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(10) $R(F_o) = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, $R(F_o^2) = \Sigma|F_o^2 - F_c^2|/\Sigma F_o^2$, $R_w(F_o^2) = [\Sigma w_i|F_o^2 - F_c^2|^2/\Sigma w_i F_o^4]^{1/2}$. σ_1, the standard deviation of an observation of unit weight, is defined by $[\Sigma w_i|F_o^2 - F_c^2|/(n-p)]^{1/2}$ where $w_i^{-1} = \sigma^2(F_o^2) = \sigma_c^2(F_c^2) + (0.02F_o^2)^2$; σ_c is determined by counting statistics, n denotes the number of observations, and p denotes the number of parameters varied during the least squares.
(11) The plane of each of the two coplanar Cr–H–Cr fragments is oriented at angles of 44.2 and 45.9° with respect to the two perpendicular mean planes each passing through the two chromium atoms and the two axial and four equatorial ligands.
(12) In contrast to the direction of $\mu(3)$ for the bridging hydrogen atom, the maximum rms components of thermal displacement for the axial carbon and oxygen atoms (*viz.*, $\mu(3)$ = 0.287(3) Å and $\mu(3)$ = 0.391(4) Å, respectively) are directed essentially parallel to the Cr–H–Cr plane and normal to the Cr–Cr line. The acute angles between the direction of $\mu(3)$ for the bridging hydrogen atom and $\mu(3)$ for the axial carbon and oxygen atoms are 86 (4)° and 87 (4)°, respectively.
(13) The estimated displacements were based upon the assumption that the two half-weighted carbon and two half-weighted oxygen atoms can be represented with isotropic thermal displacement of 0.20 and 0.24 Å, respectively; each of these values is approximately equal to the mean value of the thermal displacements of the composite peak normal to the corresponding maximum displacement, $\mu(3)$.
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Cadmium-113 Nuclear Magnetic Resonance Studies of ^{113}Cd(II)-Substituted Human Carbonic Anhydrase B

Sir:

Carbonic anhydrases (carbonate hydrolyase EC 4.2.1.1) are zinc metalloenzymes found in animals, plants, and certain bacteria, which catalyze the reversible hydration of carbon dioxide ($CO_2 + H_2O = HCO_3^- + H^+$), the hydrolysis of certain esters, and various other reactions.[1] Carbonic anhydrases from human erythrocytes (HCA) are monomeric enzymes of molecular weight ~29 000, each molecule containing a single equivalent of firmly bound Zn(II) which is required for catalytic activity. X-ray crystallographic studies of the low-activity (HCAB)[2a] and high-activity (HCAC)[2b] isozymes reveal the zinc ions near the bottoms of 12–15 Å clefts, coordinated to nitrogen atoms from three histidyl side chains in distorted tetrahedral geometry, with the fourth coordination sites presumably occupied by water molecules or hydroxide ions. Kinetic studies on HCA point to the existence of two (or more) species per isozyme in acid–base equilibrium having pK_a values near 7, with the high-pH forms producing faster hydration rates. The identities of the various active species and the detailed mechanisms of their action remain in dispute.[3]

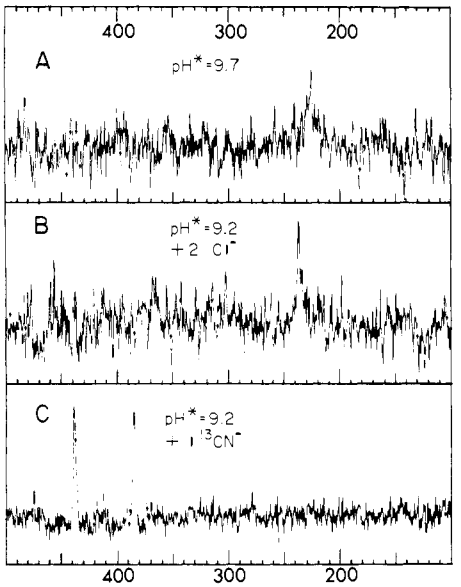

Figure 1. ^{113}Cd FT NMR spectra at 25 °C of 96% isotopically enriched ^{113}Cd^{II}HCAB in 85% H_2O/D_2O and 25 mM Tris sulfate. Chemical shifts (ppm) based on 1.0 M $CdSO_4$ at −2.8 ppm (ref 5a). No proton decoupling was employed. pH* values are uncorrected for presence of 15% D_2O. Exponential multiplication with 7 Hz line broadening was applied to the free-induction decays: (A) 5 mM enzyme, pH* 9.7, no inhibitors present, 12-h accumulation; (B) 7 mM enzyme, pH* 9.2, 2 equiv of NaCl added, 5-h accumulation; (C) above sample, plus 1 equiv of K^{13}CN (90% isotopic enrichment), 10-h accumulation.

Direct observation by NMR of the metal at the active site of a metalloenzyme is expected to provide information regarding the chemical environment of the active site, free from the background interference characteristic of ^{1}H and ^{13}C NMR spectra of proteins. Advances in sensitivity of modern Fourier transform NMR spectrometers and the use of large (15–20 mm o.d.) sample tubes have made it possible to consider direct observation of individual atom resonances for millimolar enzyme solutions.[4] However, ^{113}Cd, with its spin quantum number $I = \frac{1}{2}$, is expected to produce superior NMR spectra in a ^{113}Cd(II)-substituted enzyme over that of ^{67}Zn, with its lower gyromagnetic ratio and $I = \frac{5}{2}$, with the resultant likelihood of quadrupole broadened resonances in the ^{67}Zn(II)-enzyme. Furthermore, it has been demonstrated that ^{113}Cd, like many heavy metals, exhibits a large chemical shift range (>600 ppm), making it potentially very sensitive to changes in the active site environment.[5]

Replacement of zinc ions by other divalent metal ions has often been used to provide spectroscopic probes of the active site of carbonic anhydrase. With the notable exception of Co(II), most divalent metal ions fail to restore much catalytic activity. Recently, however, it has been shown that Cd(II)-HCAB is an effective catalyst, at least for the hydrolysis of *p*-nitrophenylacetate, with a pK_a value of ~9.1 for the activity-linked functional group.[6]

Figure 1A shows the ^{113}Cd NMR spectrum[7] at 25 °C of 4 mL of ^{113}Cd(II)HCAB[8] at pH* 9.7 in the absence of mono-

valent anions or other inhibitors. This spectrum shows a single, broad (~300 Hz) line centered at about 228 ppm from aqueous Cd(II) at infinite dilution. Under no experimental condition at 25 °C have we observed a resonance more narrow than ~250 Hz for uninhibited $^{113}Cd^{II}HCAB$. This is at variance with a recent report[9] of a rather sharp (28 Hz) resonance centered at 146 ppm for $^{113}Cd^{II}HCAB$ at pH 9.6. Our studies on complexes of Cd(II) with heterocyclic nitrogen ligands result in ^{113}Cd resonances in the range 200 to 270 ppm.[10] In the pH* range 7.3-9.7 we have consistently observed a broad peak of ~300 Hz or greater line width, generally centered at about 200 ppm at lower pH* values and about 230 ppm at higher pH* values.

Figure 1B shows the effect of addition of 2 equiv of NaCl. The resonance sharpens to ~60 Hz, and the chemical shift value is 238.6 ppm. The addition of several more equivalents of NaCl has no discernible effect. The effect of a single equivalent of NaCl has not yet been determined. Assuming the presence of a single, tight Cl^- binding site with at least 90% occupancy, we calculate a Cl^- dissociation constant of 7×10^{-4} M or less. With the reported inhibition constant $K_i \geq 2 \times 10^{-2}$ M for $Cd^{II}HCAB$,[6] it is unlikely that Cl^- binds directly to Cd(II) under the conditions of Figure 1B. There is ample evidence for the existence of two strong anion binding sites[3b,11]—one which inhibits enzyme activity, presumably by direct metal binding, and an even tighter but noninhibitory binding site which is probably within ~4 Å of the metal ion. The presence of Cl^- bound near the metal has apparently affected whatever exchange process is responsible for the peak broadening in uninhibited $^{113}Cd^{II}HCAB$.

Any ^{113}Cd resonance of line width less than ~45 Hz in the proton-coupled $^{113}Cd^{II}HCAB$ spectrum must be viewed cautiously considering the presence of five C(2) and C(4) protons[2a] with vicinal Cd-N-C-H spin-coupling constants (10-13 Hz in analogous compounds[12]).

Figure 1C shows the $^{113}Cd^{II}HCAB$ spectrum after addition of 1 equiv of $K^{13}CN$ (≥90% isotopic enrichment, Merck). The resonance splits into a doublet centered at 410 ppm with a separation $J_{CdC} = 1{,}060$ Hz and line width ~50 Hz. This is the largest known cadmium coupling constant and indicates a Cd-C bond of lifetime $>10^{-2}$ s. Addition of a second equivalent of $K^{13}CN$ produced no further change.[13] There has been considerable speculation regarding the existence of stable pentacoordinate Zn(II) in HCA.[14] Considering the larger ionic radius of Cd(II), we conclude that there is probably only one available binding site for CN^- in $Zn^{II}HCAB$. A large excess of $^{13}CN^-$ has not yet been tried on $^{113}Cd^{II}HCAB$, although this was apparently not necessary to produce the pentacoordinate species in $Co^{II}HCAB$.[14b]

Our experiments to date indicate T_1 values of 2-3 s for ^{113}Cd in Cd(II)HCAB based on flip angle optimization. In agreement with a previous report,[9] we find that proton decoupling leads to a loss of the ^{113}Cd signal. These results and our dipolar T_1 and N.O.E. calculations based on five carbon-bound imidazole protons at 2.8 Å distance from $^{113}Cd(II)$ in a molecule having a rotational correlation time of 10^{-8} s (and a negative gyromagnetic ratio for ^{113}Cd) are consistent with a purely dipolar relaxation mechanism.

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Electron Spin Exchange in Rigid Biradicals

Sir:

We have prepared six nitroxyl biradicals in which the extent of conformational change is strongly limited by the rigidity of the structure connecting the radical groups. These biradicals