

CHAPTER 4

^{67}Zn NMR, a tool for coordination chemistry problems

ATHANASSIOS G. COUTSOLELOS

*Chemistry Department, Laboratory of Bioinorganic Chemistry, University of Crete,
PO Box 2208, GR-71003 Heraklion, Crete, Greece
Fax: +30 281 054 5054; e-mail: coutsole@chemistry.uoc.gr*

and

GEORGIOS A. SPYROULIAS

*Department of Pharmacy, University of Patras, Panepistimioupoli – Rion, GR-26504
Patras, Greece
Fax: +30 261 096 9950; e-mail: gspyroul@upatras.gr*

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I. INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is a powerful and versatile analytical technique that can provide site-specific information about chemical bonding, structure and dynamics in molecular systems. NMR applications have made a major impact in a variety of disciplines ranging from materials science to molecular biology and bioinorganic

The chemistry of organozinc compounds

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chemistry. Heteronuclear NMR has found an important role when metal ions are involved in the chemical structures and for several cases has assisted in approaching solutions or solid state problems.

^1H and ^{13}C NMR spectroscopies are being widely used to characterize Zn(II) organometallic compounds with organic moieties, amino acids or amino acid synthetic analogues based on minor chemical shift differences of the nuclei of the free ligands and the Zn(II) complexes. On the other hand, ^{67}Zn nucleus could be also used to probe the metal binding of organometallic compounds or biopolymers like proteins and enzymes.

II. ^1H AND ^{13}C NMR SPECTROSCOPY OF ORGANOMETALLIC COMPOUNDS

Zn^{2+} ion is diamagnetic ($[\text{Ar}]d^{10}$), lacking any unpaired electron density, and therefore no influence on the relaxation properties and chemical shifts of the ^1H and/or ^{13}C resonances is expected. However, in an attempt to illustrate the differentiation of various Zn ligands bearing oxygen, nitrogen or sulfur donor atoms, experimental NMR data reported in the literature are collected¹⁻⁴. Ligands discussed in this section are either peptides bearing natural amino acids or synthetic organic moieties. These groups bear mainly N and S donor atoms, since the coordination of ligands through oxygen donor atoms, mainly carboxylic acids, sets the Zn metal ion in a three-bond distance from a C atom bearing protons [i.e. $\text{Zn}-\text{O}-\text{C}(\text{O})-\text{CHR}-$, where R = H or other group], thus minimizing even further the chemical shift differentiation effect arising from metal binding.

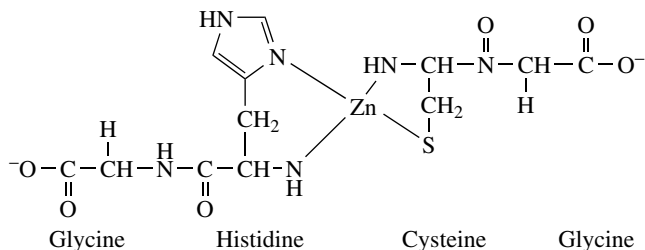


CHART 1. Zn(II) coordination to $^+\text{H}_3\text{N}-\text{His}-\text{Gly}-\text{COO}^-$ and $^+\text{H}_3\text{N}-\text{Cys}-\text{Gly}-\text{COO}^-$ dipeptides

In the former case, two dipeptides, $^+\text{H}_3\text{N}-\text{His}-\text{Gly}-\text{COO}^-$ and $^+\text{H}_3\text{N}-\text{Cys}-\text{Gly}-\text{COO}^-$, are bound to a Zn(II) ion, through the two NH terminal dipeptide groups and the imidazole ring of the histidine and the SH group of cysteine residue (see Chart 1)¹. The authors monitored the chemical shift differences of ^1H and ^{13}C nuclei between the free and metal-bound peptides by taking into account (i) the H nuclei of the $\text{C}\alpha$ and $\text{C}\beta$ carbon atoms of the cysteine residue, the $\text{C}\alpha$, $\text{C}\delta 2$ and $\text{C}\epsilon 1$ (see Chart 2) protons of the histidine residues and those sited at the $\text{C}\alpha$ carbon atoms of the glycyl residues, as well as (ii) the C nuclei of the two COO^- terminal groups and the CO carbonyl atoms of the peptide bonds, in concert with the histidine $\text{C}\alpha$, $\text{C}\beta$, $\text{C}\gamma$, $\text{C}\delta 2$ and $\text{C}\epsilon 1$ carbon atoms, the cysteine $\text{C}\alpha$ and $\text{C}\beta$ carbons and the two $\text{C}\alpha$ carbon atoms of glycines (see Chart 2). The largest ^1H chemical shift differences were observed for protons that were close to the zinc coordination sites (histidine $\text{N}\epsilon 2$ or $\text{N}\delta 2$ and cysteine S) and were measured to be only 1.12 and 1.0 ppm for cysteine βCH_2 and histidine $\text{H}\epsilon 1$ protons (see Chart 2),

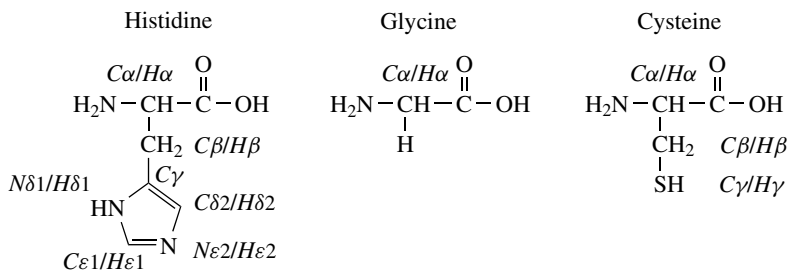


CHART 2. Proton and carbon annotations for histidine, glycine and cysteine amino acids

respectively. The ^1H chemical shift difference for the remaining non-labile protons is even smaller and varies from 0.10 to 0.75 ppm.

As far as the ^{13}C chemical shift changes are concerned, the histidine and cysteine carbons bear the most shifted protons. Thus the $\text{C}\epsilon 1$ (4.28 ppm) and $\text{C}\beta$ (4.90 ppm) carbons together with the histidine $\text{C}\gamma$ atom (which does not bear a proton) exhibit the largest variation, reaching up to *ca* 12.0 ppm. In contrast to the carbon atoms of COO^- , CONH and glycine αCH_2 groups which do not experience any chemical shift variation upon Zn(II) binding, the remaining ^{13}C resonance differences measured varied from 1.60 to 3.40 ppm. These data suggest that ^1H resonances are not considerably influenced by Zn(II) metal coordination and that ^{13}C signals are stronger indicators for monitoring the metal binding process of Zn(II) bioinorganic compounds through NMR spectroscopy.

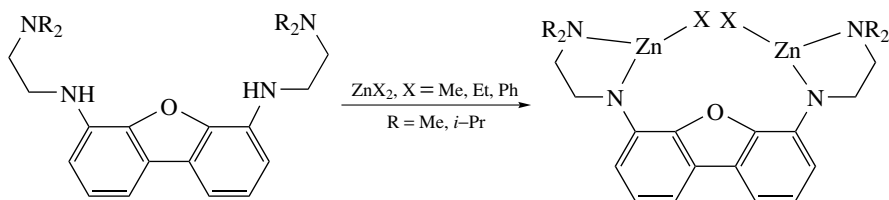


CHART 3. Zn chelating agents studied in their free and Zn(II) -bound form through $^1\text{H}/^{13}\text{C}$ NMR spectroscopy⁴

The minimal effect on proton chemical shifts in Zn organometallic compounds has been also observed through NMR studies of Zn(II) complexes with synthetic organic chelating compounds by Hlavinka and Hagadorn in a recent article⁴. In this study various tetraamino chelating agents were synthesized and used for the binding of two Zn(II) atoms per molecule. Each metal coordinates through the two nitrogen atoms of the ligand, while the other two positions of its coordination spheres are occupied by the ligands of the Zn metalation agent. Despite the nature of the ligand (different alkyl group at the terminal nitrogen atom) the chemical shift differences of the non-labile protons, sited either at the aromatic rings of the ligand or at the *N*-alkyl group ($\text{R} = \text{Me}, i\text{-Pr}$; see Chart 3), which are observed upon Zn(II) binding, vary between 0.10 and 0.55 ppm.

Other reports concerning chemically modified amino acid as metal chelating agents⁵ used for the carbonic anhydrase active site model reconstruction are in close agreement with the small contribution of Zn(II) binding to the proton chemical shift variation discussed above. NMR experiments carried out in $\text{DMSO}-d_6$, at 300 K, and the observed

chemical shift difference for histidine imidazole H ϵ 1 proton between its free and Zn(II)-bound form is measured to be only *ca* 0.50 ppm, while minimal changes for H δ 2 of *ca* 0.15 ppm have been observed as well. However, it is noteworthy that the striking difference between the ^1H NMR spectra of free and bound ligands in this study is the signal broadening. All histidine imidazole ring proton resonances in Zn(II) complexes are considerably broadened when compared with the proton signals of the free histidyl residue. Except for the two shifted signals which are also remarkably broadened, the remaining his imidazole ring signals have lost the hyperfine structure. ^1H resonance broadening usually reflects the difference in nuclei relaxation properties, providing indirect evidence for differences in the magnetic environment of the affected nuclei due to metal coordination, but it is also observed in the case of metal chelating agents with medium Zn(II) binding affinity. When Zn(II) metal is in an exchange equilibrium between its complexed (bound) and free form in solution, this effect is illustrated through signal broadening of the vicinal proton resonances.

Overall, what is reported above are some recent data from the field of the organometallic chemistry of Zn(II) compounds using a variety of metal chelating agents. All reflect the small effect of zinc binding on the NMR spectral parameters of the complexes with respect to the free organic ligands. However, a comparison between ^1H and ^{13}C data suggests that ^{13}C NMR spectroscopy provides safer evidence for the coordination of organic moieties to the Zn(II) metal than the ^1H NMR spectroscopy.

III. ^{67}Zn NMR SPECTROSCOPY OF ORGANOMETALLIC COMPOUNDS AND BIOMACROMOLECULES

From an NMR perspective, ^{67}Zn (the only NMR-active zinc isotope) is among a number of potentially important but insensitive metal nuclei such as ^{43}Ca and ^{25}Mg . However, ^{67}Zn NMR spectra of aqueous Zn^{+2} are different from ^{43}Ca and ^{25}Mg NMR spectra of aqueous Ca^{2+} and Mg^{2+} in some respects. For example, ^{67}Zn NMR spectra of aqueous Zn^{2+} have marked concentration dependences in terms of the half-band widths ($\Delta\nu_{1/2}$) compared with those of ^{43}Ca and ^{25}Mg NMR spectra of aqueous Ca^{2+} and Mg^{2+} ^{6–8}.

It has been found that the temperature effects of the ^{67}Zn NMR signals are markedly different from each other, depending upon the molecular weights of ligand molecules⁴. The intensity of ^{67}Zn NMR signals (or resonances) arises from the fact that ^{67}Zn is a quadrupolar nucleus (spin 5/2, $Q = 0.15 \times 10^{-28} \text{ m}^2$) with low natural abundance (4.11%) and a small magnetogyric ratio ($\gamma = 1.6768 \times 10^7 \text{ rad T}^{-1} \text{ s}^{-1}$). Consequently, ^{67}Zn NMR experiments are remarkably difficult. In addition to the low intrinsic sensitivity, solution state ^{67}Zn NMR is further hampered by the fact that molecular tumbling motion always induces efficient ^{67}Zn quadrupole relaxation, resulting in short lifetimes of Zeeman energy levels, which lead to broad NMR lines.

Until 1999, ^{67}Zn NMR of liquid samples was limited to studies of either highly symmetric species or unsymmetrical complexes undergoing rapid exchange with large excessive $[\text{Zn}(\text{H}_2\text{O})_6]^{2+}$ concentrations^{8–11}. Despite the biological relevance of zinc ions, the aforementioned practical difficulties have made ^{67}Zn NMR a nearly forgotten area. In the past, NMR studies of the metal binding sites of zinc-containing proteins have essentially relied on the utility of a surrogate probe, by which they replaced the native ^{67}Zn with another metal ion, e.g. $\text{Co}^{2+/3+}$, with more favorable spectroscopic properties¹². Recently, NMR-active ^{113}Cd ions were employed as a surrogate probe for ^{67}Zn ^{13,14}. Indeed, ^{113}Cd NMR has been widely employed in the spectroscopic study of metalloproteins which bear Zn(II) in their native state¹⁵. The adaptable ligand coordination, number and geometry of Cd(II) are rather similar to Zn(II) and in many cases where Cd(II) has replaced Zn(II), the catalytic activity of the metalloenzymes has been retained even to a low extent¹⁶.

In particular, in Cd(II)-Thermolysin derivative (a zinc metalloprotease with proteolytic activity similar to carboxypeptidase A), the X-ray structure has provided evidence for isostructural replacement of Zn(II) by Cd(II)^{17,18}. In general, the ^{113}Cd chemical shift is very sensitive to the nature, number and coordination type of the amino acid ligands and ^{113}Cd resonances are commonly detected by direct observation ($I = 1/2$, and 63% sensitivity compared with ^{13}C) or by inverse detection of ^{113}Cd scalar-coupled to ^1H .

A variety of inverse experiments which require a time delay for transfer of magnetization between ^1H and ^{113}Cd spins, such as ^1H - ^{113}Cd HMQC, ^{113}Cd -edited ^1H - ^1H COSY or ^1H - ^{113}Cd hetero-TOCSY experiments¹³ and 2D ^1H - ^{15}N HMQC without Cd excitation pulses, are applied for successful identification of histidines coordinated to Cd(II) metal¹⁹. The latter technique provides geometrical information for the metal coordination sphere through the determination of $^3J_{\text{H-Cd}}$ and $^1J_{\text{N-Cd}}$ for ^{113}Cd -bound imidazole rings¹⁷⁻¹⁹.

The reported data as far as ^{67}Zn NMR is concerned are sporadic, and very limited due to difficulties from a technical point of view, the nature of the ^{67}Zn nucleus, as well as all factors responsible for the latter such as concentration, the nature of the proximity ligands etc. A descriptive analysis of the last reported data concerning ^{67}Zn NMR studies will be developed with respect to the role of Zn ion or element in materials science or bioinorganic chemistry in both solution and solid state.

IV. THEORETICAL INTRODUCTION AND TECHNICAL DIFFICULTIES

Zinc is required as an integral constituent in a large number of enzymes^{20,21}. To study the catalytic or structural role of zinc ions in these biological systems, it is desirable to have a technique that can probe the chemical environment at the zinc site. In the absence of X-ray crystallographic data, direct detection of the Zn(II) ions bound to a biological macromolecule is a difficult task, because routine analytical techniques such as UV/vis, electron spin resonance (ESR) and solution NMR spectroscopy are not suitable for studying diamagnetic d^{10} Zn(II) ions. As a result, spectroscopic studies of metal-binding sites in zinc-containing proteins have reported on the utility of surrogate metal probes (e.g. Mn, Co and Cd)^{22,23}.

Recently, solid-state ^{67}Zn NMR spectroscopy has emerged as a viable method for detecting Zn(II) ions even in large molecular systems²³⁻²⁹. During these ^{67}Zn NMR studies, it has become increasingly apparent that a better understanding of the ^{67}Zn NMR properties is needed. One fundamental question is how the ^{67}Zn NMR tensors, both the chemical shift tensor and the electric field gradient (EFG) tensor, are related to the chemical environment and molecular structure. One way to decipher this NMR property/structure relationship is to use quantum-mechanical calculations. To the best of our knowledge, quantum-mechanical ^{67}Zn EFG calculations have been attempted only for simple ionic solids such as ZnO ³⁰, ZnAl_2O_4 and ZnFe_2O_4 ³¹; no theoretical study has been reported for the ^{67}Zn EFG tensors in Zn(II) coordination complexes. The fact that a reasonable amount of reliable ^{67}Zn EFG data has been accumulated in the past several years from experimental solid-state NMR studies makes the theoretical examination of ^{67}Zn EFG tensors timely and possible. Recently, Ida and Wu³², have reported a systematic quantum-mechanical investigation for the ^{67}Zn EFG tensors in six Zn(II) coordination complexes: zinc acetate dihydrate (**1**), bis(acetato)bis(imidazole)zinc (**2**), tetrakis(imidazolo)zinc perchlorate (**3**), tetrakis(thiourea)zinc nitrate (**4**), zinc formate dihydrate (**5**) and bis(acetato)bis(urea)zinc (**6**) (Figure 1).

These Zn(II) coordination complexes were chosen because both reliable X-ray crystallographic and solid-state ^{67}Zn NMR data are available in the literature. Furthermore, single-crystal ^{67}Zn NMR studies have been reported for compounds **1** and **5**, so that not only the magnitude but also the orientation of the ^{67}Zn EFG tensor is known in

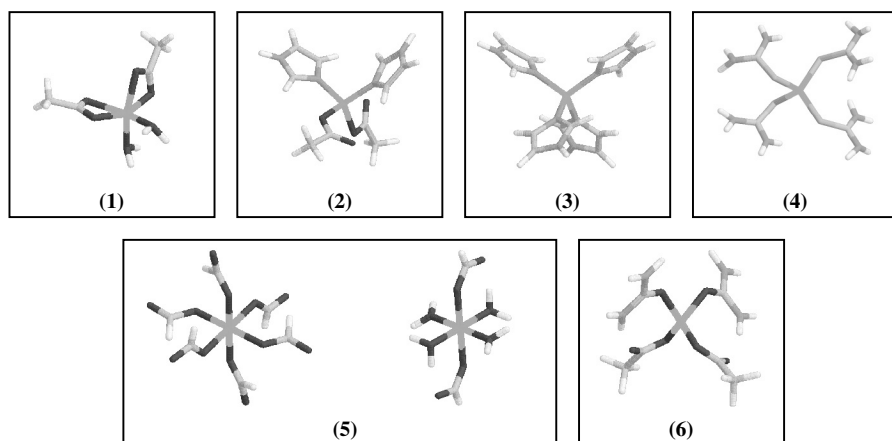


FIGURE 1. Molecular clusters models for compounds 1–6³². Reproduced with permission from Reference 32

the molecular frame of reference. This precise information is useful in determining the accuracy of the computed EFG results. The primary objective of the mentioned study is to evaluate the applicability of current computational methods for calculating ^{67}Zn EFG tensors in molecular complexes.

The authors have used a general molecular cluster approach to model the Zn(II) site in the crystal lattice. For Zn(II) coordination complexes examined in this study, the Zn(II) center is generally surrounded by ligand molecules such as imidazole and acetate groups. This is somewhat different from the ionic network solids such as ZnO and ZnAl_2O_4 , for which it is important to take into consideration the lattice periodicity. For this reason, they usually include ligand molecules only from the first coordination sphere. The only exception to this general statement is that, if the Zn center of interest is bound to water molecules and the water molecules are involved in extensive hydrogen bonding, ligands from the second coordination sphere must be included in the cluster model. This finding is important because water molecules often participate in Zn(II) ion binding in zinc-containing proteins. The implication of this study is that it is possible to predict ^{67}Zn EFG tensors for the Zn(II) sites in proteins. Although the above study is focused on EFG tensors, it should also be possible to examine the ^{67}Zn magnetic (or chemical) shielding tensors using quantum-mechanical computations. Very little is known in this latter area; however, it is hoped that the work of Ida and Wu will encourage further quantum-mechanical studies of these fundamental ^{67}Zn NMR tensors and that new reports on this issue will appear in the near future³².

V. SOLID-STATE NMR

Solid-state NMR is a branch of NMR spectroscopy that deals with solid or solid-like systems and is presently undergoing rapid expansion as a result of the significant advances in both NMR methodology and instrumentation that have occurred recently. To date, most successful solid-state NMR applications to biological systems have utilized spin-1/2 nuclei such as ^{13}C , ^{15}N and ^{31}P . For a large number of biologically important elements, however, the only NMR active isotopes are those with nuclear spins greater than one half. Such nuclei have a non-spherical charge distribution and are known as quadrupolar nuclei, for example ^{17}O ($S = 5/2$), ^{23}Na ($S = 3/2$), ^{25}Mg ($S = 5/2$), ^{39}K ($S = 3/2$), ^{43}Ca ($S = 7/2$),

^{67}Zn ($S = 5/2$) and ^{59}Co ($S = 9/2$), to mention just a few. It should be noted that all of these examples are half-integer spins. In fact, there is another type of quadrupolar nuclei for which the spin number is an integer, such as ^2H ($S = 1$), ^6Li ($S = 1$), ^{14}N ($S = 1$) and ^{10}B ($S = 3$). The integer spins have quite different NMR properties compared with those of half-integer nuclei; consequently, the techniques used to record solid-state NMR spectra of integer nuclei are unique.

In a recent study, Lipton and coworkers³³ described a new probe which has been completely redesigned in order to perform solid-state NMR spectroscopy of half-integer quadrupolar nuclides, such as Zn and Mg. This new probe includes a cross coil and variable capacitors that are operational at cryogenic temperatures. However, even with this new probe the most important issue, which had to be resolved, was the optimal sensitivity. In order to apply this technique in large molecules, such as proteins, enzymes etc., one should considerably enhance sensitivity considering that spins of interest (^{67}Zn) are found in a dilute environment. While the metal ion under investigation has an atomic mass below 100, the biopolymer has a molecular weight which ranges from 10 to 50 kDa. As the authors stated³³ after performing a number of technical modifications, the new probe reached an improvement in signal-to-noise ratio of approximately 15%. The application of this technology to a biologically interesting molecule, the carbonic anhydrase, is presented below (see Section VI).

VI. INTEREST FOR BIOINORGANIC CHEMISTRY

Zinc plays a key role in the active binding site for a range of important metalloproteins³⁴. For example, Zn^{2+} is important for the function of penicillamine⁶, insulin³⁵, carboxypeptidase A³⁴, thermolysin³⁷, phospholipase C³⁸, Angiotensin Converting Enzyme³⁹ and Anthrax Lethal Factor⁴⁰. To understand the enzymatic function of these metalloproteins, it is of interest to study the Zn^{2+} coordination environment with a variety of ligands, i.e. N, O and S donor atoms. Information on Zn^{2+} complexation may potentially be obtained from liquid-state ^{67}Zn NMR (isotropic chemical shifts, δ_{iso} ; T_1 and T_2 relaxation)^{41,42}. However, the large ^{67}Zn linewidth and poor receptivity will prevent useful data from being obtained on biological compounds via liquid-state NMR. Furthermore, the Zn^{2+} coordination is particularly reflected in the ^{67}Zn ($I = 5/2$) quadrupole coupling, an interaction which may be obtained only indirectly from liquid-state relaxation studies. Solid-state ^{67}Zn NMR is a more direct and informative probe for the local structure but is unfortunately associated with broad line shapes due to a large quadrupole moment.

To circumvent these problems it has been popular to replace Zn^{2+} with $^{113}\text{Cd}^{2+}$ ($I = 1/2$) and use empirical relations between ^{113}Cd chemical shielding anisotropy (CSA) and structure^{15–18,43,44} to obtain information about metal coordination in metalloenzymes^{45,46}. Nevertheless, ^{67}Zn NMR should be the method of choice for Zn metalloproteins. This approach removes the potential ambiguities regarding changes in local structure induced by Cd^{2+} replacement and may be used to investigate the utility of the ^{113}Cd surrogate probe strategy. Among the few ^{67}Zn solid-state NMR studies reported so far^{47,48}, one has involved the detection of a 40-kHz-wide powder pattern at 11.7 T for $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ ⁴² using the quadrupolar echo (QE) experiment⁴⁹.

For large-weight Zn^{2+} complexes with broad (50–150 kHz) second-order quadrupolar powder patterns ^{67}Zn QE NMR may be an experimental challenge. In such cases the sensitivity must be enhanced by isotope enrichment combined with, e.g., cross polarization (CP) from ^1H ⁵⁰, low-temperature acquisition⁵¹ or sampling of the free-induction decay (FID) in the presence of a train of refocusing pulses^{52–54}.

Low-temperature experiments are technically difficult and may cause the sample to be in a phase different from that at ambient temperature. Similarly, CP is demanding since it

requires the matching of an rf field amplitude on the ^{67}Zn channel of about 50 kHz with a threefold larger amplitude on the ^1H channel to obtain an efficient spin-lock. Ellis and coworkers⁵⁴ have demonstrated that ^{67}Zn QCPMG NMR represents a feasible approach to study Zn^{2+} coordination in model complexes for metalloenzyme derivatives. The QCPMG experiment⁵⁴ (equation 1)

$$(\pi/2)_x - \tau_1 - (\pi)_y - \tau_2 - \text{Acq.}(1/2\tau_a) - [\tau_3 - (\pi)_y - \tau_4 - \text{Acq.}(\tau_a)]^M - \text{Acq.}(\tau_a) \quad (1)$$

splits the QE line shape for the central transition into a manifold of spin-echo sidebands separated by $1/\tau_a$, where τ_a is the interpulse acquisition period with ^1H decoupling (M is the number of echo repetitions and τ_d an additional acquisition time to ensure full decay of the signal). Depending on the sideband separation, QCPMG may enhance the sensitivity by an order of magnitude compared to QE while maintaining information on the anisotropic interactions⁵⁴.

The applicability of the method is demonstrated using ^{67}Zn -enriched zinc formate dihydrate $\text{Zn}(\text{OOCH})_2 \cdot 2\text{H}_2\text{O}$ ⁵⁵ and zinc diimidazole diacetate $\text{Zn}(\text{OOCCH}_3)_2(\text{C}_3\text{H}_4\text{N}_2)_2$ ⁵⁶. These complexes are representatives of Zn^{2+} in an all-oxygen six-coordination sphere and in a 2-O, 2-N four-coordination sphere, respectively.

As a conclusion, they have demonstrated that ^{67}Zn QCPMG NMR, through its significant sensitivity enhancement compared to QE NMR, represents a powerful method in studies of zinc complexes. By the determination of relationships between the coordination geometry and the parameters for chemical shielding and quadrupole coupling tensors, they anticipate that ^{67}Zn QCPMG NMR will play a critical role in solid-state investigations of ^{67}Zn -enriched metalloproteins. Employing improved instrumentation, the sensitivity of the ^{67}Zn QCPMG experiment will be further improved by combination with CP and acquiring the spectra at cryogenic temperatures.

As mentioned above, the ligating units for zinc complexation in biological enzymes such as zinc fingers⁵⁷, zinc twists, zinc clusters⁵⁸, alcohol dehydrogenase⁵⁹, metallothioneins⁶⁰ and carbonic anhydrase⁶¹ are bis(cysteiny) or bis(histidiny) derivatives. These protein sequences His-X-His or Cys-Y-Cys (X, Y are 1-4 amino acids) offer N or S atoms for coordination, while in some cases residues with oxygen atom donors or H_2O were found in a coordination sphere of $\text{Zn}(\text{II})$ metal. The zinc ion is responsible for protein folding and catalytic binding of H_2O or CO_2 . Tripeptides with bis(histidiny) sequences have been investigated by Gockel and coworkers⁶². Other authors have described tripodal histidine ligands⁶³, pyrazolylborate ligands⁶⁴ and macrocyclic polyamines⁶⁵. For all these cases, NMR spectroscopy was a powerful tool in order to clarify not only the three-dimensional structure of metalloenzymes, but also the electronic and coordination properties of their metal centers.

Toward this direction, a His-X-His pseudotripeptide zinc complex (X is an *N*-alkyl glycine derivative) similar to the catalytic center of the carbonic anhydrase was computer-designed and experimentally synthesized⁶⁶. The authors applied 2D-NMR techniques in order to achieve the complete resonance assignment of all protons, carbon nuclei as well as of all nuclear overhauser effect signals. The three-dimensional structure of the complex was determined with the COSMOS (computer simulation of molecular structures) force field by applying ^{13}C bond polarization theory, chemical shift pseudoforces and restrictions for NOE distances. From molecular dynamics simulations, simulated annealing protocols and geometry optimizations, the three best structures (in terms of energy and optimized force-field parameters) were used for a final investigation by density functional theory. This detailed structural analysis of a zinc complex in combination with the profound analysis of the NMR parameters provides an excellent probe for Zn-ligand interaction through NMR. Despite the fact that no ^{67}Zn NMR experiments were performed for this

Zn-organometallic compound and the complex was synthesized with the aim of modeling the active site of carbonic anhydrase during its first catalytic step, the acquisition of the NMR properties of ^{13}C nucleus at this Zn complex could be further exploited in studies where the zinc ion would be replaced by a ^{67}Zn nucleus.

VII. INTEREST FOR MATERIAL–SOLID-STATE CHEMISTRY

A wide array of ferroelectric, piezoelectric and pyroelectric materials have titanium, zirconium and zinc metal cations as part of their elemental composition^{67–69}. Many electrical materials based on titanium oxide (titanates) and zirconium oxide (zirconates) are known to have structures based on perovskite-type oxide lattices^{67–69}. Barium titanate, BaTiO_3 and a diverse compositional range of PZT materials (lead zirconate titanates, $\text{Pb}_x\text{Zr}_y\text{Ti}_{1-y}\text{O}_3$) and PLZT materials (lead lanthanum zirconate titanates, $\text{Pb}_x\text{La}_{1-x}\text{Zr}_y\text{Ti}_{1-y}\text{O}_3$) are among these perovskite-type electrical materials.

Some materials containing the zinc cation, such as ZnO and ZnS, are also piezoelectric^{67–70}. The structural characterization of the barium, titanium, zirconium and zinc cation sites in these types of materials would aid our understanding of their chemical and physical properties, and multinuclear NMR should be helpful in this regard. The synthesis of various metal oxide ceramics often employs solid-state reactions that involve the thermal decomposition of metal hydroxide and metal chelate precursors or the use of metal alkoxide sol-gel precursor^{71–76}. In particular, PZT and PLZT ceramics have been synthesized from solid-state reactions of oxides or carbonates of the metal cations and from solutions containing $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$ and TiOSO_4 ^{74,75}. In addition to a knowledge of the metal cation chemistry, the structural characterization of the hydrogen atom chemistry of metal oxide precursor materials would be of use in helping to develop and understand synthetic pathways for the production of electrical materials, and ^1H NMR should also be the method of choice in order to address this issue.

There are a large number of scientifically and industrially important zinc products and compounds for which it would be valuable to have a characterization method, such as NMR, complementary to powder X-ray diffraction. The observation of ^{67}Zn NMR in cubic compounds, e.g. ZnS (sphaelerite), ZnSe and ZnTe^{77–80} was reported from Mössbauer^{78,79} and NQR⁸⁰ measurements, which yield a variety of coupling constants at 4.2 K, viz. 13:8.4/MHz⁷⁷, 12:45.2/MHz⁷⁶ and 13:620.8/MHz^{77–80}.

The first observation of ^{67}Zn NMR in zinc metal was by Abart and coworkers^{49a} in a measurement at 4.2 K, using a field sweeping technique, which yielded a value of $C_q = 12 : 73(4)$ MHz. No subsequent NMR observation has been reported. A measure of the temperature dependence of the ^{67}Zn nuclear quadrupole coupling in zinc metal has been obtained from time differential perturbed angular correlation (TDPAC) measurements⁸¹ using an excited state of ^{67}Zn ($I = 9/2$; 605 keV). However, the use of liquid helium temperatures and exotic short-lived isotopes precludes the adoption of these techniques for general material characterization.

The observation of ^{67}Zn NMR in zinc metal by Fourier transform NMR at a fixed frequency and around 295 K is reported by Bastow⁸². The precision in determination of edge singularities of the central ($-1/2$, $1/2$) powder lineshape, together with the sharp definition of the inner satellite transitions at 295 K, permitted an accurate determination of the isotropic Knight shift, together with the first determination of the axial component of the Knight shift. Temperature dependence for the Knight shift was also measured, for the first time, up to nearly two thirds of the melting point.

Wu²⁴ has also reported solid-state ^{67}Zn NMR spectra for ZnO, ZnS, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{Zn}(\text{CH}_3\text{COO})_2$. From the ^{67}Zn NMR spectra obtained for stationary and magic-angle spinning (MAS) powder samples, useful parameters of chemical shift anisotropy (CSA)

and nuclear coupling constant (NQCC) are determined. These results demonstrate the feasibility of natural abundance ^{67}Zn NMR studies on solids.

Recently, Ellis and coworkers²⁹ determined the crystal structure, quadrupole coupling parameters and the orientation of the electric field gradient tensors for each site of zinc formate dihydrate. According to this work there are two distinct sites in the asymmetric unit: one containing four in-plane waters with two bridging formates, the other containing six bridging formates. The solid-state NMR lineshapes have been assigned to their respective sites by using isotopic labeling and cross-polarization methods. The hydrated site corresponds to the lineshape having a quadrupole coupling constant (Cq) of 9.6 MHz and the anhydrous site has a Cq of 6.2 MHz. The absence of chemical shielding contributions to the observed lineshapes has been verified with a high-field solid-state NMR experiment performed at 18.8 T.

Finally, Bastow⁸³ used ^{67}Zn NMR as a probe to measure the electric field gradients at the metal site in the zinc halides ZnF_2 , $\gamma\text{-ZnCl}_2$, ZnBr_2 , ZnI_2 and Rb_2ZnCl_4 at room temperature. In addition, two hydration states have been detected for ZnSO_4 . For ZnF_2 and $\gamma\text{-ZnCl}_2$, an *ab initio* calculation of the electric field gradient at the Zn site using the WIEN code agrees with the experimentally observed values.

VIII. APPLICATIONS

A representative collection of various applications of ^{67}Zn NMR will be described in this section. As already mentioned in a previous section, the zinc ion is responsible for protein folding and catalytic binding of H_2O or CO_2 . For all these cases, NMR spectroscopy was a powerful tool in order to clarify the three-dimensional structure of metalloenzymes.

The basic thrust of Ellis group's developmental efforts over the past several years has been to characterize Zn^{2+} and Mg^{2+} sites in proteins using NMR spectroscopy. The simplicity of this sentence belies the fact that as little as a few years ago most of the NMR community would have described such an experiment as prohibitively difficult. The mentioned group developed a means to directly observe these metals in proteins via a low-temperature solid-state NMR experiment^{28,29}. Therefore, their interest is to exploit this new technology to define the mechanistic details of how divalent metal cations (Zn^{2+} , Mg^{2+} and Ca^{2+}) augment the chemistry of the proteins to which they bind. Moreover, they want to establish a relationship between magnetic resonance parameters for the metal and the structure of these metals in metalloproteins. The magnetic resonance parameters are sensitive measures of charge, ligand type and number, and the symmetry of the metal site. All these parameters are a reflection of the potential chemistry that occurs at the metal site. Therefore, such relationships should aid in the development and delineation of the correlation between structure and function for this important class of proteins.

Indeed, Ellis's group provided some very nice examples of the application of ^{67}Zn NMR spectroscopy when applied to proteins, such as the Minimal DNA Binding Domain of Human Nucleotide Excision Repair Protein XPA⁸⁴ and the Human Carbonic Anhydrase⁸⁵.

The former is a protein of 14.7 kDa involved in the multienzyme nucleotide excision repair (NER) pathway with a determined NMR solution structure⁸⁶. In this protein, the Zn^{2+} possesses rather a structural than a catalytic role. ^{67}Zn NMR spectra were acquired using a rather sophisticated probe (for details, see Reference 87) and operating at temperatures 5–250 K. Data acquisition was performed with the application of spin-echo methods for enhanced sensitivity^{33,84,86}. Specifically, experiments were carried out at 25 K using a combination of CP (cross-polarization)⁵⁰ and spikelet echo pulse sequences^{54,87} which provide a considerable increase in signal-to-noise ratio (of the order of 30) relative to a classical quadrupole echo pulse sequence. The proton field strength applied to the above measurements was 60 kHz with a matching field of 20 kHz for zinc and a contact time

of 30 ms. Data collection has been performed with the accumulation of 1090 transients and a selective π pulse to be set at 4 μs . Experimental data and simulations, such as line-shape parameters and isotropic chemical shifts, available for tetrakis(thiourea)zinc nitrate complex suggest that a model of a zinc metal ion coordinated to 4 sulfur atoms could account for the ^{67}Zn spectrum of XPA protein. This work of Ellis and coworkers represents the first NMR-based direct observation of the Zn^{2+} site of a metalloprotein.

The latter Human Carbonic Anhydrase Isozyme II (CAII) is a well-studied protein with a large number of crystal structures available so far⁸⁸. CAII catalyzes the reaction of CO_2 with water and the mechanism of this reaction, as any reaction catalyzed by a Zn^{2+} enzyme, is based on the water activation through ionization, where polarization accompanied by slight structural rearrangement in catalytic sites frequently involving zinc ligands' displacement. In CAII, Zn^{2+} is bound to three histidines while a hydroxide plays the role of the fourth metal ligand. His94 and His96 are coordinated to Zn^{2+} through the N ϵ 2 nitrogen atom of the imidazole ring while the third histidine, His 119, is coordinated with the other nitrogen atom of imidazole, namely N δ 1. The protonated nitrogens of these three histidines are hydrogen-bonded to the backbone or side-chain atoms of some residues found in close spatial proximity, such as Gln92, Glu117 and Asn244. The residues involved in this H-bond network seem to control the apparent pK_a of the presumed zinc–water⁸⁹ and enhance the zinc affinity in CAII⁹⁰. Ellis and coworkers⁸⁵ applied ^{67}Zn solid-state NMR spectroscopy in order to study the pH dependence of the metal site by monitoring the ^{67}Zn NMR parameters and probing the nature of the fourth ligand. Measurements were carried out for protein samples at different pH, at 9.4 T (400 MHz for ^1H) and 18.8 T (800 MHz for ^1H) with a modified probe, different than that used for the ^{67}Zn NMR investigation of the XPA protein described above, and at a temperature of 10–20 K depending on protein concentration and paramagnetic doping. Basic NMR parameters were set for proton pulse width at 5.5 μs using a ^{67}Zn Hartman–Hahn matching field 3 times less and a ^{67}Zn -selective π pulse width of 5.5 μs , and recycle time from 10 s (at 9.4 T) to 20 and 60 s (at 18.8 T). The extracted values of Cq are found to be similar (*ca* 10 MHz) despite the different pH values of protein samples, indicating that ^{67}Zn NMR parameters are not affected by the type of the zinc ligand (water or hydroxide).

The authors attempted to fit the experimental NMR data to potential conformations of CAII's active site. Therefore, they developed three possible models (representing potential coordination states of the CAII zinc site) through the application of *ab initio* electronic structure calculation to the protein's active site determined by X-ray crystallography by Liljas and coworkers⁹¹. The first minimal model considers the three zinc-bound histidines and a water molecule or a hydroxide ion and the second consists of the first models with five additional water molecules. The third, the most complicated, takes into consideration the second one adding new structural elements, which represent the side-chains of the residues (Gln92, Glu117 and Asn244, the former two represented as formic acids and the latter as formamide) proposed to be hydrogen bonded to the protonated nitrogen atoms of histidine zinc-ligands. The *ab initio* calculations fit rather well with NMR-derived Cq values, that is around 10 MHz for the second and third model but not for the first one, suggesting that the hydroxide occupies the fourth position in the Zn^{2+} coordination sphere in CAII when the enzyme exists in a pH range of 5.0–8.5. These data are found rather consistent with previous EXAFS data, which had also suggested the hydroxide as the fourth zinc ligand⁹².

The biological implication of this study is highly relevant to the fact that the enzyme's mechanism is pH-dependent and, in the case that a hydroxide is the fourth zinc ligand at pH 5, the deprotonation of the $\text{Zn}-\text{OH}_2$ could not be simply described by the acid/base equilibrium with a pK_a value close to 7.0. The combination of the ^{67}Zn NMR spectroscopy data and *ab initio* structure calculations supports the existence of a hydroxide instead of

a water throughout the pH range examined. This study on the 30 kDa CAH provides convincing evidence for the potential role and application of the ^{67}Zn NMR spectroscopy to the study of ^{67}Zn -loaded metalloproteins of rather high molecular mass, providing new insights not only for the structural determination of the enzyme's active site but also valuable information for its function mechanism.

Another interesting example is the applicability of the zinc complex of L-carnosine (L-CAZ; generic name Polaprezinc) for medical use. This is the first drug for oral administration in which zinc plays an essential role. L-CAZ was approved as an anti-ulcer drug of membrane protection type⁸⁰. Characterization of L-CAZ was achieved by various spectroscopic methods along with elemental analysis. Zinc ion coordinates with L-carnosine to form a quadridentate 1:1 complex of polymeric nature in order to maintain low strain of chelate rings. L-CAZ can remain in the stomach juice without rapid dissociation and adhere specifically to ulcerous lesion, after which L-carnosine and zinc are released to heal the ulcer. L-CAZ exhibited high efficacy in clinical use without any serious side effect. L-CAZ exhibited an inhibitory effect on helicobacter pylori. Physicochemical aspects of carnosine, zinc and the zinc complex can explain favorable features of L-CAZ as a drug.

Recent advantages in the technology of NMR instrumentation (magnets, probes, amplifiers etc.) and NMR spectroscopy methodology could probably lead to more effective studies on exotic nuclei such as ^{67}Zn and Zn-organometallic compounds or Zn-metalloproteins and enzymes, with apparent interest either for materials science or for bioinorganic chemistry. Until then, NMR studies of ^{67}Zn -enriched compounds would possibly need the cooperative use of theoretical calculations and X-ray-derived structural information in order to characterize the magnetic and electronic properties of ^{67}Zn nucleus. To this aim, the new technological and methodological achievements of the group of Ellis could be applied in new Zn-organometallic or Zn-containing polypeptides in order to accumulate additional NMR data and to expand our knowledge base about the properties and features of ^{67}Zn nuclei in a variety of coordination environments.

IX. REFERENCES

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