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Zinc Solid-State NMR Spectroscopy of Human Carbonic Anhydrase: Implications for the Enzymatic Mechanism

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Abstract: The pH dependence of the ^{67}Zn solid-state nuclear magnetic resonance spectroscopy of human carbonic anhydrase (CAII) has been investigated to characterize the nature of the fourth ligand. CAII, through the Zn^{2+}-bound hydroxide, catalyzes the deceptively simple reaction: $CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$. The accepted mechanism for CAII would predict that water would be bound to the Zn^{2+} at pH 5 and hydroxide would be bound at pH 8.5. The measured values for the electric field gradient (EFG) or quadrupole coupling constant (Cq) for CAII are independent of pH within the limits of the experimental error, i.e., 9.8 ± 0.2 MHz. The EFG interaction has been predicted by ab initio electronic structure calculations for water and hydroxide bound to the zinc, including various levels of hydrogen bonding. After comparing the predicted Cq's with the experimental values, we conclude that the species present from pH 5−8.5 is the hydroxide form. The NMR data presented here is not consistent with the accepted mechanism for CAII. We show that the NMR data is consistent with an alternative mechanism of CAII.

Introduction

Many zinc enzymes utilize zinc-bound water as a critical component of a catalytic reaction. The Zn^{2+} activates water through ionization, polarization, or simple displacement, depending upon the mechanism.[1] The mechanism is determined primarily by the influence of directly bound Zn-ligands, as well as hydrogen bonding with a secondary coordination sphere of side chains and/or bound waters within the protein. We wanted to characterize the ^{67}Zn NMR parameters of the metal with both water and hydroxide as the fourth ligand so we have chosen to study human carbonic anhydrase isozyme II (CAII). This information provides further understanding of the observed pH dependence of the activity of this well-studied protein.[2]

While CAII has been extensively studied (there are in excess of 180 crystal structures of CAII or its various mutants), it is an incompletely understood protein.[3] Carbonic anhydrase, through the Zn^{2+}-bound hydroxide, catalyzes the deceptively simple reaction:

$$CO_2 + H_2O \leftrightarrows HCO_3^- + H^+ \quad (1)$$

The other ligands to the zinc are the imidazole side chains of three histidines. Two of the histidines, His94 and His96, are coordinated through their Nϵ2 atoms, whereas the remaining imidazole, His119, is coordinated through its Nδ1 atom. The protonated nitrogens of these imidazoles are then hydrogen bonded to the Gln92 carboxamide, the backbone carbonyl of Asn244, and the Glu117 carboxyl group, respectively.[4] The hydrogen-bonding interactions are shown in Scheme 1 where X represents either zinc-bound water or -hydroxide. These "indirect ligands"[5] have been shown to fine-tune the apparent pK_a of the *presumed* zinc−water[6] as well as increase the zinc affinity of CAII.[7] In addition the hydroxyl of Thr199 accepts a hydrogen bond from the zinc-bound water/hydroxide. Moreover, His64 has been implicated as a proton shuttle in current mechanisms.[8] The accepted mechanism has the enzyme acting in several steps as indicated in eqs 2 and 3.

$$[EZn{\cdot}OH_2]^{2+} \rightleftarrows [H - EZn{\cdot}OH]^{2+} \xrightleftharpoons{B:} [EZn{\cdot}OH]^{1+} + BH \quad (2)$$

$$[EZn{\cdot}OH]^{1+} \xrightleftharpoons{CO_2} [EZn{\cdot}HCO_3]^{1+} \xrightleftharpoons{H_2O} [EZn{\cdot}OH_2]^{2+} + HCO_3^- \quad (3)$$

The most active form of the protein has a turnover rate of $\sim 10^6$ s^{-1}. The rate-limiting step seems to be the intramolecular transfer of a proton from the activated water to its destination on His64 through a water network. The mechanism of proton transport through the water most likely follows a "structured

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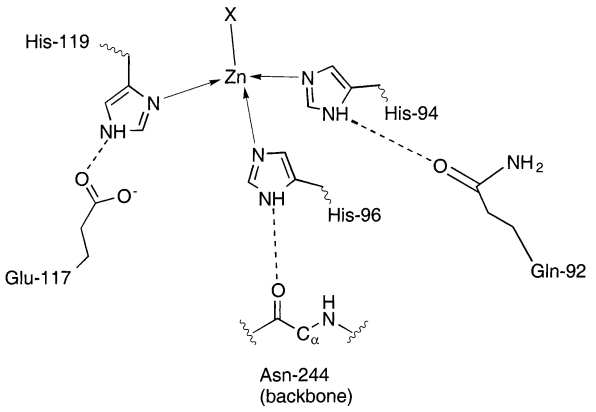
Scheme 1



diffusion" mechanism.[9] It is important to note that the zinc has a stable oxidation state of +2, while the equations above show the net charge cycling from +2 to +1 and back during the catalytic sequence. To balance these charge effects the enzyme must be a dynamic anion. It has been postulated that secondary ligand interactions are involved to counter the charge cycling, which are reflected at the zinc.[10]

NMR spectroscopy was chosen to study this zinc chemistry as most methods of direct examination find the zinc spectroscopically silent (due to its closed shell d^{10} configuration). The magnetic resonance parameters for Zn^{2+} are sensitive to the nature of bound ligands. This sensitivity arises because the dominant interaction for Zn^{2+} is the electric field gradient at the nucleus. The electric field gradient[11] originates within the electron distribution of the molecule subject to the constraint that the atom of interest is not a center of T_d or O_h symmetry. The electric field gradient at the Zn^{2+} is further augmented when ligands have different charge, depending on the ligand environment (water versus anionic hydroxide). Hence, a quadrupolar nuclide should be sensitive to changes in structure and bonding at such sites.

The principal observable in a solid-state ^{67}Zn NMR experiment is the quadrupole coupling constant, Cq. This coupling constant is directly proportional to the electric field gradient at the Zn^{2+} ion; Cq is given by

$$\mathrm{Cq} = q_{zz}\left[\frac{e^2}{(a_0{}^3h)}\right]Q \qquad (4)$$

where Q is the quadrupole moment of the nucleus in question, and q_{zz} is the zz element of the field gradient tensor. The atomic constants (e, a_0, and h) have their usual meanings.

As a direct result of these relations, the ^{67}Zn Cq values should be sensitive to the field gradient changes associated with water or hydroxide ligation to Zn^{2+}. Analysis of the ^{67}Zn NMR line shape leads to the determination of Cq and the asymmetry of the quadrupole tensor, η_q. Therefore, we have employed low-temperature (10 K) solid-state ^{67}Zn NMR[12,13] spectroscopy to directly probe the nature of the bonding at Zn^{2+} in human CAII.

Experimental Methods

Sample Preparation. Enriched $^{67}Zn(CH_3COO)_2$ was prepared by dissolving zinc-67 metal (88% enrichment from Cambridge Isotope Laboratories) in a 3:1 (v:v) mixture of glacial acetic acid and water. Crystals were obtained by slow evaporation of the excess water and acetic acid. The human CAII protein containing $^{67}Zn^{2+}$ or Co^{2+} was overexpressed from a T7 expression vector in *Escherichia coli* strain BL21 (DE3) grown in a defined media of M9 salts and either 10 μM $^{67}Zn(CH_3COO)_2$ or 10 μM $CoCl_2$.[14,15] Purification was essentially as described before except the purified protein was extensively dialyzed against deionized water and concentrated with an Amicon stirred-cell ultrafiltration unit with a YM 10 000 NMWC filter.[16] ^{67}Zn−CAII (200−300 mM) was equilibrated under a nitrogen atmosphere, and then the pH was adjusted to 8.5 or 5 with dilute NaOH or H_2SO_4 respectively. Co^{2+}−CAII mixed with an equimolar amount of the tight binding inhibitor acetazolamide was added as a dopant at 10% of total protein in each ^{67}Zn−CAII sample, and the pH was checked and readjusted if necessary. Samples were frozen in liquid nitrogen and lyophilized.[17] It was our expectation that the protein would maintain its ionization state before and after lyophilization. This has been shown for other proteins.[17] Lyophilized samples were maintained in a nitrogen atmosphere and rehydrated by vapor diffusion with 20% sample weight of water. The mass of the samples were 65, 58, and 60 mg, for the pH 5, 7, and 8.5, respectively. The NMR experiments were carried out as previously described.[13,18]

^{67}Zn NMR spectroscopy. Data collection at 18.8 T was performed on a Varian Unity^{Inova} console in an Oxford continuous flow cryostat. The NMR probe built for this system is different from the one previously described[12] in that a cross coil is now utilized and the tuning elements are now inside the cryogenic area.[18] Proton pulse width used was 5.5 μs with a ^{67}Zn Hartman−Hahn[19] matching field 3 times less[20] and a ^{67}Zn-selective π pulse width of 5.5 μs. A 30-ms contact time was used for cross polarization (CP),[21] and a proton "flip-back" pulse[22] employed to aid in recycle time (20 and 60 s for pH 5 and 8.5, respectively). Experimental conditions differed for each due to sample amounts and effectiveness of the paramagnetic doping.[13] The data for the sample at pH 5 was acquired at 10 K, needing 128 transients per frequency, and the data for the sample at pH 8.5 was acquired at 20 K with 64 transients per experiment. The frequency steps for each sample

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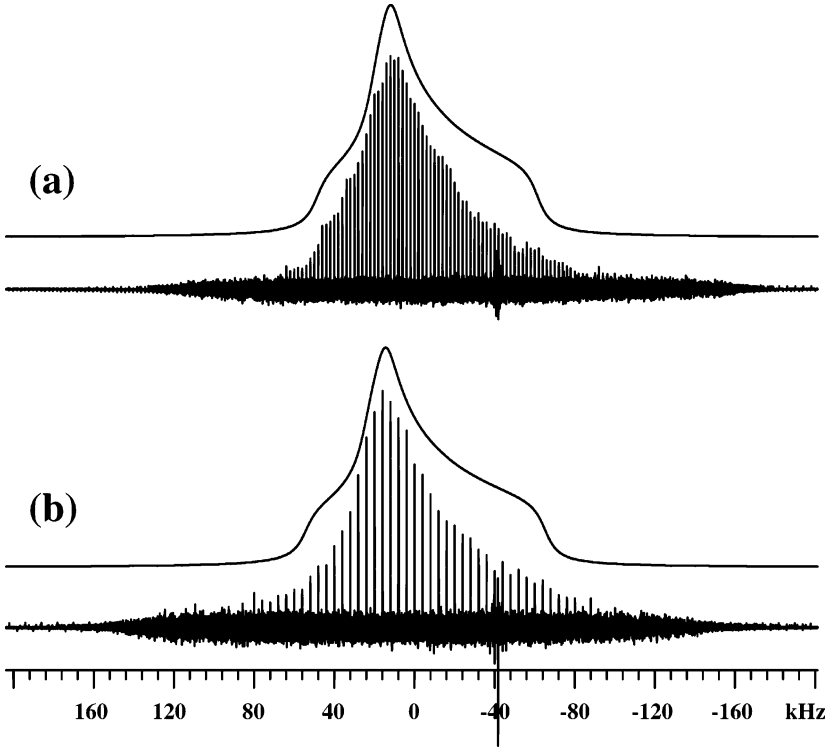

Figure 1. Presented are the low-temperature (10 K) solid-state ^{67}Zn NMR spectra of CAII at (a) pH 5 and (b) pH 8.5. Above each experimental spectrum is a simulation of the spikelet envelope. At pH 5, the extracted value of Cq is 9.6 MHz, whereas at pH 8.5 the value is 10 MHz. The spectrum at pH 5 was acquired with 32 transients and 2 min recycle delay, while the spectrum at pH 8.5 was acquired with 64 transients and 1 min recycle. Each sample was doped with 7% Co(II) CAII to manage the ^{1}H relaxation times.[13]

were 20 kHz, and the resulting data were frequency shifted in the software and then combined in a sky projection (as previously described).[13] Data collection at 9.4 T was performed at 10 K on a Varian Infinity^{Plus} spectrometer in an Oxford continuous flow cryostat with a home-built transmission line probe.[12] Proton pulse width used was 5 μs with two 30-ms contacts and a recycle time of 10 s (also employing a proton "flip-back" pulse). The zinc-selective π pulse width used was 15 μs. The number of transients collected at 9.4 T was 4096 for each 10 kHz frequency step. Data were frequency shifted in software and then combined in a sky projection. The improved S/N ratio at 18.8 T vs 9.4 T is due in large part the second order nature of the central transition quadrupole line shape. That is, the breadth of the central transition line shape depends inversely upon the resonance frequency. Therefore, the width of the central transition at 18.8 T is half of its width at 9.4 T.

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Results and Discussion

Presented in Figure 1 are the ^{67}Zn NMR spectra of CAII at pH 5, spectrum a, and 8.5, spectrum b, obtained at 18.8 T (800 MHz for ^{1}H). The NMR spectrum at pH 8.5 was acquired with 4-kHz spikelet separation. The pH 5 spectrum was acquired with a 2-kHz separation. The envelope of the spikelets is the overall NMR spectrum. Above each experimental spectrum is a simulated powder spectrum, which is a fit to the spikelet envelope. The extracted values of Cq are 9.6 and 10 MHz, for pH 5 and 8.5, respectively.[23] Essentially, the experimental Cq values are the *same* for the two pH values, Cq is 9.8 $\pm$ 0.2 MHz. We have also measured the ^{67}Zn spectrum of CAII at pH 7 at 9.4 T (400 MHz for ^{1}H's) and obtained a spectrum that is consistent with those reported here, i.e., a Cq value of 10 MHz. This spectrum and its fit are depicted in Figure 2. Hence, we can conclude the ^{67}Zn NMR spectrum of CAII is independent of pH, within our experimental error of nominally $\pm$0.2 MHz.

The data clearly indicate that either the ^{67}Zn NMR parameters are insensitive to the presence of water or hydroxide or there is something more fundamental occurring. To examine the question of sensitivity of the ^{67}Zn NMR parameters we performed ab initio electronic structure calculations[24] on progressively more complicated models for the active site of CAII. The initial geometry used for this model construction was from the protein crystallography of CAII by Liljas, et al.[4] (PDB accession number 2CBD). From this structure we developed three models for the active site of CAII. The first model simply contains the four primary ligands to the Zn^{2+} ion: the three histidines (approximated as methyl imidazoles), and either water or hydroxide. The second model includes the first model plus five additional water molecules (the OH group of Thr199 was approximated

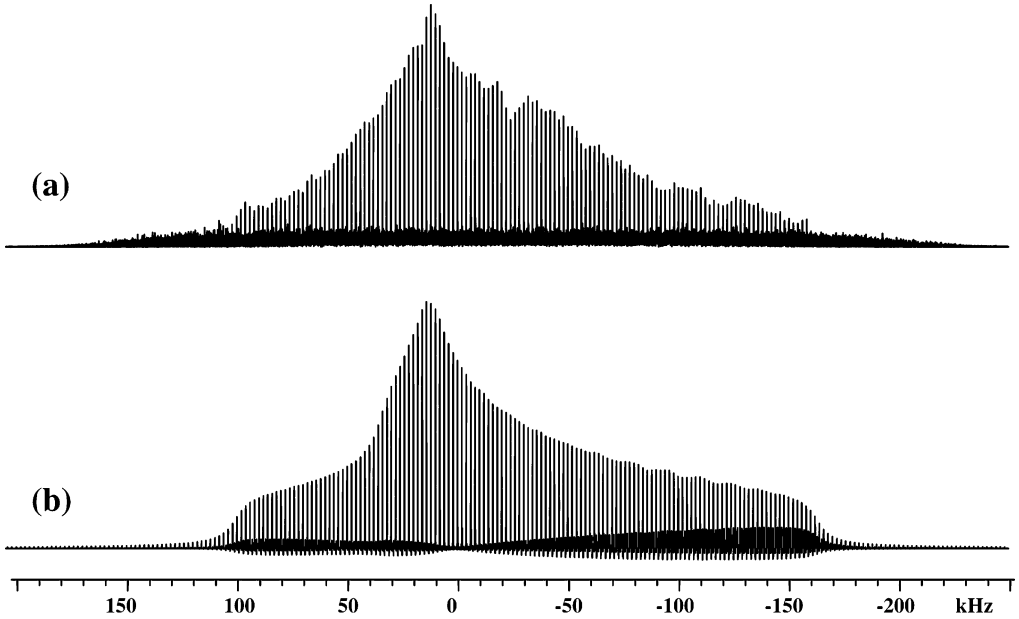

Figure 2. Shown are (a) the sky projection of the ^{67}Zn NMR spectra of CAII at pH 7 acquired at 9.4 T and 10 K and (b) a simulated QCPMG experiment using ideal pulses and a Cq of 10.5 MHz, η of 1.0, a matched apodization function of 10 kHz,[12] and conventional line broadening of 10 Hz.

Table 1. Computed Cq Values in MHz

	$N_3Zn(OH_2)$	N_3ZnOH
model 1:	37.6	29.2
model 2:	25.49	10.36
model 3:	35.16	8.72

as a water molecule). The third model contains the second model plus appropriate elements approximating the side chains that are potential hydrogen-bond acceptors to the three imidazole NH groups (Gln92 and Glu117 are each represented as formic acid and Asn244 as formamide). We added protons to each of the models and then optimized the geometry of each model with ab initio electronic theory at the double ξ level of basis set and then computed the electric field gradient tensor at the triple ξ with added polarization functions. It should be recognized at this point that the calculation is well defined at some quantum mechanical level. However, the details of the optimized geometry do not necessarily reflect the details of the protein structure. The model is simply that: a model for the various coordination states in CAII. Approximations in these models are appropriate since the models predict qualitatively different quadrupole couplings that can be compared with experiment.

The results of these are summarized in Table 1. Because of the *absence* of solvation, the first model with a hydroxide bound to Zn^{2+} provided an excellent description for the Cq of the Parkin zinc complex $[Tp^{Bu^t,Me}]ZnOH$.[25,26] However, for CAII the first model of coordination with water or hydroxide illustrates the simple point that one cannot ignore the bound waters around the active site. Clearly, model 1 does not correspond to the experimental results reported here. Hence, the active-site waters play an essential role in establishing the dynamic electric field gradient (and electrostatics) at the Zn^{2+} ion in CAII. Addition of waters (including the interaction of Thr199 as a water) in model 2 helps improve the level of agreement between theory and experiment for the hydroxide. Here the calculated results are Cq's of 25.49 and 10.36 MHz, with water and hydroxide as the fourth ligand, respectively. The addition of active-site waters has had a profound effect on the electric field gradient for both species. Addition of secondary hydrogen-bonding interaction between the histidines and appropriate hydrogen bond acceptors within model 3 has a dramatic effect on the bound water species but does not alter the calculated trend. Namely, the calculations predict that the Zn^{2+}−CAII with bound water should manifest a large value of Cq in the range of 25−35 MHz. Whereas, when hydroxide is bound, the value of Cq is smaller, in the range of 8−10 MHz. The calculations suggest the ^{67}Zn NMR parameters can reveal the presence or absence of this proton. The agreement between theory and experiment is acceptable for model 2 or 3 with hydroxide. Moreover, the results of these calculations and our experimental data suggest that hydroxide is the fourth ligand of the Zn^{2+} in CAII in the pH range from 5 to 8.5.

This observation is bolstered by prior EXAFS[27] work, which did not observe a significant change in structural parameters in carbonic anhydrase as a function of pH. Typical Zn−O bond distances are: $Zn-OH_2$ distances range from 1.98 to 2.02 Å, whereas the Zn−OH distances cluster around 1.85 Å.[26] The results of theoretical calculations (Model 3 for both water and hydroxide) support that there should be ∼0.1 Å decrease in the Zn−O bond distance in going from water to hydroxide. The EXAFS results reported by Spiro and co-workers[27a] were more focused on the pH dependence of zinc coordination number and the average bond distance of *all* of the zinc ligands. Their results indicated that the coordination number and the average zinc ligand bond distance were independent of pH. The EXAFS results of Amiss and Gurman[27b] are more to the point. They reported an average Zn−O bond distance determined at room temperature in carbonic anhydrase (isozyme I) of 1.82 ± 0.02 Å, over the pH range of 5−9 consistent with the presence of hydroxide as the fourth ligand bound to Zn^{2+}.

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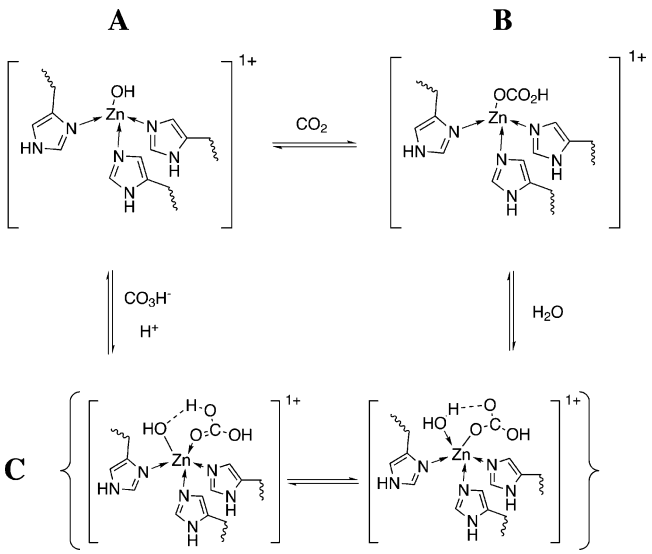
Scheme 2



Conclusions

Having a zinc-bound hydroxide at pH 5 implies that the deprotonation of the $Zn-OH_2$ is not well described as a simple acid/base equilibrium with a pK_a near 7. This has implications for the mechanism as numerous pH profiles of the activity of this enzyme have shown that the catalytic rate of this enzyme is dependent upon the ionization state of two moieties within the active site.[3] One site is His64, the so-called proton-transfer site that shuttles protons from the active site to solution.[8] The other site has been *assumed* to be the zinc-bound water/hydroxide. The NMR data presented here are not consistent with such a picture.

The NMR data are, however, consistent with certain aspects of the mechanism originally proposed by Merz, Hoffmann, and Dewar[28] and refined by Mauksh et al.,[29] for example.

Scheme 2 shows a proton leaving between step **C** and **A**, this goes to the proton shuttle (His64), while the bicarbonate remains in the buffered solution. In this mechanism, water is never bound to Zn^{2+} by itself but rather is bound to the zinc in a five-coordinate complex with bicarbonate. Our experiments do not prove or disprove the existence of these intermediates as there was never any CO_2 present in our samples. Hence, in an environment devoid of CO_2 the expected species would be the hydroxide and *not* water bound to zinc regardless of the pH of the media. The proposed mechanism suggests that addition of water to the zinc•bicarbonate complex involves a facile loss of hydrogen from the water, which eliminates the concern of the complex cycling between a charge of +2 to +1. Moreover, a concerted displacement of an anion with the deprotonation of water would predict that different anion inhibitors of CAII would alter the observed pK_a of this deprotonation event. Cobalt-substituted CAII does indeed have shifted pK_a's in the presence of various anion inhibitors.[30] This alteration to the mechanism of catalysis by CAII is speculative, yet it is in agreement with experimental studies.

The generally accepted mechanism for CAII uses a water molecule bound to Zn^{2+}. Further, it has been experimentally determined that there is a moiety within the protein with a pK_a of ~7. It has been assumed that this moiety is the Zn^{2+}-bound water. By acquiring data on the protein prepared at three pH values we have shown the ^{67}Zn NMR of CAII to be independent of pH. The resulting quadrupole coupling constants were interpreted through the use of ab initio electronic structure calculations on models of increasing complexity. The results support the existence of hydroxide and *not* water being bound to the Zn^{2+} throughout the pH range investigated. Further, these experimental results demonstrate that data collection on a ^{67}Zn-labeled protein of 30 kDa is possible as well as that there is sufficient sensitivity to observe a metal in a higher-molecular weight protein. These exciting findings on this thoroughly studied enzyme further demonstrate the usefulness of this methodology for investigating structure/function relationships in zinc metalloproteins.

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