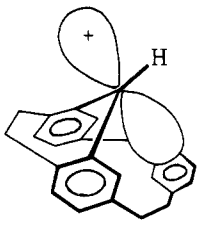

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electron cloud of the "face" benzene ring. Thus water can attack the pseudo-geminal bridge carbon only from the opposite side to this ring. This explanation is also applicable to the similar attack of hydride in the reduction of **1**. As mentioned above, there is only a slight difference (about 20°) in the inclined angles of the "equatorial" and the "axial" bonds; therefore, the present significant enhancement ($10^{14.7}$ times) of the solvolysis rate of **3**-OTf relative to **2**-OTf should be attributed to the π participation[17,18] of the "face" benzene ring with the generated carbonium ion. It is of much interest that the "face" ring locates more than 3.5 Å above the pseudo-geminal bridge carbon.

Finally, when the rate constant of **2**-OTf is compared with those of the secondary systems under the present condition, *exo*-bicyclo[3.1.0]hex-6-yl triflate (**11**), reported as the least

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reactive secondary alicyclic system,[10d] is merely 44 times less reactive than **2**-OTf. Thus, **2** is the least reactive benzhydryl system ever reported.

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(7) Compound **3**-Cl: mp 204.0–206.0 °C; M^+ 358 (21) *m/e* 323 (100); NMR δ ($CDCl_3$) 2.24–3.17 (10 H, m, –CH–), 3.78–3.99 (2 H, m, $-CH_2-$), 5.13 (1 H, s, H_{eq}), 5.51 (2 H, m, H_a), 6.14 (2 H, d, J = 1.7 Hz, H_o), 6.94 (2 H, d, J = 8.0 Hz, H_m), 7.00 (2 H, d of d, J = 1.7, 8.0 Hz, H_p), 7.07 (2 H, m, H_b); ir (cm^{-1}) (KBr) 2900, 1500, 1440, 810, 740, 610.
(8) Compound **7**-OPNB: mp 169.0–170.0 °C; NMR δ ($CDCl_3$) 2.9–3.8 (4 H, m, $-CH_2-$), 7.1–7.6 (8 H, m, Ph), 8.20 (4 H, s, NO_2PhCOO); ir (cm^{-1}) 1718, 1523, 1267, 1095, 720.
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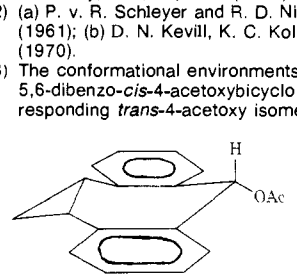

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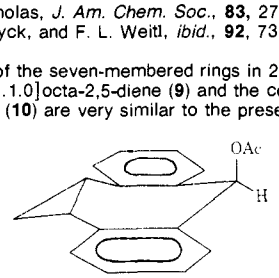

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Cadmium-113 Fourier Transform Nuclear Magnetic Resonance of Cadmium(II) Carbonic Anhydrases and Cadmium(II) Alkaline Phosphatase

Sir:

The essential nature of the 2b metal Zn(II) in maintaining the catalytically active form of a wide variety of metalloenzymes is well established.[1,2] Elucidation of the structure of the metal ion binding site and the functional role of the metal(s) in the mechanism of enzyme action by study of the native protein or analogs containing the chemically similar group 2b metal ions (Cd(II), Hg(II)) bound at the Zn(II) site is limited by the intrinsic properties of these ions (filled d shell) making them of little value as spectroscopic probes. Thus, despite differences in preferred coordination geometry and susceptibility to ligand-field induced structural distortions, the characteristics of the enzyme-bound metal have been largely inferred from the spectral properties of the enzymes in which transition metal ions have been substituted for the native Zn(II) ion.[1] Recent reports on the application of FT-NMR to metal nuclides in a variety of inorganic salts and small model complexes[3,4] suggest that this technique may be suited to the direct observation of the diamagnetic analogue of the native metal bound in the microenvironment of enzymes. In this regard, the ^{113}Cd nucleus with spin $1/2$ and its higher sensitivity to NMR detection compared to ^{67}Zn is a reasonable first candidate. The large paramagnetic contribution to the shielding constant leads to large changes in the chemical shift with changes in the nature of bonding to the metal ion as reflected in a chemical shift range of >640 ppm for common compounds of cadmium.[3] This fact coupled with its extreme sensitivity to substituent effects and a large dipolar contribution to the relaxation mechanism make ^{113}Cd an ideal NMR probe.

We wish to report on the observation and characteristics of the FT-NMR resonances of the $^{113}Cd(II)$ ion substituted for the intrinsic Zn(II) ion(s) of the metalloenzymes, bovine carbonic anhydrase B (BCAB), human carbonic anhydrase B (HCAB), and alkaline phosphatase of *E. coli* (AP).[5]

BCAB and HCAB were obtained from bovine and human erythrocytes.[6] AP was isolated from *E. coli* CW3747.[7] Zinc was removed from the purified enzymes either by dialysis

Table I. ^{113}Cd Chemical Shifts and Line Widths for Some Cd:Amino Acid Complexes and ^{113}Cd(II) Metalloenzymes

Compound[b]	pH[c]	°C	$\Delta\nu_{1/2}$ (Hz)	Chemical shift[a]
$CdCl_2$:glycine (1:2)	7.0	24	45	−160.6
$CdCl_2$:glycine (1:2)	7.0	8.5	>200	−164
$CdCl_2$:glycine (1:3)	7.0	24.5	22	−177
$CdCl_2$:glycine (1:3)	7.0	9.5	*ca.* 56	−179.6
$CdCl_2$:glycine (1:2)	8.95	26	9	−210.8
$CdSO_4$:glycine (1:2)	8.2	23	86	−108
^{113}Cd:imidazole (1:6)[d]	8.3	23	9	−133
BCAB	8.0	23	*ca.* 40	−214
HCAB	9.6	23	*ca.* 28	−145.5
AP	6.5	23	*ca.* 40	−172.2

[a] Chemical shifts are reported in ppm from external 0.10 M $Cd(ClO_4)_2$. [b] Samples prepared from natural abundance Cd salts ranged from 0.5 to 1.2 M in Cd. [c] pH adjustments were made with concentrated NaOH. [d] Prepared from a stock solution of 25 mM ^{113}Cd in 25 mM Tris-sulfate.

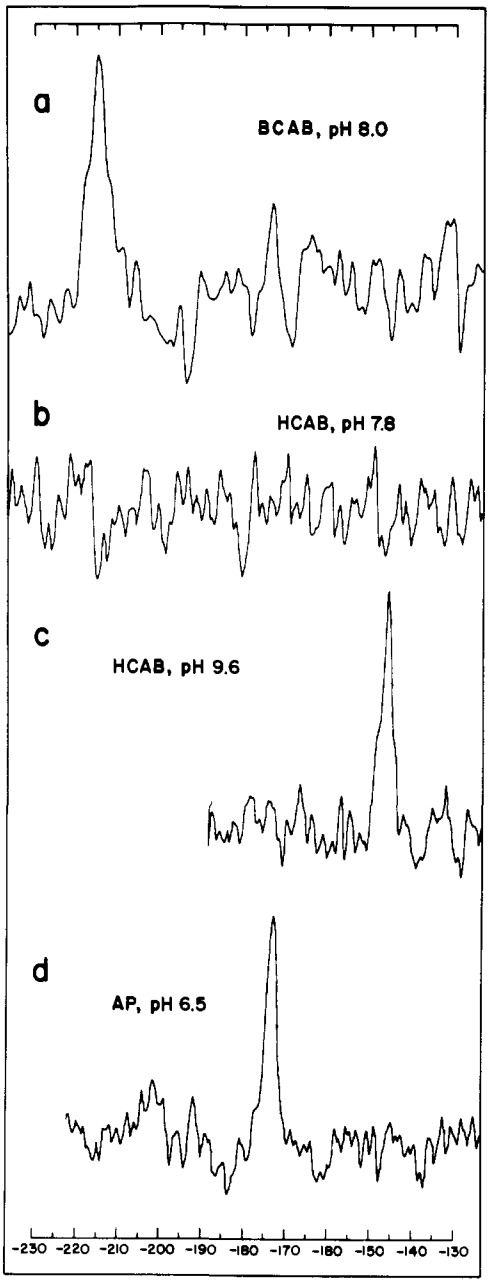

Figure 1. ^{113}Cd NMR spectra of ^{113}Cd(II) carbonic anhydrases and ^{113}Cd(II) alkaline phosphatase: (a) 3.04×10^{-3} M bovine carbonic anhydrase B, 2.92×10^{-3} M ^{113}Cd, 0.025 M Tris-sulfate, pH 8.0; (b) 1.0×10^{-2} M human carbonic anhydrase B, 9.8×10^{-3} M ^{113}Cd, 0.025 M Tris-sulfate, pH 7.8; (c) as (b) pH 9.6; (d) 3.97×10^{-3} M alkaline phosphatase, 7.70×10^{-3} M ^{113}Cd, 0.01 M NaOAc, 0.01 M Tris, 0.1 M NaCl, pH 6.5.

against *o*-phenanthroline (BCAB, HCAB)[8] or by treatment with Chelex resin (AP).[7] Cadmium metalloenzymes were prepared by addition of 96 atom % ^{113}Cd(II) salts (Oak Ridge Laboratories) to solutions of the apoenzymes, followed by concentration to a volume of ~1 ml.

^{113}Cd FT NMR spectra were obtained on a FT-Bruker HFX 90-MHz spectrometer with an operating frequency of 19.96 MHz modified for multinuclear capabilities in a manner similar to that already reported.[9] D_2O in a 3-mm coaxial capillary insert was used as an external field frequency lock. For the metalloenzyme spectra shown in Figure 1, a spectral width of 5000 Hz was used with 1024 accumulated data points and a pulse delay of 0.15 s. The Fourier transform was carried out with 2K time domain points providing a resolution of 5 Hz/point. Measurements were made at 25 ± 2° on ~1.0 ml samples contained in 10-mm sample tubes. Approximately 250 000 transients were required to obtain each spectrum. Proton decoupling was not employed because of the negative value of the nuclear magnetic moment for ^{113}Cd and the predicted dependence of the negative NOE on correlation time.

The chemical shifts and line widths for complexes of ^{113}Cd(II) with amino acids, chosen as plausible models of the metal-coordinating nuclei in the enzymes,[5] are given in Table I. The sensitivity of both chemical shift and line width to pH can be attributed to the relative instability of well-defined complexes with resulting variation in the number and rate of exchange of ligands at the central metal ion as a function of pH. The effects of variation of temperature, counterions, and ligand concentration are consistent with this interpretation. The chemical shift range (~50 ppm) observed for even this abbreviated series of ligands indicates the sensitivity of the resonance position of the ^{113}Cd(II) nucleus to subtle changes in environment. Ligand exchange mechanisms appear to be the principal contributor to line broadening, T_2, while proton-decoupling experiments show a large dipolar contribution to the longitudinal relaxation time, T_1.

The chemical shifts of the enzyme bound ^{113}Cd(II) resonances (Figure 1, Table I) fall in the range anticipated from the model complexes. The magnitude of the observed line widths are consistent with the metal bound to a macromolecular ligand, and there does not appear to be a significant contribution to T_2 from chemical shift anisotropy. Successive additions of aliquots of ^{113}Cd(II) to the BCAB sample depicted in Figure 1 do not perturb the observed resonance but result in the appearance of a very broad resonance at ca. −95 ppm consistent with the excess ^{113}Cd(II) ion exchanging between the medium and low affinity protein binding sites with relatively slow dissociation rates. In the presence of noise modulated proton decoupling, the resonance shown in Figure 1a vanishes into the noise indicating a sizeable dipolar contribution to the spin-lattice relaxation mechanism.

The activity of the carbonic anhydrases as a function of pH

has been related to changes in the immediate environment of the metal ion by a variety of spectroscopic methods.[10] The sigmoid pH functions describing these changes have been attributed to the ionization of either a coordinated water molecule or an adjacent amino acid side chain.[10a] For BCAB the apparent pK of the activity profile occurs at pH 6.9–7.4, while for HCAB the pK is 7.5–8.1, depending on substrate and conditions, for both the Zn(II) and Co(II) enzymes.[11] At pH 9.6 the resonance of ^{113}Cd(II) bound at the active site of HCAB appears as a relatively sharp line ($\delta\nu_{1/2} \simeq 28$ Hz) at −145.5 ppm. At pH 7.8 no resonance was detected under identical sampling conditions. The pH dependence of the ^{113}Cd resonance of HCAB thus appears in accord with the recently reported pH-rate profile for esterase activity of the Cd(II) enzyme (pK = 9.1) and the midpoint of the function describing the change in the nature of the Cd(II) coordination complex as detected by perturbed angular correlation of γ rays.[12] In contrast to ^{113}Cd(II) HCAB, a resonance from ^{113}Cd(II) bound to BCAB can be detected at pH 8.0 as a slightly broader line ($\delta\nu_{1/2} \simeq 40$ Hz) at −214 ppm. The variation in the relaxation of the ^{113}Cd nucleus at the active sites of the carbonic anhydrases with pH most likely reflects an alteration in the nature and exchange mechanisms of the monodentate ligand from solution. Broadening of the ^{113}Cd resonance by an exchangeable ligand (H_2O or ^-OH) from solution will be expected to be extremely sensitive to both the species of ligand and its exchange rate. ^{113}Cd NMR may prove to be a powerful tool for exploring the access of solvent or other ligands from solution to the metal binding site of Zn(II) metalloenzymes. Identification of the number and nature of the ligands contributing to the exchange phenomenon may in principle be derived from the solvent, temperature, and pH variation of the resonance and such studies are currently underway.

The appearance of a single sharp resonance ($\delta\nu_{1/2} \simeq 40$ Hz) at −172.2 ppm (Figure 1) for 2 equiv of ^{113}Cd(II) bound per AP dimer supports the postulated identity of the two metal binding sites in the absence of external ligands.[13] The effect on the ^{113}Cd(II) resonance of further metal ion addition and of alterations in the metal ion environment at one or both of the active sites arising as a consequence of allosteric interactions between the subunits accompanying phosphate binding is being determined.[14]

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The Use of ^{13}C Spin Lattice Relaxation Times for Determining the Position of the Proton in an Intramolecular Hydrogen Bond

Sir:

A particularly illuminating feature of the hydrogen bond is the location of the proton between the donor and acceptor atoms. Because of the difficulty of resolving hydrogen atoms by x-ray crystallography, the few data available have usually been obtained by neutron diffraction analysis.[1] We now describe a relatively simple method for locating the average position of the proton in the intramolecular hydrogen bond in 1-hydroxyfluorenone. This method appears widely applicable and involves measurements on liquids rather than single crystals.

Generally, the most important contribution to the spin lattice relaxation time of a ^{13}C nucleus is the dipole–dipole term (T_1^{DD}) association with neighboring protons. For a given proton, this term is proportional to $r_{C,H}^{-6}$, $r_{C,H}$ being the internuclear separation of the proton and the ^{13}C nucleus. For carbon bonded to hydrogen only the directly bound proton(s) contributes significantly to T_1^{DD}. For fully substituted carbon atoms, however, protons as far away as 3 Å may make a measurable contribution which can be used to estimate $r_{C,H}$. In general, the distances of a proton to three carbons will be necessary to define its location. In planar systems, such as 1-hydroxyfluorenone, two suffice.

The separation of the contribution of the hydrogen bonded proton to three fully substituted carbon atoms, 1, 1a, and 9, from the contributions of other protons is conveniently achieved by measuring the relaxation times in the normal molecule and the O-*d* species using samples prepared under identical conditions (simultaneous vacuum transfer of degassed solvent into two NMR tubes separately containing the same quantity of the two compounds). The contribution is then given by eq 1.[2]

$$1/T_1^{DD}(H) = [1/T_1^{obsd}(H) - 1/T_1^{obsd}(D)]/[1 - \gamma_D^2 I_D(I_D + 1)/\gamma_H^2 I_H(I_H + 1)]$$

$$1/T_1^{DD}(H) = 1.063[1/T_1^{obsd}(H) - 1/T_1^{obsd}(D)] \quad (1)$$

$$1/T_1^{DD}(H) = K/r_{C,H}^6 \quad (2)$$