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Dynamic Metal-Thiolate Cluster Structure of Metallothioneins

by Milan Vašák*

^{113}Cd-NMR studies have established that vertebrate and invertebrate metallothioneins contain two unique metal–thiolate clusters. However, it proved to be difficult to account theoretically for all features of the ^{113}Cd-NMR spectra. In a reinvestigation of these features using chromatographically homogeneous $^{113}Cd_7$-metallothionein we have identified a total of seven ^{113}Cd resonances and have confirmed the massive intensity difference among these signals. From the effects of variations in temperature, ionic strength, and magnetic field on one-dimensional ^{113}Cd-NMR spectra and from two-dimensional *J*-resolved ^{113}Cd-NMR spectrum it was concluded that the seven ^{113}Cd signals are composed of several overlapping multiplets and indicative of a dynamic organization of the metal–thiolate clusters.

The same nonrigid structure is also indicated individually by recent two-dimensional correlated (COSY) ^{1}H-NMR studies of $^{113}Cd_7$-metallothionein. While showing ^{113}Cd-^{1}H coupling for 19 of the 20 cysteine residues, these studies have evidence for only two "bridging" cysteine ligands of the clusters instead of the expected eight suggesting interferences by metal–thiolate exchange process at the ^{1}H-NMR time scale. Analogous indirect evidence for structural mobility of the clusters comes from EXAFS measurements of Zn_7-metallothionein and from measurements of the perturbed angular correlation of γ-rays (PAC) emis-sion of ^{111m}Cd-metallothionein. Thus, while the EXAFS spectra revealed back-scattering from the thiolate ligands, lattice movements within the cluster is believed to preclude back-scattering from neighboring metals. Similarly, in the PAC time spectra the damping of the major oscillatory component was attributed to inordinately large charge fluctuation in the immediate environment of the ^{111m}Cd nucleus. These results are consistent with a nonrigid cluster model in which many of the sulfur–metal bonds are temporary broken and reformed, thus giving rise to a number of interchanging cluster substates of comparable stability and overall structure.

It is now well accepted that metallothioneins are widely occurring proteins that play a fundamental role in detoxification and in the metabolism of d^{10}-metals (*1–4*). Sequencing studies have also established that metallothioneins from organism as different as mammals, anthropods, and molds are evolutionally related proteins, their principal features being the abundance of metal-chelating Cys-X-Cys sequences where X stands for an amino acid other than Cys (*5*). The occurrence and distribution of these sequences in the chain condition the formation of oligonuclear metal–thiolate clusters unique for these proteins (*6–9*).

Recently, ^{113}Cd-NMR studies (*6*) and limited proteolysis data (*10*) have led to the suggestion that metallothioneins from vertebrates and invertebrates are built up of two separate domains composed of the NH_2- and the COOH-terminal half of the polypeptide chain, respectively, which enclose in their three-dimensional folding an oligonuclear metal-thiolate cluster each. In crab metallothionein both clusters are thought to be made up of three group 2B metal ions and nine thiolate ligands of Cys (*11*). In mammalian metallothionein, the NH_2-terminal domain is believed to contain also a cluster of three metal ions and nine thiolate ligands of Cys, and the COOH-terminal domain, a cluster made up of four metal ions and 11 Cys side chains. In independent studies, these clusters were shown to have an adamantanelike structure in which each metal ion is surrounded in tetrahedral geometry by four thiolate ligands (*7–9*).

While the spectroscopic evidence for this two-domain structure is impressive, it has proven difficult to account quantitatively for all of the features. In particular, the number and the intensities of the ^{113}Cd-NMR resonances of mammalian Cd(II)-metallothionein are not completely understood (*6*). Some of these difficulties have been blamed tentatively on the heterogeneity of the protein preparations employed and on incomplete metal complexation (*6*).

Since we have succeeded recently in developing a method of isolating isoforms of rabbit liver metallothionein in high purity and of preparing homogeneously reconstituted $^{113}Cd_7$-metallothionein, such trivial explanations can now be ruled out effectively. Hence, we decided to re-examine the ^{113}Cd-NMR spectra using such well-defined material (*12*). The present review highlights our major findings and makes correlations with data from recent two-dimensional ^{113}Cd-NMR studies on $^{113}Cd_7$-metallothionein (*12*), two-dimensional correlated (COSY) ^{1}H-NMR studies on $^{113}Cd_7$-metallothionein-2

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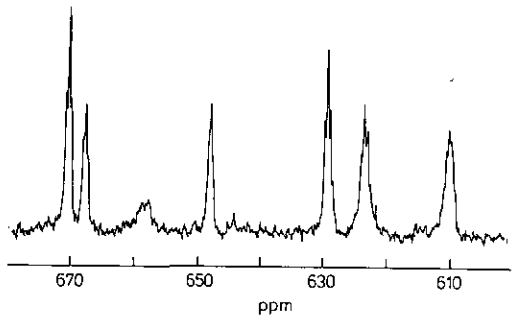

FIGURE 1. 88 MHz proton-decoupled ^{113}Cd-NMR spectrum of rabbit liver ^{113}Cd(II)-metallothionein-2 at 298°K in 50 mM Tris-HCl buffer, pH 8.6 (13'100 pulses, 3 sec total delay, line-broadening 10 Hz). From Vašák et al. (*12*).

(*13*), from the extended X-ray absorption fine structure (EXAFS) (*14,15*), and from the perturbed angular correlation of γ-ray (PAC) (*16*) measurements. The inference drawn from these studies is that some of the problems and inconsistencies encountered in the interpretation of the spectroscopic features arise from a strong inherent nonrigidity of the metal–thiolate clusters.

^{113}Cd-NMR Studies of *de Novo*-Reconstituted ^{113}Cd-Metallothionein

The typical one-dimensional ^{113}Cd spectrum of the *de novo*-reconstituted ^{113}Cd-metallothionein-2 obtained in collaboration with P. J. Sadler (London) is shown in Figure 1 (*12*). The spectrum is similar to that obtained previously by Otvos and Armitage (*6*) on ^{113}Cd-substituted rabbit liver metallothionein-2 but differs from it by containing only seven instead of eight resonances. This simplification of the spectrum does not seem to be a preparation artifact, since the material employed was homogeneous (> 90%) by HPLC criteria and was documented to contain the full complement of seven ^{113}Cd ions (*12*). The material was also shown by two-dimensional ^{1}H-NMR spectroscopy to have nearly the same structure as native rabbit liver Zn-metallothionein-2 (M. Vašák, G. Wagner, J. H. R. Kägi, and K. Wüthrich, unpublished observations). An important finding is that the ^{113}Cd-NMR spectrum still displays the massive differences in intensity of the individual ^{113}Cd resonances noted also by Otvos and Armitage (*6*). Hence, this as yet unexplained feature cannot be attributed to deficiencies of the sample but must be more complex in origin. This complexity is in fact revealed by the effects of changes in temperature, ionic strength, and magnetic field on the ^{113}Cd-NMR spectra of ^{113}Cd-metallothionein-2 which yielded additional resonances, resonance broadening, and field-dependent resonance fine structure indicative of the existence of substantial resonance overlaps and dynamic processes (*12*). The composite nature of all seven resonances was also documented by two-dimensional *J*-resolved ^{113}Cd-NMR spectroscopy (Fig. 2) (*12*). As exemplified for the 610 to 635 ppm region, the peaks in the one-dimensional spectrum (Fig. 1) result from the superposition of a number of multiplets not previously recognized (Fig. 2). In some of the one-dimensional ^{113}Cd signals, as many as eight distinct multiplets can be resolved in two-dimensional *J*-resolved spectra. This multitude of resonances and their susceptibility to perturbation are strongly suggestive of a dynamic, nonrigid organization of the metal–thiolate clusters in metallothionein in which several interchangeable structural isoforms may coexist and in which the most intense subsets of resonances arise from the most stable cluster substates.

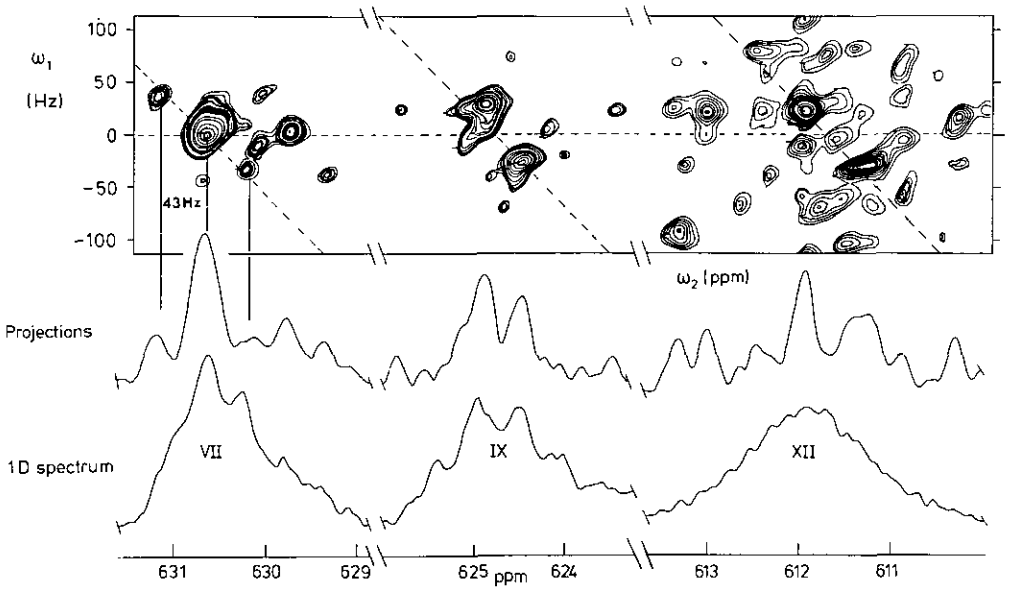

FIGURE 2. 88 MHz 2D *J*-resolved ^{113}Cd-NMR spectrum of ^{113}Cd-MT-2 (50 mM Tris-HCl buffer, pH 8.6) in the regions of ^{113}Cd peaks at 630.6, 624.7, and 611.9 ppm (see Fig. 1). Coupled multiplets lie on diagonal lines and the proton-decoupled ^{113}Cd-NMR spectrums (middle) is the projection on the chemical shift (ω_2) axis. The bottom spectrum shows the same peaks from a typical 1D experiment. Note the extensive overlap of several multiplets in the various signals. From Vašák et al. (*12*).

Two-Dimensional Correlated (COSY) ^{1}H-NMR Studies of ^{113}Cd-Metallothionein-2

Indirect support for a nonrigid model of the Cd–thiolate clusters in metallothionein comes also from recent homonuclear two-dimensional COSY ^{1}H-NMR spectra of ^{113}Cd-metallothionein-2 (*13*). In these studies, conducted in conjunction with Dr. K. Wüthrich's group in Zürich, comparison of ^{1}H chemical shift correlated spectroscopy (COSY) maps of ^{113}Cd- and ^{112}Cd-substituted rabbit liver metallothionein-2 allowed the unambiguous identification of C^α- and C^β-proton resonances for 19 out of the total of 20 cysteine residues of the polypeptide chain (Table 1). The assignments became possible since the cysteine C^β protons could be monitored specifically by their heteronuclear interaction with the vicinal ^{113}Cd nuclei yielding the heteronuclear spin–spin coupling constants ^3J ^{113}Cd-^{1}H (Table 1).

As shown by the contour plot in Figure 3 (right) there is also evidence in some instances for coupling of a C^β proton to two different ^{113}Cd nuclei as characterized by different pairs of coupling constants. As to be anticipated, these features are absent when ^{113}Cd (I = 1/2) is replaced by the NMR-inactive ^{112}Cd (I = 0) (Fig. 3, left) (*13*). These ^{1}H-NMR features constitute independent confirmation of the existence of bridging-cysteine ligands in metal–thiolate clusters inferred from the occurrence of extensive ^{113}Cd–^{113}Cd coupling (*6,12*) and the antiferromagnetic coupling observed in electron spin resonance (ESR) measurements of Co(II)-substituted metallothionein (*7,8*). However, instead of the expected total of eight bridging cysteine ligands, the ^{1}H-NMR data indicate only two such cysteines (Table 1). The failure to observe the remaining bridging ligands is not necessarily contradictive but might be accounted for by the different time scales inherent to these spectroscopic techniques. Such problems will manifest themselves especially with systems undergoing rapid time-dependent structural changes. The difficulty of observing multiple heteronuclear couplings of cysteine protons may thus be explained tentatively by the occurrence of structural interconversion within the metal–thiolate clusters. Such processes might be visualized as involving breaking and forming of metal–thiolate bonds. Such dynamic process would be in agreement with the known kinetic lability of the Cd-S bond as derived from ^{113}Cd-NMR measurements of model complexes (*17*). Thus, with $Cd_{10}(SCH_2CH_2OH)_{16}^{4+}$, a well-studied cluster model, ^{113}Cd ions undergo a rapid intramolecular exchange of Cd between CdS_4 and CdS_4O sites at the temperatures between 233 and 333°K in dimethylformamide solution (*18*) but not in solid state (*19*). Very comparable structural fluctuations leading to intramolecular ligand exchange have recently been observed in synthetic clusters of Cd(II)-, Zn(II)-, and Co(II)-thiolates with adamantane type of stereochemistry (*23*).

Extended X-Ray Absorption Fine Structure (EXAFS) of Zn-Metallothionein

Support for a nonrigid structure of the metal–thiolate clusters comes also from EXAFS studies of rabbit liver Zn and (Zn,Cd)-metallothionein (*14*). In these measurements conducted at the Synchrotron facilities at Daresbury, England, Zn(II)-metallothionein was irradiated with the intense monochromatic radiation in the K-edge region of Zn and the back-scattering profile recorded

Table 1. Chemical shifts of C^α and C^β proton resonances and ^{1}H-^{113}Cd coupling constants for 19 cysteines in ^{113}Cd-metallothionein-2 at 24°C, pH 7.0[a].

	Chemical shift, ppm			^{1}H-^{113}Cd coupling constants			
Cysteine spin system[b]	H^α	$H^{\beta a}$	$H^{\beta b}$	$J_{\beta a}{}^{113}Cd^1$	$J_{\beta b}{}^{113}Cd^1$	$J_{\beta a}{}^{113}Cd^2$	$J_{\beta b}{}^{113}Cd^2$
1	4.05jqc4.10	3.11	3.23	10	c		
2	4.16	2.99	3.64	10	5		
3	4.23	2.88	3.10	37			
4	4.27	3.01	3.25	17	5		
5	4.34	2.95	3.05	16			
6	4.39	2.91	3.01	48			
7	4.43	2.86	3.08	12	13		
8	4.44	2.63	3.14	37			
9	4.49	3.11	3.44	15	15	15	15
10	4.49	2.75	3.18	76	15		
11	4.55	3.24	3.14	48	10		
12	4.58	2.89	3.14	5	25		
13	4.63	3.21	3.28	70	c		
14	4.73	2.61	3.80	25	40	0	12
15	5.06	2.66	3.11	15	40		
16	5.14	3.53	3.59	30	20		
17	5.18	2.98	3.08	20	c		
18	5.36	3.53	3.63	20	c		
19		2.88	3.27	53	5		

[a] Data from Neuhaus et al. (*13*).
[b] Numbering of cysteine residues is arbitrary in the order of increasing chemical shift for the H^α resonances.
[c] Second order $H^{\beta a}$-$H^{\beta b}$ multiple pattern; information on coupling constants is difficult to obtain.

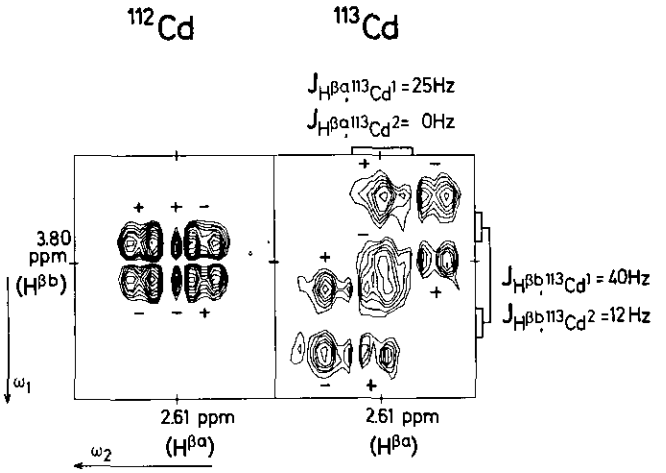

FIGURE 3. Enlarged plot of the ($H^{\beta a}$,$H^{\beta b}$) cross peak of the cysteine spin system 14 in ^{112}Cd-MT-2 (left) and ^{113}Cd-MT-2 (right). The $H^{\beta b}$ chemical shift is along ω_2, the chemical shift along ω_1; + and − indicate positive and negative contour levels. From Neuhaus et al. (*13*).

(Fig. 4). The results from Fourier transform analysis of the EXAFS spectra (Fig. 4, bottom) gave unambiguous evidence for coordination of Zn(II) to four cysteine sulfur ligands at a distance of 2.28 Å (*14,15*) compatible with a tetrahedral symmetry of the sites. There was, however, somewhat surprisingly no indication for back-scattering from the neighboring zinc atoms which are expected to lie at an average distance of 3.85 Å in the proposed adamantane type of cluster geometry (*20*). Since temperature studies also showed an amplitude increase (20%) upon lowering from 298 to 77° K with no alteration in EXAFS profile and periodicity (*14*), this failure to detect metal–metal back-scattering is thought to be caused by substantial dynamic disorder within the cluster giving rise to a large dynamic part of the Debye-Waller factor (δ_{vib}). It is of interest that similar difficulties in assessing metal–metal interactions were also encountered in EXAFS spectra of an Fe-S cluster protein and have been attributed to an inordinately large Debye-Waller factor as compared to low molecular weight model compounds (*21*).

Perturbed Angular Correlation of γ-Rays (PAC) Studies of ^{111m}Cd-Metallothionein

Fluctuations in cluster structure are also held responsible for the pronounced frequency broadening (damping) (ca. 20%) observed in PAC measurements of rabbit liver Cd(II)-metallothionein (*16*). In these nuclear spectroscopic studies which were conducted in collaboration with R. Bauer (Copenhagen), metallothionein was labeled with accelerator-produced metastable ^{111}Cd(II) which decays by a γ–γ cascade to the nuclear ground state depending on the chemical environment of the nucleus. Perturbations caused by a distorted coordination sphere give rise to oscillation in the directionality of emission of the two γ-quanta of the cascade. As shown in Figure 5, two

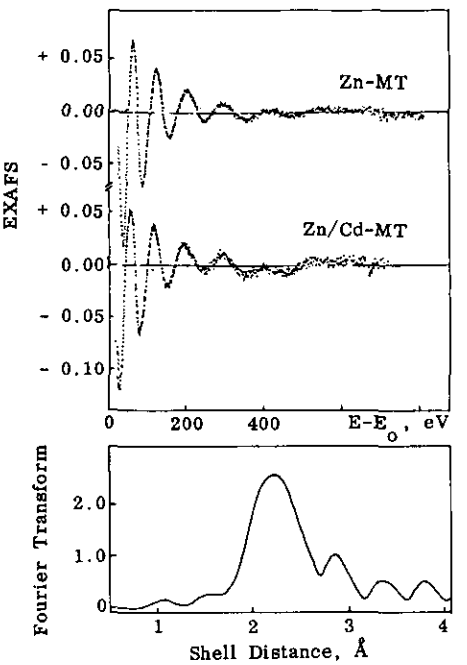

FIGURE 4. Extended X-ray fine structure (EXAFS) spectra of rabbit liver Zn- and (Zn,Cd)-metallothionein (top) and their Fourier transform (bottom). Courtesy of S. S. Hasnain; from Ross et al. (*14*).

oscillatory components can be resolved in the spectrum of ^{111m}Cd-labeled Cd_7-metallothionein (ω_1 = 120 MHz; ω_2 = 580 MHz). The predominant one was attributed to the distorted tetrahedral sulfur coordination (ω_1 = 120 MHz) in the metal–thiolate clusters and its damping is believed to be caused by dynamic charge fluctuation in the immediate vicinity of ^{111m}Cd (*16*).

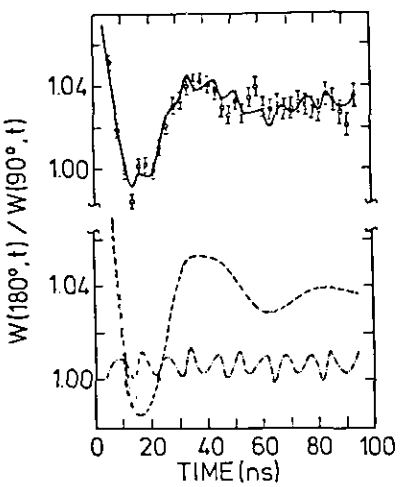

FIGURE 5. (Top) Perturbed angular correlation of γ-ray (PAC) spectra of rabbit-liver ^{111m}Cd(II)-metallothionein in 65% (weight) sucrose, 0° C. $W(180°)/W(90°)$ is plotted versus delay time. The fully drawn curve represents least-squares fit to the spectrum. The bars indicate ±1 standard deviation. The viscosity of the sucrose solution immobilized the protein within the time scale of the experiment. Fully reconstituted ^{111m}Cd(II)-metallothionein, pH 8.0. (Bottom) Resolution of the least-square fit of the spectrum into a low frequency, ω_2 = 116 MHz (dashed line) and a high frequency component; ω_2 = 579 MHz (stippled line). From Vašák and Bauer (*16*).

Besides providing independent support for the existence of metal–thiolate cluster structures in metallothionein and for their tetrahedral geometry this collection of data adds a novel perspective to the study of metallothioneins. Just as proteins are now considered to be dynamic entities undergoing continuous fluctuations in terms of statistical physics (*22*) we infer from these results that the inorganic domains in metal–thiolate cluster proteins have also an appreciable degree of dynamic freedom. Constrained by the structural organization of the polypeptide chain and the limits imposed by the preferred coordination stoichiometry and geometry of the inorganic components, such a system can be thought as interchanging between a number of related substates of comparable thermodynamic stability. In metal-thiolate cluster systems such interconversion can be visualized as involving temporary breaking and forming of coordination bonds without causing gross changes in the overall spatial organization. It remains to be seen to what extent such process relates to the still elusive function of metallothionein.

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