acid treatment, and subsequently $^{113}CdCl_2$ was added and the pH carefully raised to neutral to allow assembly of the fully reconstituted $^{113}Cd_7$ protein (see Experimental Procedures). By this method cadmium contents close to 7 mol per mole of protein were reproducibly obtained in more than 10 preparations.

Reconstitution. The accuracy of reconstitution was first assessed by obtaining the natural abundance ^{113}Cd NMR spectrum of the Cd_7-MT_2 prepared by the replacement of remaining zinc ions present in native Zn_2Cd_5-MT_2 by Cd ions (see Experimental Procedures). This method of metal replacement, commonly used for metalloenzymes, is unlikely to induce gross perturbations in protein tertiary structure and often preserves enzyme activity, e.g., Cd–LADH (liver alcohol dehydrogenase) (Drum & Vallee, 1970). The natural abundance (12.3%) ^{113}Cd NMR spectrum of Cd_7-MT_2 (Figure 1a), although weak, is almost identical with that obtained with the material reconstituted by the low pH method (see Experimental Procedures). Such an accurate reassembly of seven cadmium ions into a protein containing 20 cysteines, all of which are known to be involved in binding, suggests the presence of specific nucleation sites and cooperative metal binding (M. Vašák, G. E. Hawkes, J. K. Nicholson, and P. J. Sadler, unpublished results). The accurate incorporation of Zn and Cd into apometallothionein has also been substantiated from CD,[1] MCD,[1] electronic absorption (Vašák & Kägi, 1983), and 2D ^1H NMR measurements (M. Vašák, G. Wagner, J. H. R. Kägi, and K. Wüthrich, unpublished results).

Population of Sites. Since our samples of MT_2 were fully reconstituted with seven Cd ions per mole of protein and contained 90% of a single isoprotein as judged by HPLC analysis, we expected to observe seven ^{113}Cd NMR signals of comparable intensity corresponding to the seven metal binding sites. However, comparison of parts a and b of Figure 1 shows that the Cd_7-MT_2 samples prepared by either method (see Experimental Procedures) gave rise to four major signals, three others of about half the intensity, and a few minor resonances

Table I: Comparison of ^{113}Cd NMR Shifts of $^{113}Cd_7$-MT_2 and $^{113}Cd_7$-MT_1 at 88 MHz and 298 K in 50 mM Tris-HCl Buffer, pH 8.6

peak[a]	MT_1 (ppm)	MT_2 (ppm)	peak[a]	MT_1 (ppm)	MT_2 (ppm)
I		673.9	VII	630.1	630.6
II	670.4	671.0	VIII	629.1	629.6
III	665.6	668.8	IX	622.6	624.7
IV		657.1	X		623.2
V	648.6	648.8	XI	616.5	
VI	643.8	645.1	XII	611.9	611.9

[a] For peak numbering system see Figure 1.

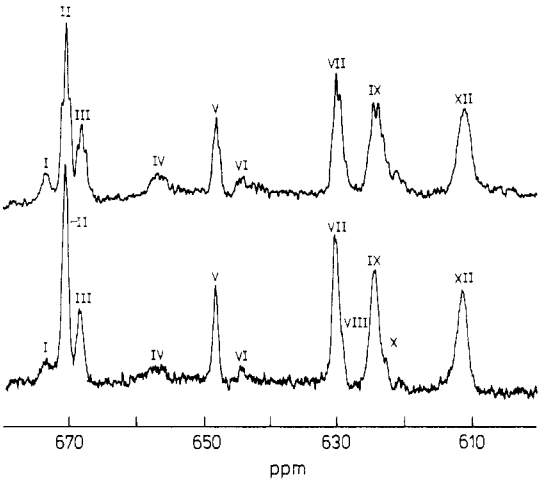

FIGURE 2: Comparison of proton-decoupled ^{113}Cd NMR spectra of $^{113}Cd_7$-MT_2 at 88 (bottom) at 55 MHz (top) and 298 K (8-Hz line broadenings were applied to both spectra).

(see Table I). The spectra shown are representative of several independent preparations, and the intensities are not believed to be affected by NOE or spin–lattice relaxation; i.e., they are fully relaxed (see Experimental Procedures).

Comparison of MT_1 and MT_2. The same low pH reconstitution method (see Experimental Procedures) as used for MT_2 was also applied to the MT_1 isoform in two independent preparations. A representative ^{113}Cd NMR spectrum of reconstituted $^{113}Cd_7$-MT_1 shown in Figure 1 can be seen to differ significantly from that of $^{113}Cd_7$-MT_2. The striking differences are (a) a splitting of the major ^{113}Cd resonance XII observed in the spectrum of MT_2 into two half-intensity peaks XI and XII in the spectrum of MT_1, (b) the reduction in intensity of signal V concomitant with the increase in intensity of resonance VI in the spectrum of MT_2, and (c) other smaller differences that include changes in the line shape of resonances VII and IX and in the shift of signal III (see Table I). However, a certain degree of correspondence in both isoproteins is also seen between resonances II, VII, IX, and XII. In spite of these apparent differences between MT_1 and MT_2 the major characteristic features, i.e., the occurrence of both major and approximately half-intensity resonances, prevail. Since both MT_1 and MT_2 are fully metal reconstituted and since significantly different HPLC traces for both isoproteins were obtained (Klauser et al., 1983), the appearance of the characteristic major and minor peaks in the ^{113}Cd NMR spectra cannot be due to metal deficiency or inhomogeneity related to the presence of isoproteins. To investigate further the problem of possible peak overlap as a cause of the nonintegral signal intensity, we have studied the frequency, pH, and ionic strength dependence of $^{113}Cd_7$-MT_2 spectra in detail.

Frequency Dependence. The spectra of $^{113}Cd_7$-MT_2 at 55 and 88 MHz are compared in Figure 2. The spectrum at 44

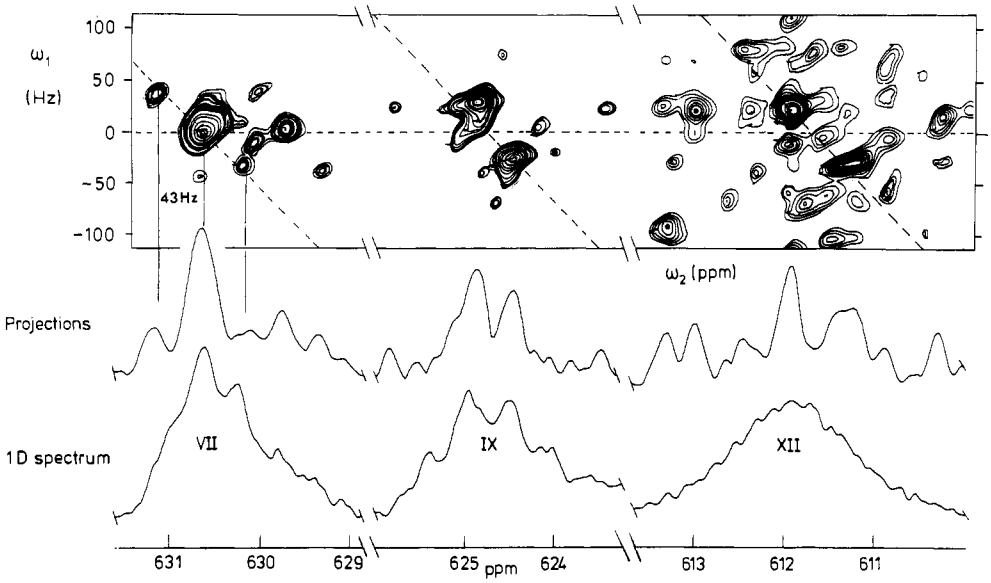

FIGURE 3: 88-MHz 2D *J*-resolved ^{113}Cd NMR spectrum of $^{113}Cd_7$-MT_2 (50 mM Tris-HCl buffer, pH 8.6) in the regions of peaks VII, IX, and XII (see Figure 1) (top). Coupled multiplets lie on diagonal lines, and the proton-decoupled ^{113}Cd NMR spectrum (middle) is the projection on the chemical shift (ω_2) axis. The bottom spectrum shows the same peaks from a typical 1D experiment. Note the extensive overlap of signals in each region of the 2D spectrum.

MHz (not shown) was almost identical with the former, except for the lower intensity of peak I. The chemical shifts, relative intensities, and line widths of resonances at 55 and 88 MHz are listed in Table II. Integrals have been calculated with the assumption that peaks VII and IX each correspond to one cadmium ion. This gives a total of about seven Cd ions per mole of protein, the value obtained also by atomic absorption.

As can be seen from Figure 2, the splittings of the resonances are less well resolved at 88 MHz than at 55 MHz, although many peaks have similar overall line widths (see Table II). The dependence of line width on frequency follows the approximate order I > IV > others. It might be expected that relaxation via the chemical shift anisotropy (CSA)[1] mechanism would be significant at the higher field. CSA relaxation increases with the square of the applied field (B_0^2) and with increasing rotational correlation (tumbling) time (Webb, 1978). However, it would appear from our data that the symmetry of most of the Cd sites in MT is high enough, i.e., tetrahedral CdS_4 (Vašák & Bauer, 1982), to make the CSA mechanism only a minor contributor to T_2 relaxation. Moreover, the earlier results show that the ^{113}Cd peaks of native rat liver MT's are also well resolved at 88 MHz (Nicholson et al., 1983). The absence of significant CSA relaxation at high field has also been reported for ^{113}Cd bound to carp parvalbumin (Forsén & Lindman, 1981).

Resonances I and IV of rabbit liver Cd_7-MT_2 broadened significantly at high field (Table II). Spectra obtained under other conditions (e.g., pH 6.4) suggest that peak IV may consist of two overlapping peaks. There may also be contributions to line widths from chemical exchange although our temperature dependence studies do not provide any clear indication for this (see below). More clearly resolved fine structure was visible at 55 or 88 MHz when Gaussian enhancements were applied. This treatment has revealed a distinct field dependence of the splittings of some resonances, e.g., II, V, and IX, thus suggesting the presence of overlapping signals. To investigate this, 2D *J*-resolved ^{113}Cd NMR experiments were carried out. Preliminary analysis of the map for Cd_7-MT_2 in the region of peaks II and III suggested that peak II contained a well-resolved triplet (J = 43 Hz), two overlapping doublets, and a further triplet. In addition, all

Table II: Effect of Various Frequencies on ^{113}Cd NMR Chemical Shifts (ppm) and Line Widths (Hz) of $^{113}Cd_7$-MT_f in 50 mM Tris-HCl Buffer, pH 8.6

	ppm ($\Delta\nu_{1/2}$)[b]		
peak[a]	55 MHz	88 MHz	44 MHz[e]
I	673.9 (72)	673.9 (213)	
II	671.0 (81)	671.0 (82)	670.4 A1, 670.1 A1′
III	668.8 (88)	668.8 (90)	665.1 B2
IV	657.0 (155)	657.1 (287)	
V	648.8 (67)	648.8 (72)	647.5 B3
VI	645.1 (89)	645.1 (82)	643.5 B4
VII	630.6 (81)	630.6 (86)	629.8 A5, 628.8 A5′
VIII	*c*	629.4[c]	
IX	624.8 (108)	624.7 (116)	622.1 A6, 622.0 A6′
X	*d*	623.2[d]	
XI			615.9 A7
XII	611.7 (126)	611.9 (140)	611.2 A7′

[a] See Figure 1. [b] ±7 Hz. [c] Overlapped with VII. [d] Overlapped with IX. [e] Taken from Otvos & Armitage (1980). Occupancy was 5.6 mol of ^{113}Cd/mol of protein; capital letters refer to the designation of either four-metal (A) or three-metal (B) clusters in MT as proposed by these authors. [f] Full metal occupancy (6.8 mol of ^{113}Cd/mol of protein).

the remaining resonances appeared to be composite in nature, each major peak containing up to six overlapping multiplets and each minor peak up to three. This is illustrated in Figure 3. The several overlapping multiplets in the regions of peaks VII and XII can be seen, and peak IX appears as a strong doublet, i.e., a ^{113}Cd coupled to single ^{113}Cd ion, perhaps the terminal ion of a chain.

pH Dependence. Over the pH range 6.4–9.5 (see Experimental Procedures), little change was observed in the ^{113}Cd NMR spectrum of Cd_7-MT_2. The only difference was the observation of the partial splitting of the broad resonance IV (not shown) at 657 ppm into two at 656 and 658 ppm at pH 6.4. This effect was absent above pH 8.0.

Temperature Dependence. The ^{113}Cd NMR spectrum of $^{113}Cd_7$-MT_2 appeared to be greatly simplified on lowering the temperature to 278 K (Figure 4d). Minor peaks I and IV appeared to broaden, and peak III shifted under peak II (Table III). The intensity of peak V relative to the major peaks decreased by ca. 50%, and clearly resolved shoulders emerged on peaks VII, IX, and XII (Figure 4). Raising the temperature

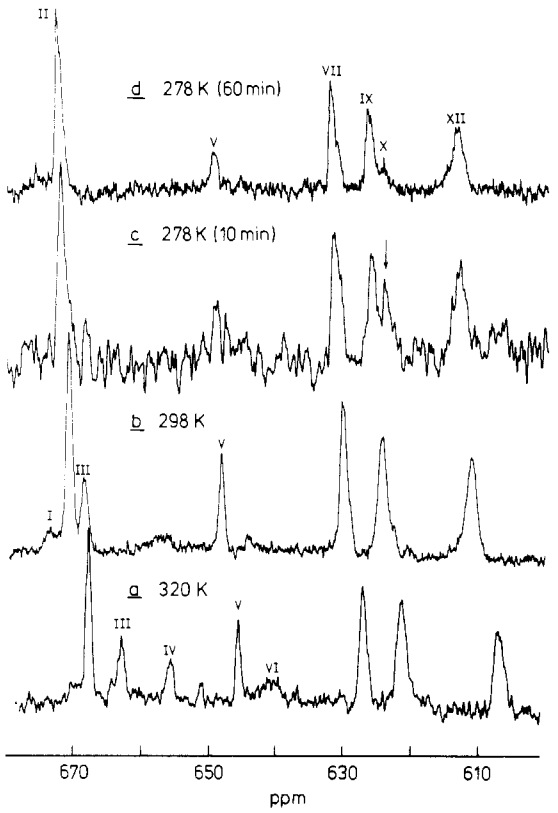

FIGURE 4: Effect of temperature on the proton-decoupled ^{113}Cd NMR spectrum at 88 MHz of $^{113}Cd_7$-MT_2 in 50 mM Tris-HCl buffer, pH 8.6: (a) 320 K; (b) 298 K; (c) 278 K; this spectrum has an average accumulation time of 10 min and was recorded between 5 and 15 min (200 pulses) after lowering the temperature from 298 K; (d) 278 K; average accumulation time of 60 min (1800 pulses). Line broadenings of 16 Hz have been applied to the spectra, except for 25 Hz in the case of (c).

Table III: Effect of Various Temperatures and Ionic Strength on Relative Integrated Areas[a] of $^{113}Cd_7$-MT_2 at 88 MHz in 50 mM Tris-HCl Buffer, pH 8.6

	no NaCl			0.5 M NaCl		
peak[b]	278 K	298 K	320 K	278 K	298 K	320 K
I	0.3	0.3		0.2	0.2	
II	2.0	1.5	1.2	1.9	1.9	1.4
III		0.5	0.6	0.5		0.6
IV		0.2	0.5[c]		0.1	0.5
V	0.3	0.6	0.6	0.5	0.5	0.6
VI		0.1	0.4			
VII	1.2	1.0	1.2	1.2	1.2	1.2
VIII		0.2				
IX	1.4	1.0	1.2	1.1	1.2	1.1
X		0.2				
XI						
XII	1.2	1.2	1.2	1.5	1.2	1.7
total	6.4	6.8	6.9	6.9	6.3	7.1

[a] All integrations were carried out manually by cutting out paper traces and weighing them. The numbers are relative to peak VII as unity (or 1.2 if overlapped with VIII). On the basis of the atomic absorption/UV determination, the protein contained 7.2 mol of Cd/mol of protein (see Experimental Procedures). [b] For the explanation of the peak numbers see Figures 1, 2, 4, and 7. [c] Sum of peaks at 651.5 and 656.1 ppm; see Figure 4a.

of solutions of Cd_7-MT_2 from 298 to 320 K leads to a distinct sharpening of many of the ^{113}Cd resonances and the appearance of a small, relatively sharp resonance at 651 ppm (Figure 4a). Both the 651 and 656 ppm peaks could originate from the broad peak IV, observed at 298 K (Figure 4b), which under different conditions (pH 6.4) shows a partially resolved doublet (see above). The appearance of the broad peak VI (641 ppm) at 320 K is conspicuous by its presence also in the MT_2 preparation used in the frequency dependence studies (Figure 2).

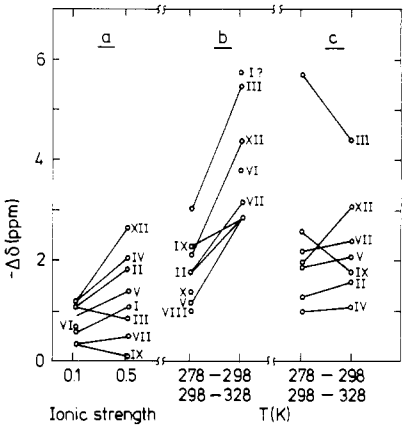

FIGURE 5: (a) Change in ^{113}Cd NMR chemical shifts of resonances on addition of NaCl (0.1 and 0.5 M) at 88 MHz in 50 mM Tris-HCl buffer, pH 8.6; (b) change in chemical shifts of resonances on increasing the temperature from 278 to 298 K or from 298 to 320 K in the absence of NaCl, at 88 MHz, in 50 mM Tris-HCl buffer, pH 8.6; (c) as for (b) but in the presence of 0.5 M NaCl.

A most striking effect in the temperature study was the emergence of a new, intense resonance X on going from 298 to 278 K (see Figure 4c,d). Within the first 10 min of accumulation the intensity of peak X was comparable with that of peak IX, but continuous accumulation for 1 h showed a marked decrease in its intensity (Figure 4d). Such variations in intensity as a function of time are indicative of a relatively slow exchange process on the ^{113}Cd NMR time scale with a half-life of several minutes. This observation appears to affect other peaks in a similar way, although the shift differences are smaller.

Figure 5b shows plots of differential chemical shifts of the resolved resonances as a function of temperature. In the temperature range 278–328 K the observed differential chemical shifts ranged from 0.1 to 0.01 ppm/K. Interestingly, the slopes of individual resonances are found to be nearly constant. All spectral changes observed at high and low temperature are reversible.

Ionic Strength Dependence. It was thought that ionic strength might play an important role in stabilizing the structure of MT, especially in view of the large number of charged residues, eight Lys, three (Asp + Glu), the COOH-terminal, and six additional negative charges arising from the complexation of seven Cd^{2+} ions to twenty cysteine thiolates (Kojima et al., 1976). When the ionic strength of a ^{113}Cd-MT_2 sample was raised from 0.02 to 0.5 M, changes in ^{113}Cd NMR spectra were observed. Thus, peak II shifted on top of peak III, and the major resonances VII, IX, and XII became more symmetrical and approached equal intensities (Figure 6 and Table III). The chemical shift changes experienced by the individual resonances with increasing ionic strength are shown in Figure 5a. With the exception of resonances III and IX, which show decreasing differential shifts with increasing salt concentration, the remaining resonances show the opposite trend.

In the presence of 0.5 M NaCl resonance XII in $^{113}Cd_7$-MT_2 exhibits a notable sharpening at 328 K (Figure 7). Differential chemical shifts (Figure 5c) of the individual ^{113}Cd resonances plotted as a function of rising temperature show almost the same behavior as on raising the salt concentration alone at 298 K (Figure 5a,c). Interestingly, the temperature-induced shifts of resonances over the range of 298–320

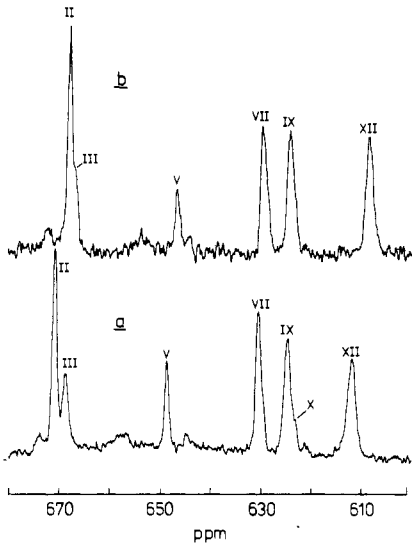

FIGURE 6: Effect of ionic strength on the proton-decoupled ^{113}Cd NMR at 88 MHz of $^{113}Cd_7$-MT_2 in 50 mM Tris-HCl buffer, pH 8.6, at 298 K in the absence (a) and presence (b) of added NaCl (0.5 M). Note the sharpening of peak XII relative to peaks VII and IX and the shift of II on top of III.

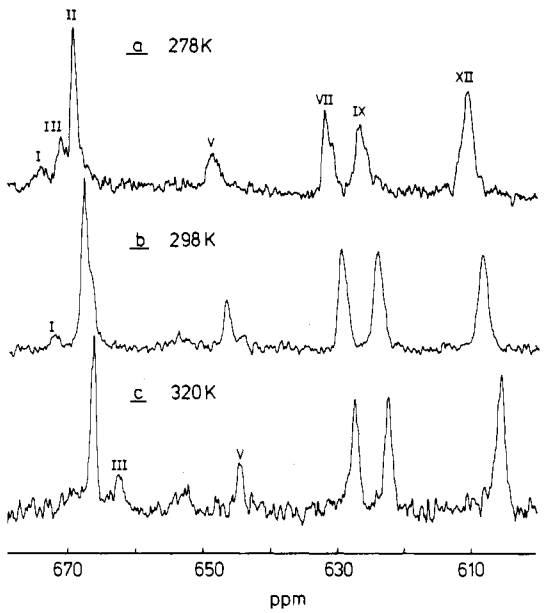

FIGURE 7: Effect of temperature on the proton-decoupled ^{113}Cd NMR spectrum at 88 MHz of $^{113}Cd_7$-MT_2 in the presence of 0.5 M NaCl in 50 mM Tris-HCl buffer, pH 8.6, at (a) 320, (b) 298, and (c) 278 K. Note the differential shifts of peaks, e.g., III compared to II and the appearance of distinct shoulders on VII and IX at low temperature.

K in the absence of salt are markedly diminished (ca. 50%) in its presence.

Discussion

This report represents the first detailed study of the effects of various perturbants (applied field, temperature, pH, and ionic strength) on ^{113}Cd NMR spectra of MT. These results, together with our preliminary 2D *J*-resolved ^{113}Cd NMR data, yield a number of new facts concerning the metal binding site organization and the gross structural features of MT molecules.

Armitage and co-workers have previously reported ^{113}Cd NMR spectra on rabbit liver MT. The ^{113}Cd-MT samples used in their studies were isolated from rabbit livers in response to ^{113}Cd salt administration. On the basis of the existence of the ^{113}Cd–^{113}Cd homonuclear couplings in MT_2 with heterogeneous (^{113}Cd, Zn) metal content, a metal–thiolate cluster structure in this protein was inferred (Otvos & Armitage, 1979). Their recent ^{113}Cd NMR studies were concerned with the structural features of MT using the metal-homogeneous ^{113}Cd-MT forms. In this case the remaining two to three zinc atoms in the protein were replaced in vitro by adding an excess of $^{113}CdCl_2$ to the liver homogenate prior to chromatography (Otvos & Armitage, 1980). Their results can be summarized briefly as follows: (a) ^{113}Cd NMR spectra of the isoproteins MT_1 and MT_2 were identical; (b) the seven metal binding sites of ^{113}Cd-MT_2 gave rise to eight prominent resonances, three of which were of double intensity. On the basis of this and of ^{113}Cd homonuclear decoupling connectivities, the authors inferred the presence of two four-metal clusters (A and A′) and only one three-metal cluster (B) (see Table II). Two reasons were given to account for the apparent discrepancy: (a) a possible heterogeneity of primary structure, i.e., the presence of isoproteins in both electrophoretically distinguishable forms, MT_1 and MT_2; (b) the possible lability of the three-metal cluster (B) resulting in the loss of metal during isolation.

In the present study we were able to rule out both of these reasons as a cause for the unequal peak intensities. We have demonstrated, moreover, that the ^{113}Cd NMR patterns of both isoproteins, MT_1 and MT_2, are not identical. However, the major features (see Figure 1) of the respective NMR spectra were retained, indicating that they were an integral part of these systems. The persistence of nonintegral intensities prompted further investigations. The explanation of this effect was provided by the 2D *J*-resolved spectrum of $^{113}Cd_7$-MT_2 (Figure 3) in which the composite nature of all ^{113}Cd resonances was revealed. While this spectrum has, on one hand, confirmed the existence of the homonuclear ^{113}Cd–^{113}Cd couplings in MT (Otvos & Armitage, 1979), it has, on the other hand, provided clear evidence for the existence of numerous resonances under each ^{113}Cd peak. Thus, ^{113}Cd peaks observed in all 1D ^{113}Cd NMR spectra represented an envelope of several (three to seven) resonances.

Further support for the complex nature of ^{113}Cd signals came from the observed frequency dependence of the fine structure concomitant with the broadening of some peaks (Figure 2). In the absence of CSA (see Results), the observed results suggest that, as well as ^{113}Cd–^{113}Cd homonuclear coupling, a certain amount of peak overlap must be present.

Indirect evidence for the above conclusions was provided by our failure (in three independent attempts) to observe cross-peaks in the 2D (COSY)[1] spectra. The cross-peaks in the $^{113}Cd_7$-MT_2 COSY spectrum would be expected to establish the through-bond connectivities among metal sites. On the other hand, the composite nature of resonances would result in a distribution and further lowering of the intensity of cross-peaks. Thus, the fact that no distinct cross-peaks appear in our ^{113}Cd-MT_2 COSY spectrum strongly supported the suggestion that the observed resonances in 1D spectra were composite in nature. On the basis of these results we infer the simultaneous presence in solution of several isomeric forms of this protein. Additional support for their presence stems from temperature, ionic strength, and pH dependence studies.

Unambiguous evidence for the existence of various configurational and/or conformational substates of MT was provided by the observation of a relatively slow exchange process in the $^{113}Cd_7$-MT_2 spectra on lowering the temperature from 298 to 278 K (Figure 4b,c). This effect can be viewed as a redistribution of the population among the individual substates.

The effect of ionic strength can be interpreted in a similar manner, as is seen from the similarity between the spectrum

of $^{113}Cd_7$-MT_2 at low temperature (278 K) and that obtained at 298 K in the presence of 0.5 M salt (Figures 4d and 6b).[2] Furthermore, the temperature-induced differential chemical shift changes in the presence of salt (0.5 M NaCl) are markedly lowered (ca. 50%) as compared with those in the absence of salt.

Other evidence suggests that native as well as reconstituted MT's undergo well-defined structural changes to a more spherical shape in the presence of salt. The Stokes radius decreased from 17.8 to 15 Å when the ionic strength was increased from 0.015 to 0.5 M, indicating the large changes in the molecular shape of this protein. CD spectra obtained under similar conditions showed changes in the CD band at 227 nm, possibly attributable to a conformational transition of the polypeptide chain (Vašák et al., 1984).

No dramatic changes of protein structure as a function of pH were expected due to the absence of charged amino acids in MT with pK_a's in the pH range studied (6.4–9.5) (Kojima & Nordberg, 1979), and the $^{113}Cd_7$-MT_2 NMR spectrum showed only one minor difference at the low pH limit. Under these conditions (pH 6.4) the partial splitting of the 657 ppm resonance (peak IV) into two peaks was observed, and as the spectrum at pH 8.6 and 320 K shows two distinct resonances (Figure 4a), it was inferred that the broad feature of peak IV probably originates from two independent resonances.

^{113}Cd NMR shifts are known to be very sensitive to the nature of the Cd coordination sphere and its coordination geometry (Ellis, 1983). The lack of large changes in chemical shift in the pH and ionic strength studies, therefore, implies that the Cd^{2+} ions in MT do not have bound H_2O molecule(s) with lowered pK_a's of the type found in the catalytic sites of zinc enzymes such as liver alcohol dehydrogenase and carbonic anhydrase (Eklund et al., 1978; Quicho & Lipscomb, 1971). Also, unlike ^{113}Cd alkaline phosphatase where Armitage et al. (1978) observed a 50 ppm shift when the salt concentration was increased from 0 to 0.1 M NaCl, no binding of Cl^- to the metal occurs in MT. Already previous ^{19}F NMR and IR work has shown that the Cd and Zn ions in native MT_1 and MT_2 do not have open binding sites for F^- or N_3^- (Vašák and Galdes, unpublished results).

In conclusion, ^{113}Cd NMR results clearly show that MT does not possess a unique rigid structure but that a great deal of structural variability and flexibility is present in this protein. The ^{113}Cd NMR spectra appear to reflect simultaneous contributions from various conformational and/or configurational substates. The population distribution of these substates is then perturbed by changes in ionic strength and/or temperature.

Model Considerations. The ^{113}Cd NMR results appear to indicate that several stable metal–thiolate cluster structures of comparable energy can be formed from seven Cd ions and twenty cysteinyl residues. Cluster structures consisting of a linear oligomer (Sadler et al., 1978), independent clusters of three and four Cd ions (Otvos & Armitage, 1980), or clusters of the adamantane type of unit (Vašák & Kägi, 1983) have already been proposed. In view of the data presented here, any new model of MT metal-cluster organization needs to incorporate the following structural requirements. First, the ^{113}Cd NMR results should be considered: (a) several signals of nonintegral intensity are seen even for fully metal occupied Cd_7-MT_2; (b) there is a relatively slow exchange process present; (c) there are large differential temperature and ionic strength effects; (d) most resonances appear to be composed of several overlapping multiplets, many of which are doublets and triplets, i.e., Cd ions coupled to one or two other Cd ions, respectively, but with little clear indication of quartets or of pairs of triplets. Second, the results from other spectroscopic and structural studies should be considered, too: (a) tetrahedral-tetrathiolate metal coordination (Vašák, 1980); (b) the participation of all 20 cysteine residues in metal binding via mercaptide linkages (Kägi & Vallee, 1961; Weser et al., 1973); (c) the capability of the COOH-terminal part of the protein to bind four Cd^{2+} ions (Winge & Miklossy, 1982); (d) the marked change of the shape of the MT molecule on raising the ionic strength (Vašák et al., 1984).

As doublets and triplets were resolved clearly in our 2D *J*-resolved NMR of MT, it is inferred that in the metal–thiolate cluster(s) Cd ions linked to one or two other Cd ions occur. So far, no evidence has been found for Cd ions linked to three or more others. In order to accommodate both the nonintegral ^{113}Cd resonance intensities and their composite nature and the marked change in the protein's shape on raising the ionic strength in the model, the bridging thiolate bonds connecting two Cd ions would have to be broken and re-formed during structural fluctuation of the protein. This idea is supported, at least in part, by the recent 2D ^1H NMR study on $^{113}Cd_7$-MT_2 (Neuhaus et al., 1984) in which 19 cysteinyl residues were clearly identified via ^{113}Cd–^1H heteronuclear coupling. Only two of these residues showed couplings to two ^{113}Cd ions. The failure to see the other ligand bridged metal pairs by this method is presumably indicative of a rapid motional process in which such bridges are broken and reformed. This is in agreement with the very high kinetic lability of Cd^{2+}–thiolate bonds, which may promote an intramolecularly ready interchange between the various configurational substates of the cluster(s). The large number of ^{113}Cd resonances obtained for native Zn,^{113}Cd-MT's may be a further manifestation of this effect (Otvos & Armitage, 1982). Exchange rates are very fast for all the model complexes that have been studied by ^{113}Cd NMR (Ellis, 1983) except the recently reported dicysteinyl-EDTA derivatives (Bulman et al., 1984). In the ^{113}Cd NMR studies of the cluster species $Cd_{10}(SCH_2CH_2OH)_{16}(ClO_4)_4$, for example, a rapid intramolecular exchange of Cd ion between CdS_4 and CdS_4O sites is observed at temperatures between 233 and 333 K in dimethylformamide solution (Haberkorn et al., 1976) but not in the solid state (Murphy et al., 1981).

It is possible that a model of MT as a flexible structure that can stabilize several interchangeable Cd thiolate cluster configurations of comparable energy, in the manner discussed in this paper, may be relevant to its biological function. The protein is probably involved in the controlled uptake and release of metal ions, a process that may require fine thermodynamic and kinetic tuning. Although X-ray crystallographic studies may yield detailed information about the protein structure and metal-cluster organization of MT in the near future, crystals were reported to form at very high ionic strength (Melis et al., 1983). Under these conditions, one configurational substate may crystallize preferentially. Further NMR studies along the lines described here would, therefore, appear to be essential in achieving a more complete understanding of this unusual protein.

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[2] These spectra have the same appearance as that of cluster A in the previously proposed metal cluster model (Otvos & Armitage, 1980). Hence, great care should be taken in the interpretation of NMR studies of MT in which the temperature and/or ionic strength have been varied.

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