Chapter 6
Use of ^{113}Cd NMR to Probe the Native Metal Binding Sites in Metalloproteins: An Overview

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Contents

Abstract Our laboratories have actively published in this area for several years and the objective of this chapter is to present as comprehensive an overview as possible. Following a brief review of the basic principles associated with ^{113}Cd NMR methods, we will present the results from a thorough literature search for ^{113}Cd chemical shifts from metalloproteins. The updated ^{113}Cd chemical shift

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figure in this chapter will further illustrate the excellent correlation of the ^{113}Cd chemical shift with the nature of the coordinating ligands (N, O, S) and coordination number/geometry, reaffirming how this method can be used not only to identify the nature of the protein ligands in uncharacterized cases but also the dynamics at the metal binding site. Specific examples will be drawn from studies on alkaline phosphatase, Ca^{2+} binding proteins, and metallothioneins.

In the case of *Escherichia coli* alkaline phosphatase, a dimeric zinc metalloenzyme where a total of six metal ions (three per monomer) are involved directly or indirectly in providing the enzyme with maximal catalytic activity and structural stability, ^{113}Cd NMR, in conjunction with ^{13}C and ^{31}P NMR methods, were instrumental in separating out the function of each class of metal binding sites. Perhaps most importantly, these studies revealed the chemical basis for negative cooperativity that had been reported for this enzyme under metal deficient conditions. Also noteworthy was the fact that these NMR studies preceeded the availability of the X-ray crystal structure.

In the case of the calcium binding proteins, we will focus on two proteins: calbindin D_{9k} and calmodulin. For calbindin D_{9k} and its mutants, ^{113}Cd NMR has been useful both to follow actual changes in the metal binding sites and the cooperativity in the metal binding. Ligand binding to calmodulin has been studied extensively with ^{113}Cd NMR showing that the metal binding sites are not directly involved in the ligand binding. The ^{113}Cd chemical shifts are, however, exquisitely sensitive to minute changes in the metal ion environment.

In the case of metallothionein, we will reflect upon how ^{113}Cd substitution and the establishment of specific Cd to Cys residue connectivity by proton-detected heteronuclear ^{1}H-^{113}Cd multiple-quantum coherence methods (HMQC) was essential for the initial establishment of the 3D structure of metallothioneins, a protein family deficient in the regular secondary structural elements of α-helix and β-sheet and the first native protein identified with bound Cd. The ^{113}Cd NMR studies also enabled the characterization of the affinity of the individual sites for ^{113}Cd and, in competition experiments, for other divalent metal ions: Zn, Cu, and Hg.

Keywords alkaline phosphatase • calbindin • calcium-binding proteins • calmodulin • metallothionein • ^{113}Cd NMR methods • ^{113}Cd NMR chemical shifts from metalloproteins

1 Introduction

Speaking on behalf of myself (IMA) and my co-authors, it was a delight to accept the invitation from the Editors to write a chapter for Volume 11 of this series. This provided us with the opportunity to do a thorough review of the use of ^{113}Cd NMR to probe the structure and dynamics at the metal binding sites in metalloproteins that we were actively involved in pioneering some 30 plus years ago [1–4] following up on the early study on the ligand dependence of the ^{113}Cd chemical shift in small molecules [5]. Several reviews devoted to ^{113}Cd NMR studies in

biological systems have appeared since in the literature [6–12]. In this chapter, we focus exclusively on solution ^{113}Cd NMR studies on biological systems. For solid state ^{113}Cd NMR studies we would refer the reader to the pioneering work of Ellis and co-workers [13] and the section on solid state ^{113}Cd NMR studies in the Summers review [9]. While the preponderance of these ^{113}Cd NMR studies report on Zn- or Ca-binding proteins, there are a handful of examples where ^{113}Cd has been used to probe the native Fe, Mn, Mg, and Cu sites in metalloproteins [14–17]. It is not surprising that Cd is a good surrogate for Zn as both are in the same group in the periodic table with a d^{10} valence electron configuration and similar ligand and coordination number preferences, although the ionic radius of Cd^{2+} is larger than Zn^{2+}, 0.98 Å *versus* 0.74 Å, respectively [18]. It may be somewhat more surprising that Cd is also a good substitute for Ca even though their coordination chemistry is quite different, as Cd strongly prefers nitrogen and sulfur over oxygen ligation, which is the preference for Ca. However, the ionic radius of Cd (0.98 Å) is very close to that of Ca (0.96 Å). The most unlikely metal ion, of the ones mentioned above, to be replaced by Cd is Mg which is much smaller than Cd and has a preference for oxygen ligands.

As will be discussed in Section 2, chemical exchange broadening may play a major role in the detection of the bound ^{113}Cd metal ion, which is a consequence of the extreme sensitivity of the Cd chemical shift to pertubations in its ligand environment. The simple illustration that we offer to explain ^{113}Cd sensitivity to chemical exchange broadening is the difference between ^{113}Cd NMR studies of a metalloprotein *versus* a metalloenzyme. In the former, the metal binding site is frequently there to establish the integrity of the protein, e.g., zinc finger proteins, metallothionein [12], or α-lactalbumin [19], where the metal coordination shell is usually filled with protein ligands, which helps suppress effects from chemical exchange. However, in the latter case there is frequently an open coordination site that is required for the binding of the substrate and catalysis. This situation can lead to the exchange of solvent counterions at the open coordination site that in many cases can result in an exchange-broadened and undetectable ^{113}Cd resonance, *vide infra* alkaline phosphatase. The four proteins that we have selected to highlight the biological applications of ^{113}Cd NMR provide a clear illustration of these fundamental differences (see Section 4).

2 General Considerations and Basic Principles

The preponderance of biological systems whose function depends on the binding of the native metal ions Zn, Mg, and Ca provided the impetus behind our efforts to develop and apply spin $I = 1/2$ ^{113}Cd NMR methods to characterize the structural and/or functional role of these native metal ions. While these native metal ions are not strictly spectroscopically silent, they all have isotopes with non-zero spin quantum number $I > 1$ which makes them difficult to use but not totally intractable for studies of macromolecules [7,20].

The foundation for developing the biological applications of ^{113}Cd NMR traces back to the early ^{113}Cd NMR studies on small molecules, which identified the extreme sensitivity of the chemical shift and relaxation properties to the ligand environment [5,21,22]. There are two spin ½ isotopes of Cd, ^{111}Cd and ^{113}Cd, with a natural abundance sensitivity about eight-fold that of natural abundance ^{13}C. Isotopic enrichment (96%) provides an additional eight-fold enhancement of sensitivity, which enables studies at a few tenths millimolar concentration of metalloprotein in most cases. An overwhelming majority of the Cd NMR studies have been on ^{113}Cd, which is slightly more sensitive than ^{111}Cd.

Chemical shifts/nuclear screening: Electrons around the nucleus shield it from the applied magnetic field giving rise to NMR's perhaps most ubiquitous parameter, the chemical shift. Electrons in s-orbitals have spherical symmetry and result in an upfield or diamagnetic shift while p- and d-orbitals result in a deshielding or paramagnetic shift. For protons, naturally the diamagnetic term dominates while for elements beyond the second row in the periodic table, the paramagnetic term usually dominates the screening. In this chapter, we have made our utmost effort to retrieve and list all the solution ^{113}Cd NMR studies of biological systems and present them with references in Section 3 and Figure 2. For the electron-rich ^{113}Cd ion complexed to a host of different metallo-proteins/enzymes, this translates into a chemical shift scale of ~950 ppm!

Coupling constants: The observation of through bond scalar coupling from/to ^{113}Cd provides, among other things, insight into the degree of covalency of the ^{113}Cd-ligand bond. For representative examples of ^{1}H, ^{13}C, ^{31}P, and ^{15}N scalar couplings to ^{113}Cd, which date back to the mid 70s and early 80s, the reader is referred to Table II in Armitage and Boulanger [8]. The significance of the observation of coupling from ^{113}Cd to the protein ligands will be clearly illustrated in the alkaline phosphatase and metallothionein studies presented in Section 4. Before leaving this section, we would be remiss by not mentioning the following. Many years ago, the dihedral angle dependence of three bond ^{1}H-^{1}H coupling was reported [23], which has subsequently been established for numerous three bond coupling constants and as such provided an important additional structural constraint in protein structure calculations. In 1994, the Vašák lab demonstrated the dihedral angle dependence of the three bond ^{113}Cd-^{1}H coupling [24].

Chemical Exchange: A phenomenon that is especially important in ^{113}Cd NMR is the effect of chemical exchange. The importance of this stems from the extreme sensitivity of the ^{113}Cd chemical shift (~950 ppm), which makes it sensitive to exchange rates that are faster by orders of magnitude compared to the conventional exchange rates affecting nuclei like ^{13}C and ^{1}H. In the Introduction, this has been alluded to with the analogy between metalloproteins and metalloenzymes.

There are at least three different kinds of exchange that can significantly affect the ^{113}Cd NMR spectrum: (i) Direct metal ion exchange between the free and protein bound states; (ii) Exchange of metal ion ligands and; (iii) Exchange between two or more protein conformations, which perturb the ^{113}Cd chemical shift with no direct change in metal coordination. The second type of exchange is not likely to appear in metalloproteins where the metal coordination shell is

usually filled with protein ligands. For metalloenzymes, on the other hand, an open coordination site is often essential for the function of the enzyme. The other two types of exchange could occur in any kind of metalloprotein and have been observed.

In view of the potential role of this exchange affecting the chemical shift, it is important to conduct the appropriate experiments (varying temperature, pH, solvent counterions, etc.) to rule in or out the presence of chemical exchange whenever the shift is being used to report on the nature of the protein ligands. Specific examples are: the shift of the A-site Cd in alkaline phosphatase (AP) from 122 ppm in Tris-acetate to 169 ppm in Tris-chloride and in human carbonic anhydrase B (HCAB) where the replacement of the Cd-bound H_2O or ^-OH with Cl^- results in a shift from ~145 ppm to ~ 240 ppm. Both of these examples illustrate the deshielding affect of the metal bound Cl^-.

Relaxation Properties: A knowledge of the spin relaxation properties is of interest for at least two reasons. The first is related to the use of acquisition parameters for optimum signal-to-noise acquisition times and the second is to use the relaxation data to provide important information concerning motional dynamics in and around the metal binding site. Unfortunately, to our knowledge there has been no comprehensive ^{113}Cd NMR relaxation study on macromolecular systems, which limits our discussion of this topic to early, preliminary investigative studies dating back to the mid to late 80s.

Our field-dependent ^{113}Cd NOE and T_1 measurements at 2.1 T and 4.7 T at that time revealed about an equal contribution from the chemical shift anisotropy (CSA) and dipolar relaxation mechanisms when analyzed by the standard isotropic rigid rotor expressions [6,25]. A strong contribution from CSA was not surprising but an almost equal dipolar contribution was, considering the relatively long distance ($\geq$ 3.5 Å) to the nearest ligand protons. However, the measured NOEs revealed a dipolar modulation process at a more effective relaxation frequency than overall protein reorientation was necessary to account for the experimental NOE. To explain the large dipolar contribution, we proposed a mechanism which involved the contribution from the facile exchange rate (10^8 to 10^9 s^{-1}) of ionizable protons on solvent molecules in the immediate vicinity of the metal ion. Support for this model comes from studies of the exchange rates of water molecules (10^8 to 10^9 s^{-1}) in the vicinity of the metal ion [26–28]. This model, and the supporting T_1 and NOE plots, was presented in a review on ^{113}Cd NMR by Armitage and Boulanger [8]. However, in the process of compiling the material for this chapter, IMA was made aware of an error that was made in the ^{1}H-^{113}Cd dipolar relaxation plots presented in this latter reference and in two earlier publications [6,25]. The error involved not properly taking into account both the sign of the gyromagnetic ratio and the opposite sense of the angular precession of the ^{113}Cd spins. This error was discovered after reading a paper by Kay et al. [29], which referred to two papers by Werbelow [30,31]. Subsequent communication with Lewis Kay and especially Larry Werbelow proved invaluable to the explanation and to our understanding of the miscalculation. Re-evaluating the interpretation of the relaxation rate and NOE values in the slow motion limit using the corrected plots provides a new

understanding of the data. For reference, Figure 1a shows the corrected NOE *versus* correlation time plot at three different field strengths with the incorrect plot at 4.7 T for comparison. Note the dramatic change in the slow motion limit NOE values, which was also reflected in this regime for the exchange NOE plot shown in Figure 1b.

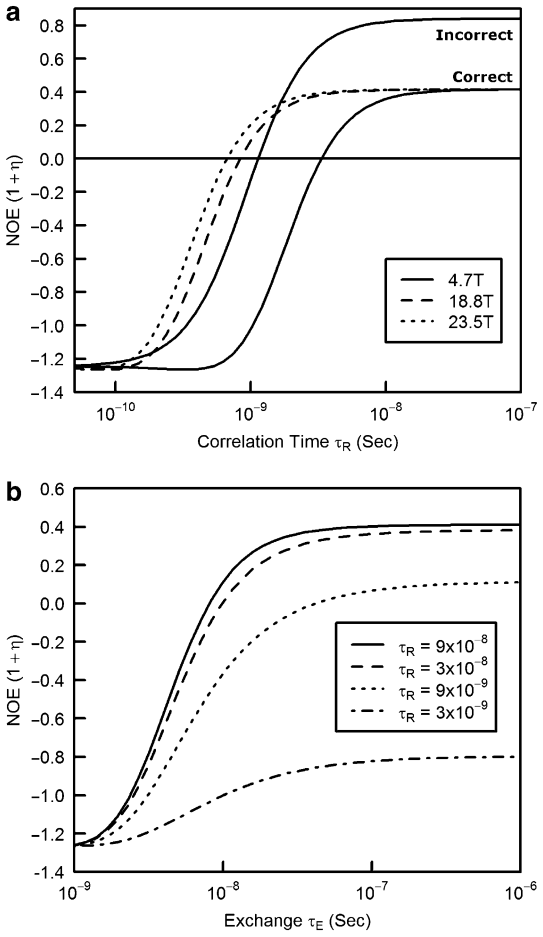

Figure 1 (**a**) Theoretical plots of the ^{1}H-^{113}Cd NOE *versus* correlation time, τ_R, in seconds at different field strengths. See [6,8,25] for the description of the model and parameters used. (**b**) Theoretical plots of the ^{113}Cd NOE *versus* exchange time, τ_e, at 2.1 T for a range of τ_R values. See [8] for a description of the model and parameters used.

3 ^{113}Cd NMR Chemical Shifts from ^{113}Cd-Substituted Metalloproteins

Figure 2 [1–4,13–17,19,24–26,32–187] illustrates that oxygen ligands provide the greatest shielding with a progressive deshielding upon substitution with nitrogen ligands to the most deshielded state containing all sulfur ligation from cysteines. In the case of mixed ligands (O, N, S), we would draw your attention to the excellent correlation in the deshielding with the number of N and S ligands. Starting from all oxygens, there is an approximate downfield shift of 100 ppm per N (from histidine) and 200 ppm per S (from cysteine). Also, in the case of all cysteine S ligation, there is a clear discrimination in the ^{113}Cd shift with the participation of terminal versus bridging cysteines with the former being the most deshielded, at about 700 ppm or lower field for 4S. There is also a clear difference between cysteine and methionine sulfur ligands with participation of the latter being more shielded by about 100 ppm. In addition to the shielding contributions originating from the identity of the ligands, there is a contribution from both the coordination number and geometry as is best seen for the all oxygen binding sites in the calcium binding proteins. More shielding results from increasing the coordination number and deshielding correlates well with the number of charged ligands.

All of the ^{113}Cd chemical shifts for cadmium bound to calcium sites are to high field of the 0.1 M $Cd(ClO_4)_2$ [$Cd(H_2O)_6^{2+}$] reference standard at 0 ppm and are, therefore, negative. The standard is in effect itself an all oxygen octahedral complex. They span almost 200 ppm with as yet no definitive consensus as to what causes the large spread. Contributing factors are definitely the number of charged ligands, the total number of ligands, and the symmetry of the site.

All Ca^{2+} binding sites for the proteins in Figure 2 are either hexa- or hepta-coordinated. The two hexa-coordinated structures, trypsin [188] and insulin [189], have ^{113}Cd shifts clearly downfield from the hepta-coordinated proteins in agreement with the notion that a higher coordination number will result in an upfield shift. The truly hepta-coordinated structures, α-lactalbumin [190], lysozyme [191], and peroxidase [192], differ by only one charge in the first coordination sphere, but the ^{113}Cd shift difference is more than 100 ppm between peroxidase and the other proteins. We have presently no explanation for this large shift difference unless the structure around the cadmium ion differs from that of calcium, for example, being octa-coordinated in peroxidase.

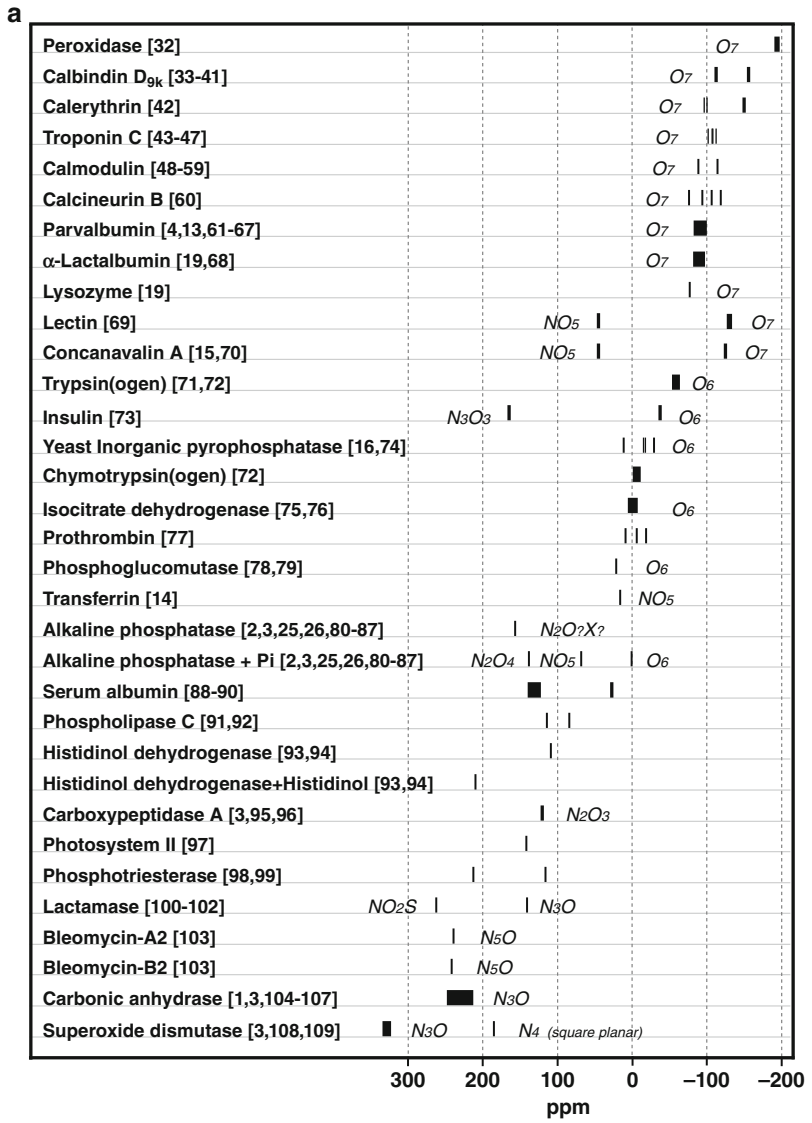

Figure 2 The ^{113}Cd NMR chemical shifts from ^{113}Cd-substituted metalloproteins. The chemical shifts are represented as bars. For proteins with representatives from different sources, like parvalbumin and metallothionein, the width of the bar indicate the spread in shift, including two for each parvalbumin and seven for each metallothionein. For most of the entries metal ligands are shown: S for cysteine, S* for methionine, N for histidine, and O for all types of oxygen ligands. Numbers within brackets refer to references in the list of references.

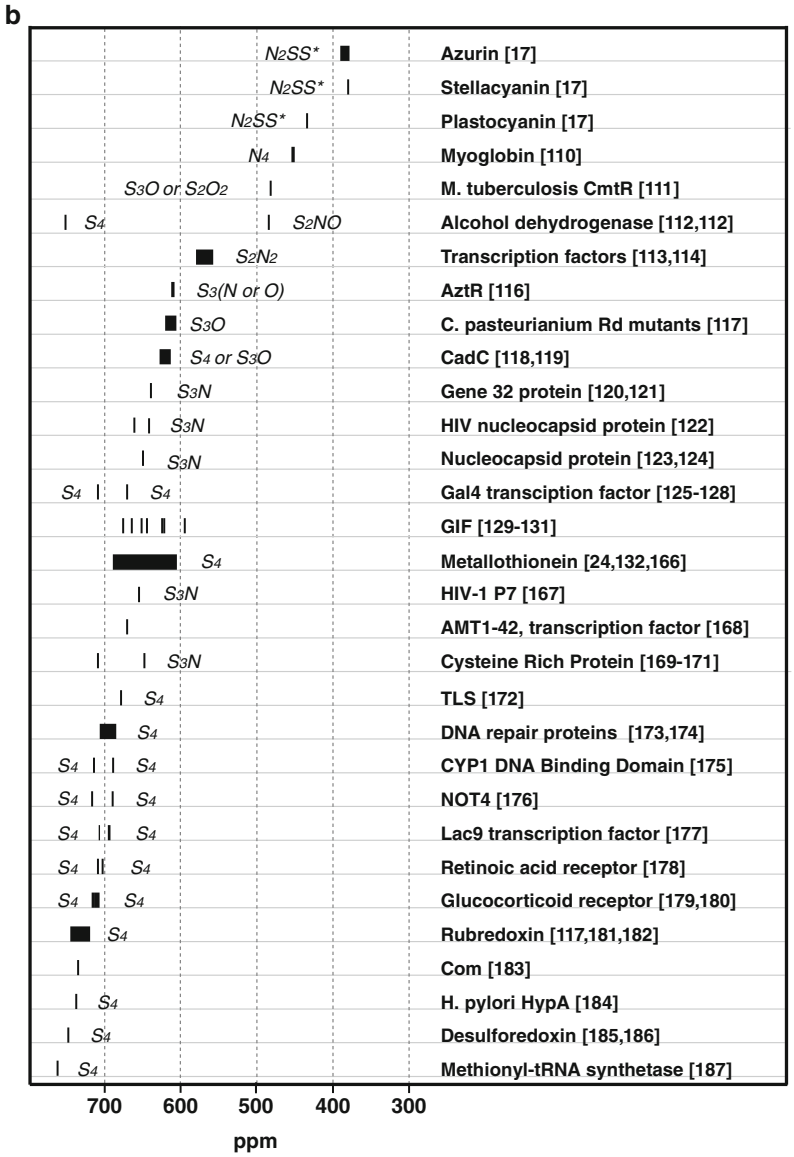

Figure 2 (continued)

The remainder of the calcium binding proteins in Figure 2 are formally hepta-coordinated but with bidentate cordination from a single carboxylate group which will make a distorted pentagonal bipyramidal structure that may more resemble octahedral symmetry with the bidentate group seen as a single ligand [193–200]. For this group of proteins, including almost forty ^{113}Cd shifts, a linear correlation is observed over 70 ppm for the ^{113}Cd chemical shift versus the number of charged ligands (Figure 3).

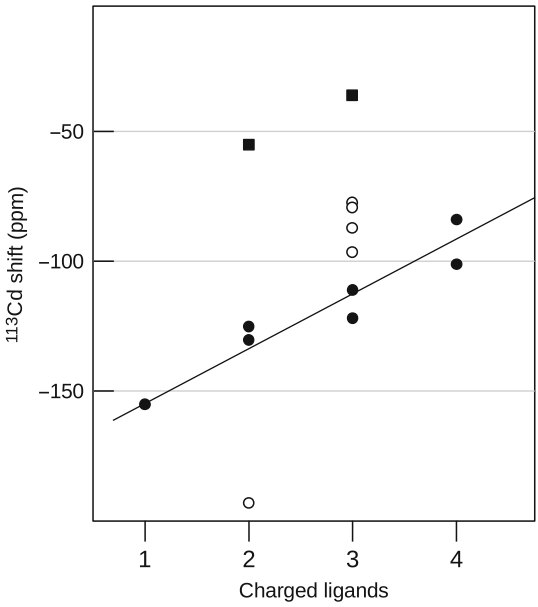

Figure 3 ^{113}Cd chemical shifts for calcium binding proteins with only oxygen ligands plotted *versus* the number of charged ligands. ■, hexa-coordinated, octahedral symmetry; ○, hepta-coordinated with only monodentate ligands; ●, hepta-coordinated with one bidentate and five monodentate ligands.

4 Specific Highlights of Studies on Alkaline Phosphatase, Calcium Binding Proteins, and Metallothioneins

Each of the examples discussed below in this section illustrate, in different ways, how ^{113}Cd NMR methods have been used to provide new insight into the structural, dynamics and functional role of the metal binding site.

4.1 ^{113}Cd NMR and Alkaline Phosphatase

Escherichia coli alkaline phosphatase, a dimeric zinc metalloenzyme (~95,500 Da), binds 2 Zn ions and 1 Mg ion per monomer and functions in the non-specific hydrolysis of phosphate monoester. ^{13}C NMR on ^{13}C labeled histidine biosynthetically incorporated into AP in conjunction with substrate ^{31}P NMR and ^{113}Cd NMR methods were used for the assignment of the three ^{113}Cd resonances to specific sites per monomer and their role in substrate binding. A full account of these studies can be found in the following references [6,25,80,82,201].

^{113}Cd titration of the apoenzyme showed that the first two equivalents of added ^{113}Cd gave a single sharp resonance at 169 ppm consistent with the selective binding to a pair of symmetrically disposed A sites on the dimer. Continued titration with ^{113}Cd to produce the saturated $^{113}Cd_6$ enzyme resulted in the progressive loss of the resonance at 169 ppm with no new ^{113}Cd resonances appearing. Chemical exchange broadening obliterates the ability to detect any ^{113}Cd resonance from the

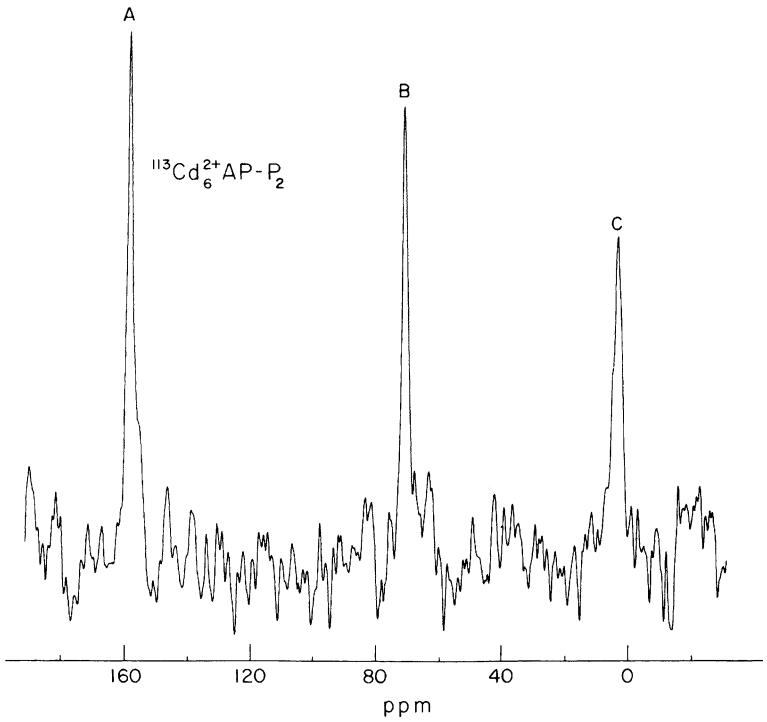

Figure 4 ^{113}Cd NMR spectrum at 44.4 MHz of 1.5 mM $^{113}Cd_6^{2+}$ diphosphoryl alkaline phosphatase, pH 6.2. Reproduced from [6] with kind permission from Springer Science + Business Media B.V.; copyright 1982.

six equivalents of bound ^{113}Cd. Phosphorylation of the active site serine residue, $Cd_6AP\text{-}P_2$, inhibits the exchange broadening with the appearance of three ^{113}Cd resonances at 160 ppm (A site), 70 ppm (B site), and 2 ppm (C site) (Figure 4). Concurrent ^{13}C NMR studies on the [γ-^{13}C]-histidine labeled $^{113}Cd_6AP\text{-}P_2$ and $^{112}Cd_6AP\text{-}P_2$ produced the spectra shown in Figure 5A and B [6,82].

Separate resonances are observed for each of the ten histidyl resonances per monomer in this symmetric dimer. The collapse of the doublet for resonances 7, 8, and 9 in Figure 5A, in Figure 5B with the spin I = 0 ^{112}Cd isotope provided unequivocal proof of three bond ^{113}Cd-^{13}C J coupling from Cd coordinated to $N_{\varepsilon 2}$ of three imidazole rings. Figure 5C shows the ^{13}C spectrum after the addition of eight equivalents of $^{112}Cd^{2+}$ to the sample in 5A and removal of the excess Cd^{2+} by ultrafiltration. The ^{113}Cd NMR spectrum of this sample, not shown, indicated a significant loss in the A and C site ^{113}Cd resonances through ^{112}Cd displacement, but none for ^{113}Cd at the B site. Histidyl resonance number 9 is therefore assigned to the B site metal and histidyl resonances number 7 and 8 to the A site metal. The assignment of the two low field histidyl resonances labeled 1 and 2 to ^{113}Cd ligands with coordination through the $N_{\delta 1}$ of the imidazole ring was based on the following: these resonances did not titrate, relaxation time measurement and, the absence of observable coupling, which would be consistent with a smaller 2 bond ^{113}Cd-^{13}C J

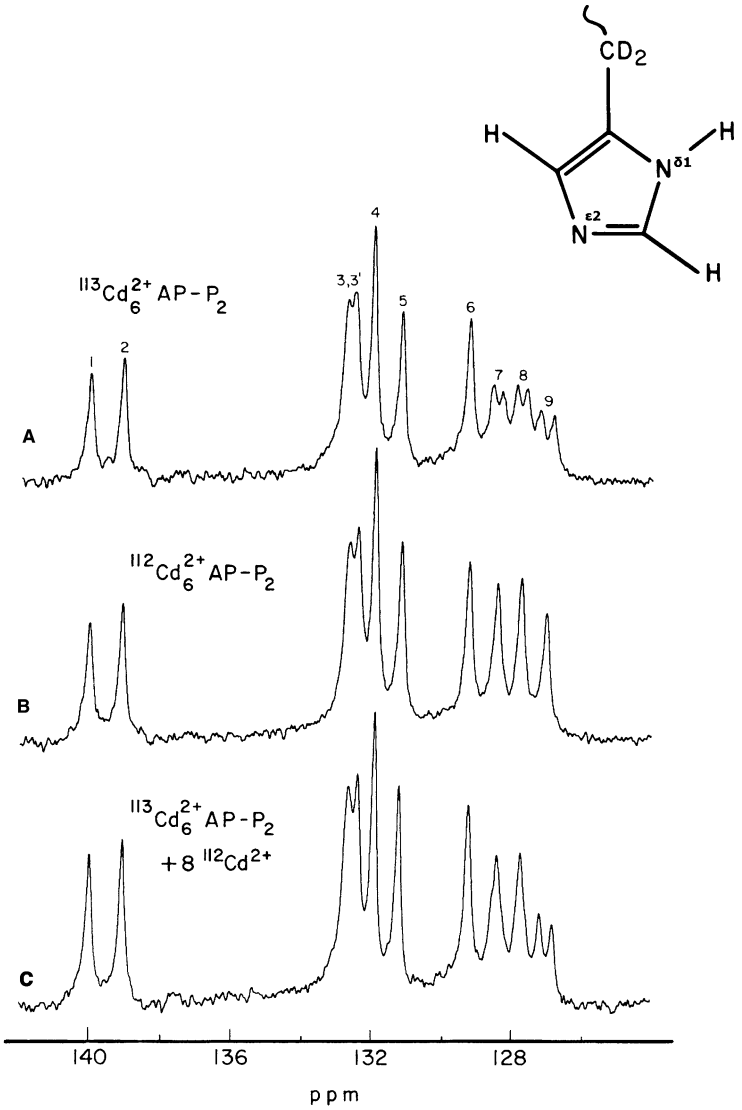

Figure 5 The effect of selective Cd^{2+} isotope exchange at the A and C sites on the 50.3 MHz ^{13}C NMR spectrum of the [γ-^{13}C]-histidine labeled Cd_6^{2+} diphosphoryl alkaline phosphatase. **(A)** $^{113}Cd_6^{2+}$ AP-P_2; **(B)** $^{112}Cd_6^{2+}$ AP-P_2 with spin I = 0 ^{112}Cd; **(C)** sample A after addition of 8 equivalents of $^{112}Cd^{2+}$ and removal of excess Cd^{2+} by ultrafiltration. The ^{113}Cd NMR spectrum of this sample, not shown, indicated an almost complete loss of the ^{113}Cd resonance at the A and C site through exchange with ^{112}Cd with no change of the ^{113}Cd resonance at the B site. On top at the right the imidazole ring is shown. Reproduced from [6] with kind permission from Springer Science+ Business Media B.V.; copyright 1982.

coupling [82]. However, these circumstantial pieces of evidence had to be retracted when the X-ray structure became available approximately 10 years later [202,203]. From the X-ray structure of AP (1ALK), the A site is a 6-coordinate octahedral with His412 $N_{\varepsilon 2}$, His331 $N_{\varepsilon 2}$, two O from Asp327 and two O from phosphate; the B site is a highly distorted octahedral with His370 $N_{\varepsilon 2}$, two O from Asp51, two O from Asp369 and Ser102. This is the serine that is phosphorylated, which is the form of the Cd enzyme being examined here which probably means the two oxygens from phosphate coordinated to the A site are replaced with waters and the B site Ser-OH is replaced with phosphate. The C site is the Mg site, which is a slightly distorted octahedron, with the ^{113}Cd resonance at 2 ppm arising from a bound Asp51 (1 bond), Thr155 OH, Glu322(1 bond), and three water molecules.

An unexpected bonus came from the above NMR studies using a combination of ^{113}Cd, ^{13}C, and ^{31}P NMR to monitor the disposition, occupancy, and role of the individual metal binding sites in substrate phosphate binding and serine phosphorylation. Specifically, these studies showed that substrate binding and phosphorylation requires a minimum occupancy of the A and B site on a monomer. Thus, while the first two equivalents of Cd added bind to the symmetric A sites on each monomer, addition of phosphate results in the relocation of the metal ions to give a distribution where 50% are apo monomers and 50% are di-metal monomers. This is the chemical explanation for the elusive half of the sites reactivity and negative cooperativity reported in some studies on this enzyme! Further addition of metal to saturate the 3 sites/monomer results in a symmetric diphosphorylated dimer giving rise to the spectra shown in Figures 4 and 5.

4.2 ^{113}Cd NMR and Calcium Binding Proteins

The most common Ca binding motif, the EF-hand or helix-loop-helix motif, consists of a 12 amino acid long loop with a helix of approximately 10 amino acids on each side. The loop contains all the metal ligands, in positions 1, 3, 5, 7, 9, and 12. Position 1 is always an Asp and position 12 always a bidentate Glu. There are two more side chain ligands that may or may not be charged. The remaining two ligands are either backbone C=O or water. It is, however, not a single calcium binding site that is the active unit but a pair of sites. The sites are connected via a short anti-parallel β-sheet, with amino acids 7 to 9 in each loop supplying one strand each.

4.2.1 Calbindin D_{9k}, a Study of Mutants

Calbindin D_{9k}, previously known as intestinal calcium binding protein, is the smallest (75 amino acids) in the calmodulin superfamily of proteins. A special characteristic of this EF-hand protein is that the first metal binding loop is modified compared to the typical EF-hand loop, a pseudo EF-hand, a common feature for

proteins belonging to the S100 family. The sites will be referred to as site I and site II for the pseudo site and the typical site, respectively. The pseudo loop is two residues longer than the typical EF-hand due to insertion of one residue after the first and one after the sixth residue in the loop. There is only one side chain group that coordinates the metal ion, the glutamate at position 14 which uses both carboxylic oxygens. The remaining ligands are backbone carbonyls and one water [195]. It looks like the site has turned itself inside out. Residues 9 to 11 are involved in the β-sheet connecting it to the other EF-hand. Despite these differences between the two calcium binding sites, the cooperativity in calcium binding is preserved.

The ^{113}Cd NMR spectrum for wild type bovine [34] and porcine [33] calbindin D_{9k} show the same feature when titrated with ^{113}Cd. First appears a resonance with a chemical shift of about –105 ppm that increases in intensity and stays with this shift up to one equivalent of added Cd. Thereafter, the resonance shifts towards higher field and broadens. A maximum broadening is observed at about 1.5 equivalents of added Cd. After addition of at least 2 equivalents of Cd, the shift has changed to about –110 ppm and the line width is back to what it was at low levels of added Cd. The first signal to appear has been assigned to site II, with a chemical shift in the same range as for other EF hands. A second signal observed only at lower temperatures has a shift of –155 ppm and is assigned to site I. This high field shift can, as seen in Figure 3, be explained as being due to the fact that there is only one negatively charged ligand. The broad signal not observed at room temperature is an example of exchange broadening due to direct metal ion exchange between free and protein bound ^{113}Cd. An off-rate for the bound metal ion of about 2000 s^{-1} would explain both the fact that no signal is observed at room temperature for site I ^{113}Cd and the broadening of site II ^{113}Cd at 1.5 equivalents of ^{113}Cd added.

The ^{113}Cd chemical shift is known to be very sensitive to even subtle changes in the immediate surrounding of the Cd ion. ^{113}Cd NMR has therefore shown itself to be an easy to use and sensitive monitor of structural changes due to mutations. It has been observed that mutations outside the metal binding loops have no effect on the ^{113}Cd shift. On the other hand, it has been found that charge mutants, where charges in loop residues whose side chains are not metal ligands have been removed, have diverse effects. Only the mutant Asp19Asn, and double and triple mutants containing Asp19Asn, show some effect on the ^{113}Cd shifts (Figure 6).

The ^{113}Cd shift of cadmium in site I has moved downfield by 20 ppm, as much as by adding a charged ligand according to Figure 3. Within this group of mutants there is no change in the ^{113}Cd shift of cadmium in site II with site I empty, however, the change in ^{113}Cd shift for site II cadmium upon cadmium binding to site I has diminished or totally disappeared for the triple mutant, Glu17Gln + Asp19Asn + Glu26Gln. This indicates that the interaction between the sites has decreased. This is also seen as a decreased cooperativity in the calcium binding. In fact there is a correlation, though weak, between the cooperativity in calcium binding and shift in site II ^{113}Cd caused by binding of Cd to site I [38,40,41].

A more pronounced effect on the ^{113}Cd spectrum was seen for mutants directly affecting the metal binding. Changing loop I into a normal EF-hand loop, either by

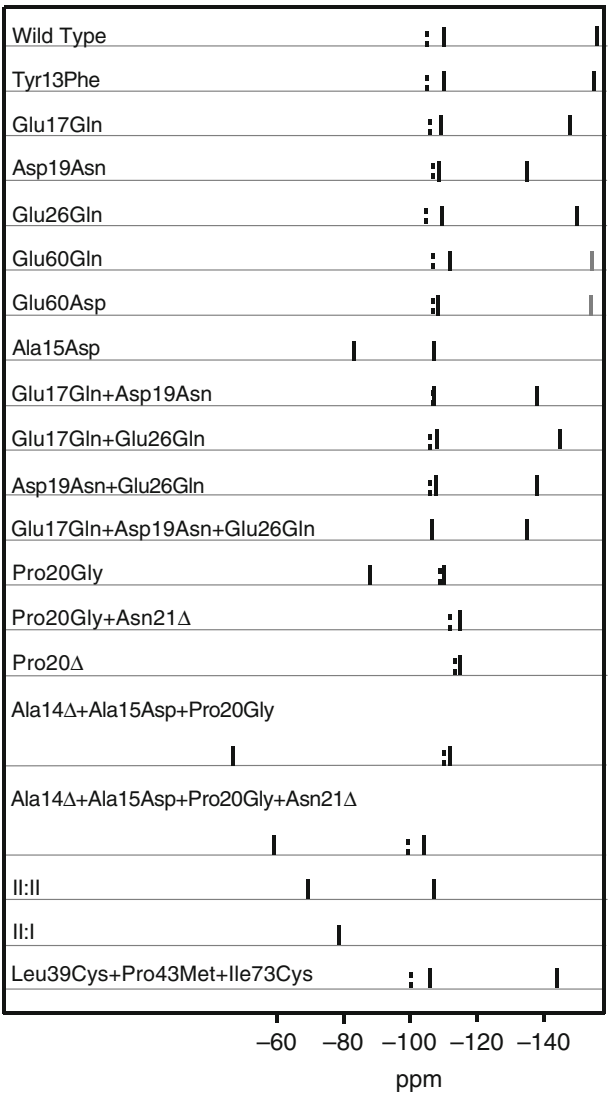

Figure 6 Bars indicating the chemical shifts for the two ^{113}Cd resonances in calbindin D_{9k} and for a set of mutants. Note that the typical EF-hand ^{113}Cd signal has an almost constant shift, –105 to –110 ppm. The dashed bars refer to proteins with only one bound $^{113}Cd^{2+}$ ion and the solid bars refer to proteins with two bound $^{113}Cd^{2+}$ ions. The entry II:II denotes the mutant with site two loop sequence also in site one, and II:I the mutant with interchanged loop sequences. Δ denotes a deletion. The mutant Leu39Cys + Pro43Met + Ile73Cys has been cleaved after Met43 and the S-S bond between Cys39 and Cys73 has been formed.

inserting the sequence of loop II or by the minimum number of replacements and deletions, did not result in a ^{113}Cd spectrum expected for two typical EF-hands, but a shift for site I ^{113}Cd of 20–30 ppm downfield from the expected value (Figure 6). This shows that the general structure around loop I does not easily adopt a typical EF-hand loop [34,37].

We hope that these examples show how useful ^{113}Cd NMR can be in studies of mutants to detect not only major changes but also very small changes in the structure of the studied protein.

4.2.2 Calmodulin, Target Peptide Binding

Calmodulin (CaM) is a small, 148 residue acidic protein that has four pair-wise oriented EF-hands. The amino acid sequence is strictly conserved among vertebrates, indicating that every amino acid is important for some function. CaM is a Ca^{2+} sensor protein that has no enzymatic activity by itself but has been shown to control the activity of hundreds of different functions in a Ca^{2+}-dependent manner [204]. Ca^{2+} binding studies have shown that there are two classes of metal binding sites with two metals each. Early ^{113}Cd NMR studies showed that when ^{113}Cd is titrated into a CaM solution two resonances, –88 and –115 ppm, appear and increase contemporaneously up to two added equivalents of Cd^{2+} [48]. Nothing more happened to the spectrum until more than four equivalents of Cd^{2+} had been added when a signal from free Cd^{2+} appeared. Two ^{113}Cd ions are evidently not seen by NMR due to some intermediate exchange process. By limited trypsin cleavage of CaM, two halves can be generated, TR_1C (residues 1-77) and TR_2C (residues 78-148). When ^{113}Cd is titrated into a solution of TR_2C a ^{113}Cd spectrum very similar to that of intact CaM is observed, showing that the two strong binding sites are in this half of CaM. When ^{113}Cd is titrated into a solution of TR_1C nothing happens until a signal from free ^{113}Cd appears when more than two equivalents have been added [51]. Evidently there is an exchange process, different from direct metal exchange, that broadens the ^{113}Cd signals beyond detection both for intact CaM and TR_1C.

The first crystal structure of Ca_4CaM, 5 years after our first ^{113}Cd NMR study [48], showed a dumbbell structure with two similar domains containing two EF-hands each and connected by a long helix [198,200]. SAXS data showed, however, that the solution structure deviates from the crystal structure in the distance between the two halves [205], interpreted as due to a flexibility in parts of the central helix. This was later confirmed in an NMR relaxation study [206]. NMR studies have shown that apo-CaM has a flexible dumbbell structure [207–209] similar to the structure of Ca_4CaM. The structures of the individual lobes differs, however, significantly between the apo- and Ca-forms of CaM. Ca^{2+} binding causes a rearrangement of the helices from a “closed conformation” in the apo-form to an “open conformation” in the Ca-form, where a hydrophobic patch has been exposed, similar for both lobes. Our present interpretation of the unseen ^{113}Cd signals from TR_1C is that Cd binding has not resulted in a dominating “open conformation” and that there is an intermediate exchange rate between the two conformations. It has more recently been shown for a mutant TR_2C fragment of CaM, Glu140Gln, that in the fully calcium-loaded form there is an exchange occurring between two conformers seemingly similar to the open and closed forms [210].

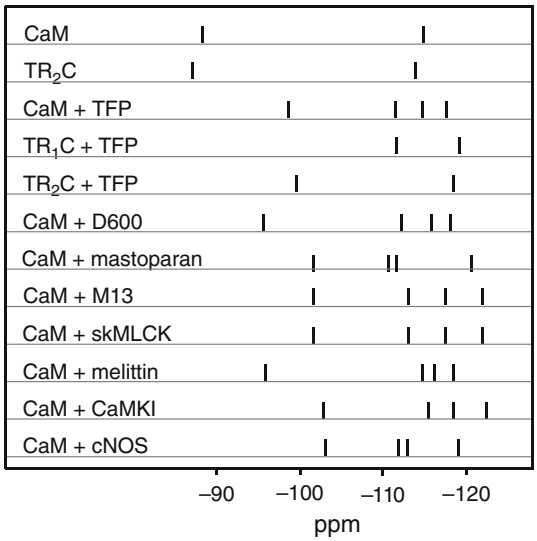

Figure 7 Bars indicating the ^{113}Cd chemical shifts of ^{113}Cd bound to calmodulin and fragments thereof as well as for CaM complexes with various natively occurring peptides or peptides derived from CaM-activated enzymes are shown. The resonance with a shift between –88 and –103 is from site IV, which has four negatively charged ligands, whereas the other three sites have three negative charged ligands. CaMKI, calcium-calmodulin-dependent protein kinase I; cNOS, constitutive nitric oxide synthase; D600, α[3-{[2-(3,4-dimethoxyphenyl)-ethyl]-methylamino}propyl]-3,4,5-trimethoxy-α-(1-methylethyl)-benzene-acetonitrile; M13, KRRWKKNFIAVSAANRFK-KISSSGAL; skMLCK, skeletal myosin light chain kinase.

Fortuitously, the two missing ^{113}Cd signals in CaM can be made visible by the addition of hydrophobic molecules, like trifluoperazine (TFP), that are thought to bind to the hydrophobic patches made solvent-exposed upon metal binding [198]. The binding of two molecules of, for example, TFP per CaM are needed to complete the change in the ^{113}Cd spectrum to show all four signals. For the TR_1C fragment one TFP molecule is sufficient [52,53].

As mentioned above, CaM interacts with a great number of different enzymes. The interaction is in most cases strictly calcium dependent. A few natively occurring small peptides that bind to CaM in a calcium-dependent manner have also been found. Before any crystal structure information was available it was possible to show by means of ^{113}Cd NMR that drug molecules like TFP bind to CaM and cause the same structural changes as the peptides, as judged from the ^{113}Cd shifts. However, one peptide molecule is able to cause the same, or at least very similar, effect as two TFP molecules (Figure 7) [54,55].

For one peptide to bind to both halves of CaM and affect all four metal binding sites as seen from the change in shifts for all four ^{113}Cd resonances, the distance between the two hydrophobic patches, one in each half of CaM, has to be much shorter than seen in the structure reported for Ca_4CaM [198]. A major structural change could therefore be postulated based on ^{113}Cd NMR alone and was later

confirmed when structures were determined in solution with NMR [211] and in crystals with X-ray crystallography [212,213]. These structures show CaM "grabbing" the peptide, however, with no detectable change in the structures of the individual halves of CaM. This compact structure was in fact first observed experimentally in SAXS studies [214]. We can thus see that even though the whole domain containing two EF-hands does not appear to change its structure there is a change in the ^{113}Cd shifts of up to 15 ppm upon peptide or drug binding. Thus again emphasizing the extreme sensitivity in the ^{113}Cd chemical shift to even very subtle changes in the structure surrounding the metal ion, structural changes so far not detected by other means.

4.3 ^{113}Cd NMR and Metallothionein

Metallothioneins (MTs) are small (6000–7000 Da), intracellular, cysteine-rich (~33% of the amino acids are cysteines) metal binding proteins that were first discovered in 1957 in equine kidney cortex. The subsequent purification of this protein identified it as the only known native cadmium-containing protein. Further studies showed this protein to bind both essential (e.g., Cu and Zn) and nonessential (e.g., Cd and Hg) metal ions and to be truly ubiquitously distributed in nature [215–218]. Additionally, MT biosynthesis is induced at the transcriptional level by a wide range of factors, which includes heavy metal ions that were subsequently found bound to the protein. All of the above factors suggest a role for this protein in heavy metal homeostasis, transport and detoxification [215–218]. Despite 55 years of extensive studies on MTs, which has included the high resolution characterization of the 3D structure and metal binding properties [134,136,138,151,154,218–222], the essential physiological functional role(s) of MT remains elusive. Of particular note in these studies was the determination of the NMR solution structure of a mammalian MT in a *tour de force* effort by the Wüthrich lab which resulted in the reinterpretation and correction of the only mammalian MT crystal structure currently available [223,224].

The objective in this section of this chapter is to illustrate how ^{113}Cd NMR methods were used to provide the first definitive evidence for the existence of polynuclear Cd clusters in MT, which was followed by the elucidation of the 3D solution structure of a variety of MTs and the establishment of the relative affinities of the multiple metal binding sites for Cd, Zn and Cu [132,136,138,143,154,219,220,225]. For a more in depth discussion on metallothioneins in general, the reader is referred to Chapter 11, "Cadmium in Metallothionein" by Freisinger and Vašák in this volume.

The existence of a naturally occurring cadmium binding protein with multiple, presumed isolated, metal mercaptide sites prior to our ^{113}Cd NMR studies, offered an irresistible challenge! From the very first ^{113}Cd NMR spectrum on the native ^{113}Cd MT obtained from the $^{113}CdCl_2$ induced protein isolated from rabbit liver (Figure 8) [132], we observed homonuclear ^{113}Cd scalar coupling in all of the ^{113}Cd resonances that could only arise from the existance of polynuclear Cd-thiolate

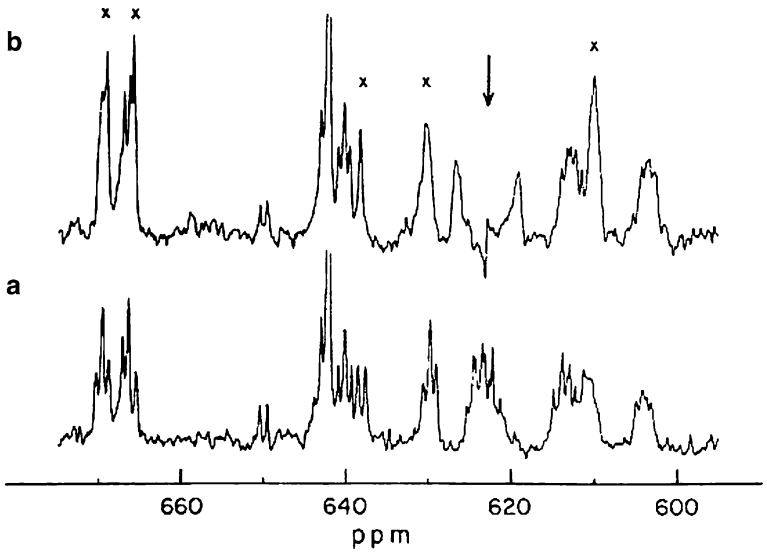

Figure 8 ^{113}Cd NMR spectra at 44.37 MHz of ^{113}Cd-induced native rabbit liver MT2 containing 4.4 g-atoms of ^{113}Cd and 2.6 g-atoms of Zn per mol protein. (**a**) Proton-decoupled. (**b**) Proton-decoupled with selective homonuclear decoupling at the position of the arrow with coupled resonances identified with an x. Reproduced from [132] by permission from the American Chemical Society; copyright 1979.

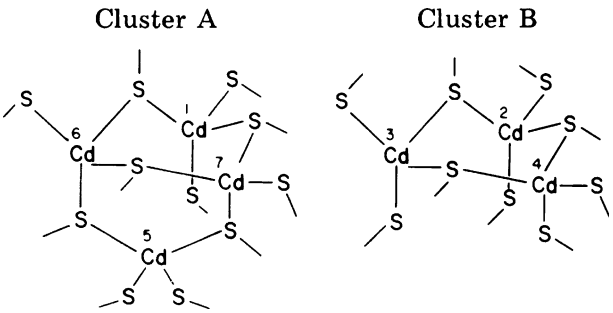

Figure 9 Structures of the 4-metal and 3-metal cluster located in the α- and β-domain respectively, of rabbit liver metallothionein deduced from spin coupling information. The numbers beside each Cd refers to the corresponding resonance position in the ^{113}Cd spectrum proceeding from low field to high field. Reproduced from [6] with kind permission from Springer Science + Business Media B.V.; copyright 1982.

metal cluster(s)! This feature immediately dispelled the prevailing model, which consisted of multiple, separate metal binding sites. Shortly thereafter the size and structures of the metal clusters in the mammalian protein were determined by selective homonuclear ^{113}Cd decoupling techniques (Figure 9) [133].

^{1}H-^{113}Cd HMQC experimental methods were then developed which enabled the establishment of the specific coordination of each of the ^{113}Cd resonances to the sequentially assigned cysteines in the 2D ^{1}H NMR spectrum [142,226]. As it turned

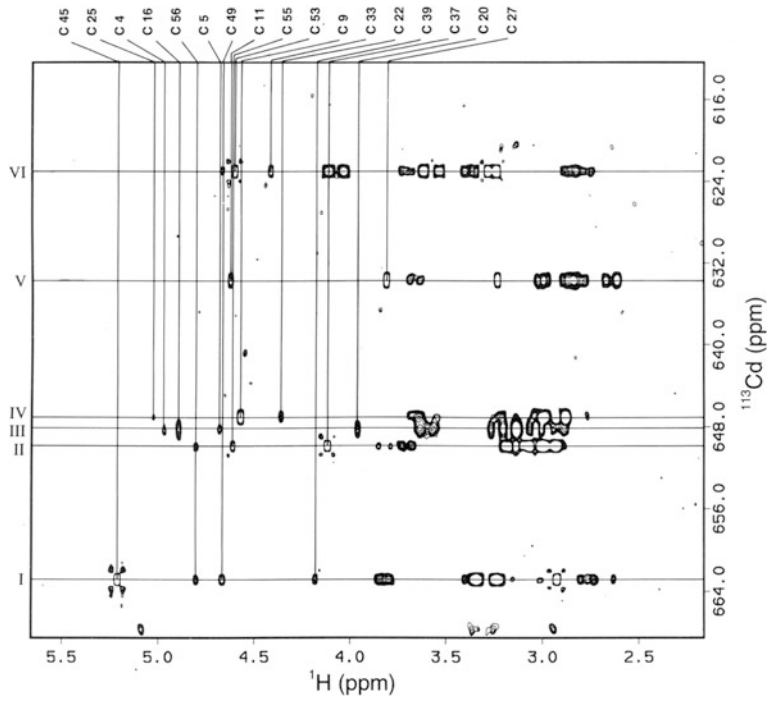

Figure 10 ^{1}H-^{113}Cd HMQC spectrum of $^{113}Cd_6$ blue crab MT1. The six ^{113}Cd resonances along the vertical axes are labeled by roman numerals according to their decreasing ^{113}Cd chemical shifts. Resonances on the horizontal axis are from the α and β protons of cysteine with the former assigned to specific cysteines in the primary sequence. Reproduced from [149] by permission from John Wiley & Sons Ltd., Chichester, UK; copyright 1993.

out, these latter structural constraints were absolutely essential for the determination of the 3D fold of MT where an insufficient number of ^{1}H-^{1}H dipolar constraints were observed in the early 2-dimensional ^{1}H NMR studies to calculate the 3D structure. This resulted from the lack of regular secondary structural elements, α-helix and β-sheet, in MT and their associated diagnostic dipolar constraints. A representative example of this experiment is shown for the $^{113}Cd_6$MT from blue crab *Callinectes sapidus* in Figure 10 with the 3D structure of both three-metal binding domains of this protein shown in Figure 11 [149,151].

As was the case with the solution structural studies on the mammalian proteins [154,219–222], there were no dipolar constraints between the two metal domains to permit their respective orientation. This was a limitation when working with MTs isolated from natural source at the time, which today could be overcome by the expression of recombinant MT with isotopic ^{15}N labeling and the use of residual dipolar coupling methods [227]. Another advancement in the 3D structural studies on this protein has arisen from the increased sensitivity of newer, higher field NMR instruments. NMR studies on MT at 800 MHz now provide a sufficient number of ^{1}H-^{1}H NOE dipolar constraints to permit the calculation of the tertiary fold of the protein backbone in MT *without* the need for ^{113}Cd substitution and the

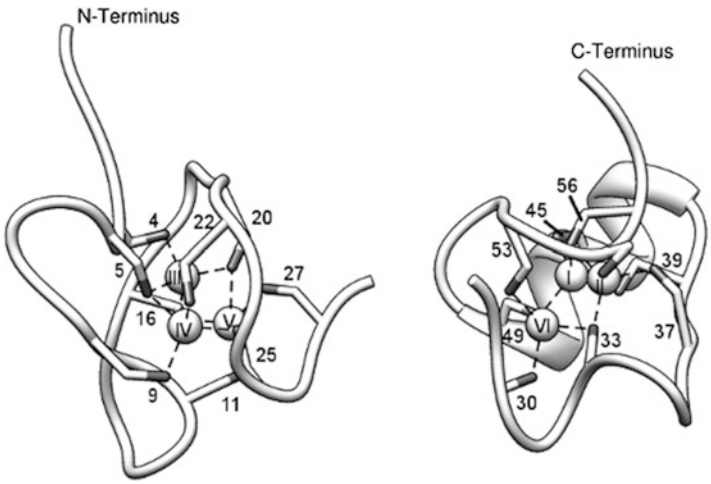

Figure 11 The 3D NMR structure of the two domains in blue crab MT1. The Cd ions are numbered according to their resonance position from low field to high field along with the sequence number of the liganding cysteines (PDB entries 1DMC and 1DME).

establishment of specific Cd to Cys residue number connectivities [228]. The reader is referred to this reference where the method is illustrated with the determination of the 3D fold of the protein backbone in Cu_6MT from the fungus *Neurospora crassa* with validation provided by the comparison of the calculated structure of the MT1 protein with and without ^{113}Cd-Cys restraints. The implication from this study is that with 800 MHz NMR data, accurate 3D protein backbone folds are readily obtainable for MTs from any species containing the bound native copper and/or zinc metal ions.

5 Conclusions and Outlook

While we don't foresee a resurgence in the use of ^{113}Cd NMR methods to the levels in the 80s, we sincerely hope that the material presented in this chapter provides a useful overview and guide for the use of ^{113}Cd NMR methods to potential new users. While we have attempted to be comprehensive in this overview, we hope that you will accept our apologies if your contribution in this area has not been highlighted. The excellent correlation in chemical shift *versus* ligand type and nature of coordination shown in Figure 2 provides information about the metal binding sites in metallo-proteins/enzymes that is not readily available by other methods. Additionally, the extreme sensitivity of the ^{113}Cd chemical shift provides one with a very sensitive monitor of dynamic changes around the metal binding site(s). Hopefully, illustration of the use of ^{113}Cd NMR with the select examples here will arouse new interest and applications.

Abbreviations

AP	alkaline phosphatase
AztR	*aztR* gene product
CadC	*cad* operon transcription regulating protein
CaM	calmodulin
CaMKI	calcium-calmodulin-dependent protein kinase I
cNOS	constitutive nitric oxide synthase
Com	bacteriophage MU *com* gene product
CSA	chemical shift anisotropy
D600	α[3-{[2-(3,4-dimethoxyphenyl)-ethyl]-methylamino}propyl]-3,4,5-trimethoxy-α-(1-methylethyl)-benzene-acetonitrile
GIF	neuronal growth inhibitory factor
HCAB	human carbonic anhydrase
HMQC	heteronuclear multiple quantum correlation
M13	KRRWKKNFIAVSAANRFKKISSSGAL
MT	metallothionein
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser enhancement
p7	HIV-1 nucleic acid binding protein
R1	spin-lattice relaxation rate
Rd	rubredoxin
RDC	residual dipolar coupling
SAXS	small angle X-ray scattering
skMLCK	skeletal myosin light chain kinase
TFP	trifluoperazine
TR_1C	N-terminal half of calmodulin
TR_2C	C-terminal half of calmodulin

Acknowledgments IMA would like to express his sincere appreciation to all the students and postdocs who have conducted the ^{113}Cd NMR studies in his lab over the years and whose names can be found on the publications in the reference list. A special thanks is extended to his first postdoc, Dr. James D. Otvos, who embarked upon these studies at their outset in the mid 70s. A special thank you is also extended to Lewis Kay and Larry Werbelow for clarifying the correct treatment of relaxation rates and NOE for gyromagnetic ratios of opposite sign. We all thank Dr. Melanie Rogers for her assistance with both editing and formatting this chapter.

TD would like to dedicate this chapter to his long time colleague, Hans Lilja, who passed away last summer (2011). He was an electronics wizard and instumental to all our early NMR work. Hans you are dearly missed.

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