I (β-cyclodextran) column, 0.025 M Tris-OAc (pH 7.5):MeOH (70:30), 1.0 mL/min, 285 nm) of the acetates. Authentic 7- and 8-acetoxyquinolines were prepared by standard acetylation (pyridine/acetic anhydride) of commercially available phenols (retention times (min): 8-acetoxy (6.0), 7-acetoxy (8.5)). Structures of the standards were confirmed by NMR. The only compound detected from the acid hydrolysis reaction was 8-acetoxyquinoline. A mixture of the 8-acetoxyquinoline and *trans*-7,8-diacetoxy-7,8-dihydroquinoline obtained from base hydrolysis was chromatographed on the cyclodextran column eluted with 85:15 (0.025 M Tris-OAc (pH 7.5):MeOH). This resulted in a separation of the enantiomers of the 7,8-diacetoxy derivative from 8-acetoxyquinoline (retention times (min): enantiomer 1 (8.5), enantiomer 2 (9.5), and 8-acetoxy (10.5)).

Base hydrolysis was carried out under the standard kinetic conditions in 0.1 M NaOH (3.2 mg of oxide in 50 mL) for 66 h ($5.7t_{1/2}$). The solution was then neutralized, saturated with salt, and extracted with EtOAc. The organic phase was dried (Na_2SO_4), solvent was removed under reduced pressure, and the products were acetylated. The 300-MHz ^1H NMR spectrum (CD_3COCD_3) of the mixture revealed a product ratio of 55:45 (8-acetoxyquinoline:*trans*-7,8-diacetoxy-7,8-dihydroquinoline).

***N*-Methylquinoline 5,6-Oxide Cation (4). Synthesis.** Reaction of the 5,6-oxide with excess dimethyl sulfate in 1 M phosphate buffer at pH 7.4 for 1.5-2.0 h gave a product with a UV spectrum very similar to that of the protonated but unmethylated oxide. Mass spectral data were obtained on a Californium-252 PDMS after dissolving in MeOH and electrospraying onto an aluminized Mylar film: m/z 160 (M^+), 144 ($-CH_4$ or O), and 142 ($-H_2O$).

Kinetics. Acid hydrolysis of **4** was attempted at pH 1.85 and 4.10 under standard conditions and was monitored spectrophotometrically (220-400 nm) as illustrated in Figure 2. The spectrophotometric experiment at pH 1.85 showed a very small increase in absorbance at 250 nm, which was attributed to a 12% contamination with unmethylated oxide. For solutions with an initial absorbance of 1 at the λ_{max} ca. 300 nm, the change in absorbance at 250 nm was 1.16 for unmethylated **2** and 0.14 for **4** contaminated with **2**; equal extinction coefficients at λ_{max} for **4** and protonated **2** were assumed. As further evidence that the N-methylated oxide (**4**) was stable at acidic pH (1.82), the reaction was monitored by HPLC (4.6 × 250 mm Du Pont Zorbax phenyl column, eluted with 0.05 M NH_4OAc (pH 5.00):CH_3CN, gradient 80:30 to 20:70 in 25 min, at 1 mL/min, 301 nm). No significant change (≤6%) in the ratio of N-methylated oxide (rt 5.8 min) to *p*-nitrobenzyl alcohol (internal standard, rt 12.4 min) was observed over 66.5 h, indicating that the N-methylated oxide is essentially stable in acid ($k_{obsd} < 4 \times 10^{-7}$ s^{-1}). Base hydrolysis was carried out over a pH range of 9.64 to 12.35 and monitored spectrophotometrically at 270 nm.

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Hydrogen/Deuterium Fractionation Factors of the Aqueous Ligand of Cobalt in $Co(H_2O)_6^{2+}$ and Co(II)-Substituted Carbonic Anhydrase

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Abstract: We have measured the hydrogen/deuterium fractionation factor for the rapidly exchanging, water ligands of cobalt in $Co(H_2O)_6^{2+}$ and in two Co(II)-substituted isozymes of carbonic anhydrase. The fractionation factor was determined from NMR relaxation rates at 300 MHz of the protons of water in mixed solutions of H_2O and D_2O containing these complexes. In each case, the paramagnetic contribution to $1/T_2$ was greater than to $1/T_1$, consistent with a chemical shift mechanism affecting $1/T_2$. The fractionation factors obtained from T_1 for $Co(H_2O)_6^{2+}$ and for the isozymes of Co(II)-substituted carbonic anhydrase were close to the fractionation factor for bulk water which is unity. The fractionation factors obtained from T_2 were 0.73 ± 0.02 for $Co(H_2O)_6^{2+}$ and 0.72 ± 0.02 for Co(II)-substituted carbonic anhydrase I, with a value of 0.77 ± 0.01 for isozyme II. We concluded that fractionation factors in these cases determined from T_1 and T_2 measured isotope preferences for different populations of ligand sites. We suggest that since T_2 has a large contribution from a chemical shift mechanism, the fractionation factor determined from T_2 has a large contribution of the fractionation of inner-shell ligands. The fractionation factors determined from T_1 are close to unity, the value of the fractionation factor of bulk water, and contain a larger contribution of the fractionation of outer-shell water. Although not yet helpful in interpreting the complex contributions to the isotope effects in the enzymatic catalysis, these hydrogen/deuterium fractionation factors for the water bound to cobalt in carbonic anhydrase can be significantly different from the fractionation factor for solvent water and appear not to be sensitive to the active site environment in these two isozymes of carbonic anhydrase.

The hydrogen/deuterium fractionation factor, ϕ, of a metal-bound water is the equilibrium constant for this isotope exchange reaction:

$$M(HOH) + (HOD) \rightarrow M(HOD) + (HOH)$$

$$\phi = \frac{[M(HOD)]/[M(HOH)]}{[HOD]/[HOH]}$$

This fractionation factor measures the tendency of deuterium to accumulate at the aqueous ligand of the metal relative to the deuterium content of bulk solvent. Fractionation factors are valuable in interpreting the effects of deuterium on kinetic and equilibrium constants because they represent individual contributions to isotope effects measured relative to the common reference of water. Values of the fractionation factor for various functional groups are known,[1,2] and the fractionation of hydrogen isotopes in water bound to metal ions has been considered.[1] However, there are few reports of the fractionation factor of the aqueous ligands of a metal. Using NMR methods, Silverman[3] has measured such a factor for the water ligands of cobalt in cobalt(II)-substituted carbonic anhydrase II ($\phi = 1.05 \pm 0.17$) and Melton and Pollack[4] for $Cr(H_2O)_6^{2+}$ ($\phi = 1.00 \pm 0.03$). They relied on the properties of a paramagnetic metal to enhance the

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relaxation rate of water exchanging rapidly between bulk solvent and the hydration shell of the metal. By measuring the proton relaxation rate as a function of the deuterium content of solvent the fractionation factor of the metal-bound site can be determined.

The aqueous ligand of the metal at the active site of carbonic anhydrase is believed to have a role in the catalytic pathway. Direct nucleophilic attack by the metal-bound hydroxide on CO_2 is a likely step in the production of bicarbonate,[5] and an intramolecular proton transfer involving the metal-bound water is proposed to be a rate-limiting step in catalysis by isozyme II.[6] The solvent hydrogen isotope effect associated with the turnover number for CO_2 hydration catalyzed by carbonic anhydrase II is 3.8.[6] To interpret fully the solvent hydrogen isotope effects observed for catalysis by carbonic anhydrase requires knowledge of the fractionation factor of the aqueous ligand of the metal.

We have determined the fractionation factors of the aqueous ligands of Co^{2+} in $Co(H_2O)_6^{2+}$ and in two mammalian isozymes of Co(II)-substituted carbonic anhydrase using the proton relaxation rates $1/T_1$ and $1/T_2$ at 300 MHz. In each case the paramagnetic contribution to $1/T_2$ was greater than to $1/T_1$, consistent with a chemical shift mechanism in $1/T_2$. The values of the fractionation factor that we determined from T_1 for $Co(H_2O)_6^{2+}$ and each isozyme of Co(II)-substituted carbonic anhydrase were close to the fractionation factor for bulk water which is unity. The fractionation factors obtained from T_2 were less than unity and close to the fractionation factor for H_3O^+ which is 0.69. We conclude that fractionation factors in these cases determined from T_1 and T_2 measured isotope preferences for different populations of ligand sites. We suggest that since T_2 has a large contribution from a paramagnetic chemical shift, the fractionation factors determined from T_2 have a large contribution of the hydrogen/deuterium fractionation of inner-shell ligands. The fractionation factors determined from T_1 are close to unity, the value in bulk water, and contain a larger contribution of the fractionation of outer-shell water.

Methods

Enzymes. Human carbonic anhydrase I was purified from human red blood cells by an affinity chromatography method.[7] Bovine carbonic anhydrase II was obtained from Sigma Chemical and used after extensive dialysis to remove paramagnetic impurities. The concentration of carbonic anhydrase I and II was estimated at 280 nm using $\epsilon = 4.7 \times 10^4$ and 5.4×10^4 M^{-1} cm^{-1}, respectively.[8]

The apoenzymes of carbonic anhydrase I and II were prepared according to the procedure of Hunt et al. by dialysis against dipicolinic acid.[9] Co(II)-substituted carbonic anhydrase I and II were prepared by the addition of 1.1 equiv of $CoCl_2$ followed by dialysis against several changes of large volumes of deionized water.

NMR Measurements. The NMR relaxation times T_1 and T_2 of water protons were measured at a frequency of 300 MHz on a Nicolet NT-300 spectrometer at 23 °C. T_1 was measured by the inversion recovery method according to Freeman et al.,[10] and T_2 was measured by the Carr-Purcell-Meiboom-Gill sequence.[11] Some measurements of T_1 of water protons in solutions of Co^{2+} were performed at frequencies between 0.01 and 50 MHz on a field cycling "relaxometer" at the IBM T. J. Watson Research Center, Yorktown Heights, NY.[12] The observed relaxation of water protons in a solution of a paramagnetic ion is the sum of the relaxation due to the paramagnetic ion, $1/T_{ip}$ (i = 1, 2), and the relaxation in the absence of the ion, $1/T_{io}$,

$$1/T_i = 1/T_{ip} + 1/T_{io} \quad (1)$$

The contribution of the paramagnetic ion to the longitudinal relaxation is given by[13]

$$1/T_{1p} = p'[1/(T_{1m} + \tau_m)] \quad (2)$$

and the paramagnetic contribution to the transverse relaxation is given by[14]

$$1/T_{2p} = p'\left[\frac{(1/T_{2m})(1/T_{2m} + 1/\tau_m) + \Delta\omega_m^2}{\tau_m((1/T_{2m} + 1/\tau_m)^2 + \Delta\omega_m^2)}\right] \quad (3)$$

where T_{im} is the relaxation time of a proton in the hydration shell of the metal and is described by the Solomon-Bloembergen equations,[15,16] τ_m is the lifetime of a proton in the hydration shell, and p' is $q[M]/55.5$ where q is the hydration number and [M] is the concentration of the metal ion. $\Delta\omega_m$ is the chemical shift difference between a proton in the primary hydration shell and a proton in the free solvent site. If $\Delta\omega_m^2$ is small relative to the other terms, eq 3 reduces to eq 2. In the case of $Co(H_2O)_6^{2+}$, $1/T_{io}$ was taken as the relaxation of water containing only buffer, with no added metal ions. In the cases of Co(II)-substituted carbonic anhydrase I and II, $1/T_{io}$ was taken as the relaxation of solutions of the Co(II)-substituted enzyme in the presence of a molar excess of the inhibitor acetazolamide, which is known to displace the aqueous ligand of the metal at the active site. Enough acetazolamide was present to bind to 99.9% of the active sites. Alternatively, $1/T_{io}$ was taken as the relaxation of solutions of the native zinc enzyme, which has no paramagnetic contribution. These two methods were equivalent at a frequency of 300 MHz for Co(II)-substituted carbonic anhydrase I and II. R_i, the relaxivity, is defined as the paramagnetic relaxation per millimolar concentration of metal ion.

$$R_i = (1/T_{ip})/([M]1000) \quad (4)$$

Solutions of $Co(H_2O)_6^{2+}$ for NMR measurements contained ultrapure $CoCl_2$ (Aldrich Gold-Label) with 5×10^{-4} M acetic acid buffer at pH = 4.4. This pH was necessary to preclude the formation of multinuclear hydrolysis products.[17] When EDTA was present no acetic acid was used. Solutions of enzyme for relaxation measurements were buffered with 50 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] at pH 8.5. At this pH the relaxivity of solutions of Co(II)-substituted carbonic anhydrase I and II is greatest and in a region that is independent of pH.[18] The values of pH reported here are uncorrected pH meter readings. The correction of a pH meter reading in 100% D_2O (pD = meter reading + 0.4) is approximately offset by the change in ionization state of the buffer in D_2O ($pK_{D_2O} - pK_{H_2O} = 0.5 \pm 0.1$ for almost all acids with pK values between 3 and 10).[19] Water used for solution preparations was distilled and passed through two ion-exchange resin cartridges (Cole-Palmer 1506-35). D_2O (99.8%) was stirred with activated charcoal, the charcoal was filtered out, and then the D_2O was distilled.

Measurement of the Hydrogen/Deuterium Fractionation Factor. The measurement of the relaxation of water protons in solutions of varying deuterium content and containing paramagnetic metal ions is given by

$$1/T_{ip} = p'[X]/(1 - n + n\phi) \quad (5)$$

where n is the atom fraction of deuterium in solvent water, ϕ is the hydrogen/deuterium fractionation factor of the water ligands of the metal, and [X] is the term in brackets in eq 2 and 3 for i = 1 or 2, respectively. This equation is derived considering the fractionation of isotopes that arises when there is a deuterium content in water. In this case the ratio of protons in the hydration shell of the metal to total protons in solution is $p'/(1 - n + n\phi)$.[1] Equation 5 can be rearranged to

$$p'T_{ip} = [X]^{-1} - n(1 - \phi)[X]^{-1} \quad (6)$$

Thus $p'T_{ip}$ vs n gives a slope of $-(1 - \phi)[X]^{-1}$ and an intercept of $[X]^{-1}$, and ϕ = 1 + slope/intercept.

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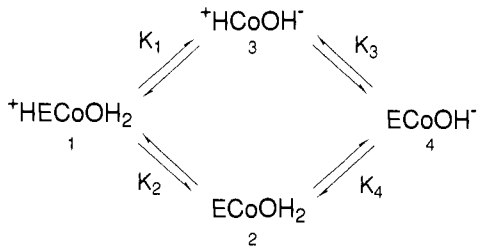
Scheme I. A Diagram of Two Significant Ionizations At and Near the Active Site of Co(II)-Substituted Carbonic Anhydrase II[a]



[a] One is the ionization of metal-bound water and the second is suggested to be the ionization of His-64,[22] the imidazole ring of which is about 7.5 Å from the metal. For Co(II)-substituted carbonic anhydrase I the second ionization could be that of either His-64 or His-200.[35]

Table I. Paramagnetic Contribution to the Relaxivities of Water Protons Observed for Aqueous Solutions of $CoCl_2$ and Co(II)-Substituted Carbonic Anhydrase I and II

	freq, MHz	R_1, $mM^{-1}\ s^{-1}$	R_2, $mM^{-1}\ s^{-1}$	T_{1p}/T_{2p}
$Co(H_2O)_6^{2+}$	20[a]	0.18	0.21	1.2
$Co(H_2O)_6^{2+}$	300[b]	0.16	2.0	12.5
Co(II) carbonic anhydrase I	300[c]	0.28	2.3	8.2
Co(II) carbonic anhydrase II	300[c]	0.26	1.4	5.3

[a] From Bernheim et al.[25] for an unbuffered solution of 0.5 M $CoCl_2$ at 25 °C. [b] Determined in this work at 23 °C for 10 mM $CoCl_2$ which contained 10% D_2O by volume and 5×10^{-4} M acetic acid buffer. The pH was 4.4. [c] Measurements were done at 23 °C for solutions of enzyme buffered at pH 8.5 by 50 mM Hepes. Solutions contained 20% D_2O by volume.

Determination of Equilibrium Dissociation Constants. The equilibrium dissociation constants describing the binding of iodide ion to Co(II)-substituted carbonic anhydrase were determined from visible absorption measurements.[20] The pH dependence of the absorbance at 640 nm in solutions of Co(II)-substituted carbonic anhydrase I and II was measured on a Beckman DU-7 spectrophotometer. Stock solutions of enzyme at low pH contained 12.5 mM Hepes and 25 mM Mops (4-morpholinepropanesulfonic acid) buffer, and at high pH 0.5 M of triethylenediamine. The stock solutions had equal concentrations of enzyme. The titrations were performed by adding small increments of the high pH enzyme solution to the low pH enzyme solution. In this way the enzyme concentration stayed the same though the volume increased slightly. These titrations were done in H_2O and D_2O in the presence and absence of iodide ion which has been shown to bind to the metal site in carbonic anhydrase II.[21]

In the absence of anion, the data were fit (RS1, BBN Software, Cambridge, MA) to a scheme involving 4 micro equilibrium constants.[22,23] In this scheme, only 3 of the 4 microconstants are independent, that is $K_1K_3 = K_2K_4$ (Scheme I). When anion was present, Scheme I was expanded to include the binding of the inhibitor, and it was assumed that the anion binds only to species **1** and **2** (see ref 24 for an estimate of the accuracy of this assumption). Thus two separate binding constants were determined. $K_1^{I^-}$ is the equilibrium dissociation constant of the anion complex with species **1** of Scheme I, and $K_2^{I^-}$ is the equilibrium dissociation constant of the anion complex with species **2**.

Results

Relaxation in Solutions of Co^{2+} and Co(II)-Substituted Carbonic Anhydrase. The paramagnetic contribution to the relaxivity R_2 of water protons in a solution of Co^{2+} at 300 MHz was greater than that obtained at 20 MHz by previous investigators (Table I).[25] For Co^{2+}, R_1 at 300 and 20 MHz were close in value. In solutions of Co^{2+} as well as Co(II)-substituted carbonic anhydrase the paramagnetic contribution to the relaxivity of water protons at 300 MHz was greater for R_2 than for R_1 (Table I).

Table II. Calculated Paramagnetic Relaxivities of Water Protons in Solutions Containing Co^{2+}[a]

freq, MHz	R_1, $mM^{-1}\ s^{-1}$	R_2, $mM^{-1}\ s^{-1}$	T_{1p}/T_{2p}
20	0.18	0.19	1.1
300	0.16	1.8	11.2

[a] Calculated with eq 2 to 4 in the text. The calculations used $\tau_m = 7.4 \times 10^{-7}$ s from ref 14, the 20-MHz relaxation data from Bernheim et al.,[25] and the 300-MHz relaxation data and chemical shift determined in this work. A further description is in the text.

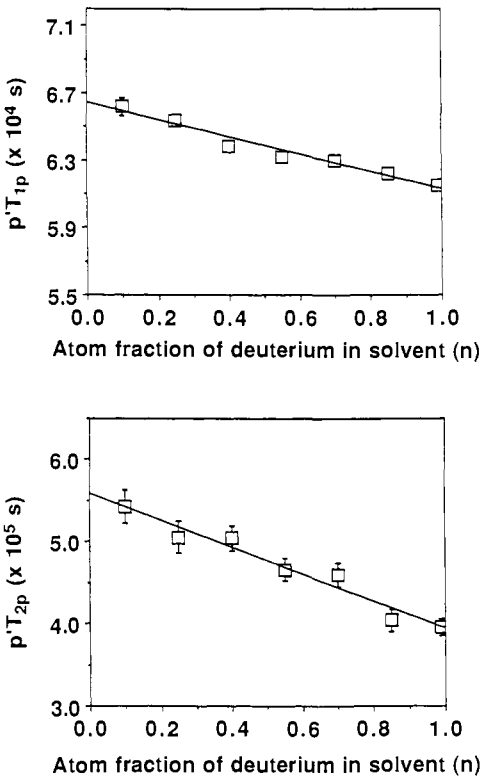


Figure 1. The dependence of $p'T_{ip}$ (i = 1, 2) at 300 MHz on n, the atom fraction of deuterium in solvent water, for a 10 mM solution of $CoCl_2$ at 23 °C. The solutions were buffered at pH 4.4 with 0.5 mM acetic acid. Data are the means and standard errors propagated from NMR measurements.

The addition of EDTA to the solutions of $CoCl_2$ (10 mM) reduced R_1 and R_2. When an equimolar amount of EDTA was added to these solutions of Co^{2+}, R_1 was reduced to 47% of the value of R_1 in the absence of EDTA and R_2 was reduced to 5% of the value of R_2 in the absence of EDTA. With this equimolar amount of EDTA T_{1p}/T_{2p} was 1.2 measured at 300 MHz. The remaining paramagnetic relaxivity due to the CoEDTA complex was $R_1 = 0.075\ mM^{-1}\ s^{-1}$ and $R_2 = 0.091\ mM^{-1}\ s^{-1}$. (These measurements were made with an 0.5 atom fraction of deuterium in solvent water.)

The observed change in the chemical shift caused by the paramagnetic metal, $\Delta\omega$, at 300 MHz for a 10 mM solution of $CoCl_2$ was measured to be 158 Hz. The measured change in the chemical shift, $\Delta\omega$, is the difference between the chemical shift of water protons in water containing only buffer and the chemical shift of water protons in a solution of a paramagnetic metal. This was measured with tetramethylsilane as an external reference using coaxial tubes. We have estimated the influence of this chemical shift on the transverse relaxation of $Co(H_2O)_6^{2+}$ solutions at 20 and 300 MHz using eq 3 (Table II). In the calculations, we used $\tau_m = 7.4 \times 10^{-7}$ s determined by Swift and Connick[14] using ^{17}O exchange. Also, we used $T_{1m} = T_{2m}$ as predicted by the Solomon-Bloembergen equations for $Co(H_2O)_6^{2+}$ at 20 and 300 MHz.

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Table III. Hydrogen/Deuterium Fractionation Factors Determined from T_1 and T_2 at 300 MHz for the Aqueous Ligands of the Paramagnetic Metal in $Co(H_2O)_6^{2+}$ and Two Isozymes of Co(II)-Substituted Carbonic Anhydrase[a]

	ϕ_{T_1}	ϕ_{T_2}
$Co(H_2O)_6^{2+}$ [b]	0.95 ± 0.01	0.73 ± 0.02
Co(II) carbonic anhydrase I[c]	1.10 ± 0.02	0.72 ± 0.02
Co(II) carbonic anhydrase II[c]	0.95 ± 0.02	0.77 ± 0.01

[a] Data were obtained from the least-squares slope and intercept and standard error in a plot such as Figure 1 according to eq 6. [b] Measurements were made at 23 °C for 10 mM $CoCl_2$ containing 5×10^{-4} M acetic acid buffer at pH 4.4. [c] Measurements were made at 23 °C for solutions of enzyme buffered at pH 8.5 by 50 mM Hepes.

Table IV. Solvent Hydrogen Isotope Effects on the Acid–Base Microequilibrium Constants Observed for Co(II)-Substituted Carbonic Anhydrase I and II[a]

	Co(II) carbonic anhydrase I		Co(II) carbonic anhydrase II	
	value in H_2O	ΔpK[b]	value in H_2O	ΔpK[b]
pK_1	7.55 ± 0.05	0.54 ± 0.06	5.65 ± 0.04	0.42 ± 0.05
pK_2	7.58 ± 0.15	0.45 ± 0.19	5.73 ± 0.07	0.37 ± 0.10
pK_3	8.11 ± 0.15	0.45 ± 0.19	6.95 ± 0.08	0.38 ± 0.12
pK_4	8.09 ± 0.22	0.54 ± 0.28	6.87 ± 0.12	0.43 ± 0.17

[a] The microconstants were determined from the variation of the absorbance at 640 nm with pH at 23 °C and fit to Scheme I. The solutions were buffered with Mops, Hepes, and triethylenediamine with no other added ions as described in the methods. [b] $\Delta pK = (pK_i)_{D_2O} - (pK_i)_{H_2O}$.

From the observed T_1 at 300 MHz, T_{1m} was calculated (from eq 1 and 2) to be 6.62×10^{-4} s. From the data of Bernheim et al.[25] $T_{1m} = 5.95 \times 10^{-4}$ s at 20 MHz. Since $T_{1m} \gg \tau_m$, the fast exchange limit applies at 20 MHz and at 300 MHz. $\Delta\omega_m$ was calculated from $\Delta\omega$ at 300 MHz using $\Delta\omega = p'\Delta\omega_m$, which is valid in the fast exchange limit.[14] We determined $\Delta\omega_m$ to be 1.46×10^5 Hz at 300 MHz. Since $\Delta\omega_m$ is directly proportional to the precessional frequency, $\Delta\omega_m$ at 20 MHz is 9.73×10^3 Hz. The results obtained with use of these values with eq 3 are given in Table II and are in good agreement with the experimental results in Table I.

Fractionation Factors of $Co(H_2O)_6^{2+}$ and Co(II)-Substituted Carbonic Anhydrase. The relaxation of water protons in solutions of 10 mM $CoCl_2$ as a function of the atom fraction of deuterium in solvent water is shown in Figure 1. From these data and with use of eq 6, the hydrogen/deuterium fractionation factor, ϕ, for $Co(H_2O)_6^{2+}$ was obtained (Table III). The value of the fractionation factor for $Co(H_2O)_6^{2+}$ was independent of the concentration of metal in the range 0.010 to 0.025 M but was dependent on whether it was obtained from T_1 or T_2. The fractionation factor from T_1 for $Co(H_2O)_6^{2+}$ was determined at frequencies between 0.01 and 50 MHz on a field cycling "relaxometer" at the IBM T. J. Watson Research Center and found to be virtually field independent (data not shown).

We have also measured the relaxation of water protons in solutions of two isozymes of Co(II)-substituted carbonic anhydrase as a function of the atom fraction of deuterium in solvent water. Typical results for isozyme I at pH 8.5 are shown in Figure 2. The hydrogen/deuterium fractionation factors calculated with use of T_2 are significantly less than unity for isozymes I and II (Table III).

Isotope Effects on Equilibrium Dissociation Constants. In the absence of anion, the visible pH titration data fit Scheme I very well (Table IV). The constants K_1, K_2, and K_3 were determined from a fit to the data, and then K_4 was calculated from K_1, K_2, and K_3. The 4 microconstants determined in H_2O for Co(II)-substituted carbonic anhydrase II agreed very well with those obtained by Simonsson and Lindskog,[22] and the values determined in H_2O for Co(II)-substituted carbonic anhydrase I also agreed fairly well with those obtained by Bertini et al.[23] In the presence of iodide the pH dependence of the absorbance at 640 nm was shifted to higher pH. Iodide binds with greater affinity to the

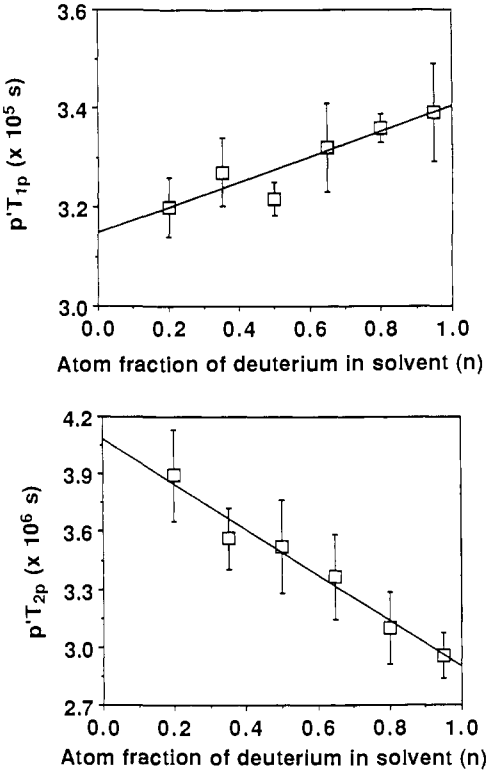

Figure 2. The dependence of $p'T_{ip}$ (i = 1, 2) at 300 MHz on n, the atom fraction of deuterium in solvent water, for 1.5 mM solutions of Co(II)-substituted carbonic anhydrase I. Solutions contained 50 mM Hepes buffer at pH 8.5 and 23 °C. Data are the means and standard errors of three measurements.

Table V. Solvent Hydrogen Isotope Effects on the Equilibrium Dissociation Constants of Iodide Ion with Co(II)-Substituted Carbonic Anhydrase I and II[a]

	Co(II) carbonic anhydrase I		Co(II) carbonic anhydrase II	
	value in H_2O	ΔpK^{I-}[b]	value in H_2O	ΔpK^{I-}[b]
pK_1^{I-}	2.95 ± 0.10	−0.09 ± 0.17	3.48 ± 0.13	0.47 ± 0.19
pK_2^{I-}	2.35 ± 0.06	−0.07 ± 0.11	2.79 ± 0.02	0.27 ± 0.02

[a] The dissociation constants were determined from the pH dependence of the absorbance at 640 nm in the presence of 10 mM I^- with conditions as in Table IV. K_1^{I-} is the dissociation constant of I^- binding to species **1** of Scheme I, and K_2^{I-} is the dissociation constant of I^- binding to species **2** of Scheme I. [b] $\Delta pK = (pK_i^{I-})_{D_2O} - (pK_i^{I-})_{H_2O}$.

diprotonated species **1** in Scheme I than to the monoprotonated species **2** (Table V). This is similar to the iodide inhibition results of Simonsson and Lindskog for native zinc carbonic anhydrase II determined from activity measurements[22] and the results of Bertini et al. for nitrate inhibition of Co(II)-substituted carbonic anhydrase II determined from spectrophotometric titrations.[23] The change in solvent from H_2O to D_2O has no measurable effect on K_1^{I-} and K_2^{I-} for Co(II)-substituted carbonic anhydrase I, but for Co(II)-substituted carbonic anhydrase II the change in solvent from H_2O to D_2O results in a decrease in these equilibrium constants (Table V).

Discussion

The results at 300 MHz of T_{1p}/T_{2p} much greater than unity for solutions of both $Co(H_2O)_6^{2+}$ and Co(II)-substituted carbonic anhydrase suggest that a relaxation mechanism different from the dipole–dipole mechanism is present at this frequency. The data in Table I indicate that this mechanism primarily, if not exclusively, affects the transverse relaxation. Melamud and Mildvan[26] observed a field-dependent increase in $1/T_{2p}$ for water protons in solutions of $Co(H_2O)_6^{2+}$ and concluded that the $\Delta\omega_m^2$

(26) Melamud, E.; Mildvan, A. S. *J. Biol. Chem.* **1975**, *250*, 8193–8201.

term in eq 3, a chemical shift mechanism, made a major contribution at the higher frequencies. We support this conclusion in two ways. First, we have calculated the magnitude of the chemical shift contribution to the transverse relaxation of $Co(H_2O)_6^{2+}$ solutions at 20 and 300 MHz using eq 3. These calculations demonstrate that the observed value of T_{1p}/T_{2p} at 300 MHz is consistent with a chemical shift mechanism that affects T_{2p} (compare Tables I and II). The chemical shift does not have an effect at 20 MHz at which frequency $\Delta\omega_m^2 \ll 1/T_{2m}\tau_m$, $1/T_{2m}^2$. The large chemical shift at 300 MHz due to the addition of Co^{2+} is the result of contact interactions that result from the delocalization of unpaired electron spin density. At lower magnetic field strengths this mechanism has been observed to affect T_2 for ^{17}O and ^{19}F in cobalt complexes.[14,27] The effect on the relaxation when EDTA is added to solutions of $Co(H_2O)_6^{2+}$ is the second way we support the chemical shift mechanism. The chemical shift mechanism will affect T_{2p} to the greatest extent when the protons are in the primary hydration shell of the ion because it is a contact interaction. EDTA binds to Co^{2+} occupying all coordination sites and thus excludes water molecules from the inner shell. Thus the addition of EDTA would prevent the water protons from experiencing the chemical shift mechanism. The ratio T_{1p}/T_{2p} was 1.2 for water protons at 300 MHz when an equimolar amount of EDTA was added to solutions of Co^{2+}, consistent with the absence of a chemical shift relaxation mechanism and the dominance of a dipole–dipole relaxation mechanism.

When EDTA was added to solutions of Co^{2+}, the percent decrease in the relaxivity R_2 at 300 MHz was much greater than R_1. The residual paramagnetic relaxivity of the cobalt–EDTA complex is most likely due to water protons in an "outer shell" of the complex. This residual relaxivity makes up a much larger part of R_1 than R_2 observed for $Co(H_2O)_6^{2+}$ solutions at 300 MHz. This suggests that the relaxation of outer shell water contributions to a greater extent to R_1 than to R_2 in $Co(H_2O)_6^{2+}$, and R_2 has a greater contribution from inner-shell water than does R_1. Additional support for this is discussed below because the fractionation factor ϕ of $Co(H_2O)_6^{2+}$ obtained from T_1 is much closer to that for bulk water than is ϕ from T_2. A similar situation in which the outer-shell relaxation has a larger contribution to R_1 than to R_2 has been reported by Kushnir and Navon[28] for water protons in solution with Mn(II)-substituted carbonic anhydrase II.

The definition of the hydrogen/deuterium fractionation factor we have given assumes the rule of the geometric mean is valid;[1] that is, the fractionation factor of one particular hydrogenic site is independent of the isotopic composition of any other site. We will assume that the rule is valid in our system and this is supported by the high degree of linearity in plots as in Figures 1 and 2. A value of $\phi < 1.0$ means that the ligand position prefers the light isotope of hydrogen and the O–H bond is weaker in the ligand relative to that bond in the solvent, while a $\phi > 1.0$ means that deuterium will preferentially accumulate in the ligand position, indicating that the O–H bond is stronger in the ligand relative to that bond in the solvent.

The values of the fractionation factor for the water ligands of cobalt in $Co(H_2O)_6^{2+}$ and in the Co(II)-substituted isozymes of carbonic anhydrase were dependent on whether they were determined from T_1 or T_2 (Table III) and are a weighted value of all the protons that experience the paramagnetic relaxation. An explanation for this difference in ϕ_{T_1} and ϕ_{T_2} is based on the analysis of the contributions to the relaxation at 300 MHz. The paramagnetic contribution to the transerse relaxation of water protons has a predominant contribution of water in the inner coordination shell suggesting that ϕ_{T_2} is measuring more of the fractionation of inner-shell water. Since T_{1p} has a larger contribution of outer-shell water, ϕ_{T_1} is more of the fractionation of outer-shell water. This interpretation is consistent with expectations for the fractionation factor of metal-bound water. The fractionation factor for H_3O^+ is 0.69,[29] presumably due to the positive charge on the oxygen. It has been suggested that a metal-bound water site ought to have a fractionation factor approximated by $(0.69)^\delta$, with δ measuring the degree of positive charge transferred from the metal to the oxygen.[1] The fractionation factor from T_2 for $Co(H_2O)_6^{2+}$ being 0.73 supports this suggestion in resembling the fractionation factor for H_3O^+. Pure water has by definition a fractionation factor of 1.0. Outer-shell water, being much more loosely bound than inner-shell water, should be much more like bulk water in its hydrogen/deuterium fractionation properties. The observation of ϕ_{T_1} for $Co(H_2O)_6^{2+}$ being close to unity is consistent with this fractionation factor having a large contribution of outer-shell water.

The fractionation factors measured from T_1 for the two isozymes of Co(II)-substituted carbonic anhydrase were also close to unity (Table III), consistent with these values having a large contribution from the fractionation of outer-shell water. This is reasonable from distance considerations, as it can be calculated by using a $1/r^6$ dependence that the paramagnetic contribution to the longitudinal relaxation rate for a cobalt-bound hydroxide with a cobalt–proton distance of 2.8 Å is about the same as the paramagnetic contribution of the protons of three water molecules at an average distance of 3.8 Å. Also, it is known from crystallographic data that there are a large number of water molecules within the active site cavity. Specifically, in human carbonic anhydrase II there are an estimated 9 water molecules between histidine 64 and the zinc, a distance of about 7.5 Å from the X-ray coordinates.[30] The fractionation factors measured from T_2, ϕ_{T_2}, were near 0.72 for both Co(II)-substituted isozymes I and II and reflect a larger contribution of the hydrogen/deuterium fractionation of the inner-shell aqueous ligand. It was found that ϕ_{T_2} was the same for $Co(H_2O)_6^{2+}$ as for the Co(II)-substituted carbonic anhydrase isozymes, a result that could mean that ϕ_{T_2} is not sensitive to the environment of the aqueous ligands of the metal but is determined more by the ligand–metal interaction. Although isozymes I and II have very similar amino acid sequences, there are significant differences in their structure, residues in the active site cleft, and catalytic activities,[5] none of which apparently have a significant effect on the fractionation factors that are nearly identical for isozymes I and II.

These relaxation measurements were made at pH 8.5, a pH for which the relaxation rate of water protons in solutions of Co(II)-substituted carbonic anhydrase is greatest.[18] This pH is above the value of the pK for the ionization of the active site group as determined in many experiments (see Table IV), especially spectrophotometric titrations and activity measurements, and the ligand of cobalt is believed to be a hydroxide ion at this pH. This presents some uncertainty as to which protons are actually being relaxed at pH 8.5 since a tetracoordinate cobalt-bound hydroxide ion would not be expected to exchange rapidly with water in solution. This issue has been discussed by Koenig et al.,[12] who proposed that ligand exchange with solvent involved a pentacoordinate intermediate having both OH^- and H_2O as ligands. Proton exchange can be rapid between these two ligands so that the departing H_2O can contain the oxygen of the initially bound OH^-. On the basis of ^{18}O-exchange kinetics, Tu and Silverman[31] suggested that the NMR relaxation observed at pH 8.5 is that of a water molecule hydrogen bonded to the cobalt-bound hydroxide yet close enough to the metal to be strongly relaxed. Yet another possibility is the rapid exchange of the hydrogen of the metal-bound hydroxide without exchange of the oxygen. Because of these unresolved issues, we cannot state specifically which of the hydrogens near the metal are the main contributors to the observed fractionation factors.

We have discussed above the fractionation factor for the exchangeable hydrogen at the active site in its high-pH form. We now describe the calculation of the fractionation factor of the active

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Table VI. Values of the Hydrogen/Deuterium Fractionation Factor for the Low-pH Forms of Co(II)-Substituted Carbonic Anhydrase[a]

	ϕ_{T_1}	ϕ_{T_2}
Co(II)-substituted carbonic anhydrase I	1.12 ± 0.05	0.90 ± 0.04
Co(II)-substituted carbonic anhydrase II	0.90 ± 0.03	0.81 ± 0.03

[a] The ϕ for the low pH form was calculated by using the isotope effect on the ionization constant of the cobalt-bound water and the ϕ for the high pH form measured directly from NMR and given in Table III.

site in its low-pH form. Since the paramagnetic contribution to the relaxivity decreases to a very small value below pH 6.0[18] (except at very low ionic strength),[32,33] we cannot measure the fractionation factor directly from the relaxation rates at low pH. Instead, this is calculated from the fractionation factor of the high-pH form and the solvent hydrogen isotope effect on the ionization constant of the active site, metal-bound water.

The visible pH titration data of isozymes I and II indicate that more than one ionization at or near the active site governs the absorbance at 640 nm.[33] Previous investigators[22,23] have attributed this pH dependence to the ionization of two groups, the metal-bound water and another group near the active site that may be His-64 for isozyme II and probably His-64 or His-200 in isozyme I.[34,35] We have measured the pH dependence of the absorbance at 640 nm for isozymes I and II in H_2O and D_2O and fit the data according to Scheme I to determine the solvent hydrogen isotope effect on the microequilibrium constants (Table IV). In Scheme I, K_1 and K_4 are the constants for the metal-bound water, and K_2 and K_3 are the constants for the other active site group. Since the solvent hydrogen isotope effects on these equilibrium constants are all similar, within experimental uncertainty, it appears that the protonation state of one of the groups does not affect the hydrogen/deuterium fractionation properties of the other (consistent with our conclusion that active site properties do not seem to influence the fractionation factors of the aqueous ligand of cobalt). Then the calculation to determine the fractionation factor of the water ligand of the metal can be performed neglecting the ionization state of the second ionizable group.

The isotope effect on an equilibrium constant is the product of all of the reactant state fractionation factors divided by the product of all of the product state fractionation factors. For this ionization

$$\text{>Co-OH}_2 + \text{H}_2\text{O} \rightleftharpoons \text{>Co-OH}^- + \text{H}_3\text{O}^+$$

the solvent hydrogen isotope effect can be written

$$\frac{K_{H_2O}}{K_{D_2O}} = \frac{(\phi_{Co\text{-}OH_2})^2(\phi_{H_2O})^2}{(\phi_{Co\text{-}OH})(\phi_{H_3O^+})^3}$$

Thus, from this measured isotope effect, the measured fractionation factor for the cobalt-bound hydroxide, the fractionation factor for H_3O^+ which has been measured to be 0.69,[29] and the fractionation factor for H_2O which is 1.0, the fractionation factor values for the exchangeable hydrogens at the active site in its low pH form can be calculated and are presented in Table VI. We have done the calculations of Table VI using both ϕ_{T_1} and ϕ_{T_2} measured for the high-pH form of the aqueous ligand of cobalt in Co(II)-substituted carbonic anhydrase I and II. However, the calculation from T_1 may not be meaningful, since it contains a large contribution of outer-shell water. The value calculated by using ϕ_{T_2} represents more closely the fractionation factor value of the inner-shell cobalt-bound water.

To apply the fractionation factors to an equilibrium solvent hydrogen isotope effect associated with the enzyme, we have studied the equilibrium binding of I^- to Co(II)-substituted carbonic anhydrase I and II. I^- is known to bind to the metal in Co(II)-substituted carbonic anhydrase I and II[21] displacing the coordinated water molecule. Also, I^- binds fairly tightly (Table V) and its size is comparable to that of a water molecule. Thus the isotope effect on the binding of I^- to Co(II)-substituted carbonic anhydrase may be simple enough to be accounted for by the fractionation factor of the metal-bound water.

The solvent hydrogen isotope effect on the binding of the iodide ion to the active site of carbonic anhydrase is given by

$$\text{>Co-I}^- + \text{H}_2\text{O} \rightleftharpoons \text{>Co-OH}_2 + \text{I}^-$$

$$\frac{(K_i^{I^-})_{H_2O}}{(K_i^{I^-})_{D_2O}} = \frac{(\phi_{H_2O})^2}{(\phi_{Co\text{-}OH_2})^2}$$

where $(K_i^{I^-})_{H_2O}$ is the equilibrium dissociation constant describing the binding of I^- to species i of Scheme I in H_2O. Because it is known that $\phi_{H_2O} = 1.0$, with knowledge of $\phi_{Co\text{-}OH_2}$ it should be possible to estimate an isotope effect. Using ϕ_{T_2} for $\phi_{Co\text{-}OH_2}$ from Table VI, we thus predict that $\Delta pK^{I^-} = 0.09 \pm 0.04$ for Co(II) isozyme I, and 0.18 ± 0.03 for Co(II) isozyme II. The predicted value for isozyme I is in rough agreement with the measured value (Table V), and the predicted value for isozyme II is slightly below the measured value. We have considered other interactions that may be taken into account for I^- binding. One of these is the composite fractionation factor of the solvation shell of I^- ($\phi = 0.59$).[36] Including this in the denominator of the above equation for the isotope effect increases the predicted value, which is then closer to the experimental value for Co(II) isozyme II, but farther from the experimental value for Co(II) isozyme I. This implies that we have incomplete knowledge of the fractionation properties of each hydrogenic site that is affected when iodide ion binds to the enzyme. However, to include the fractionation factors of any other groups in the equation requires much speculation about the fractionation factors near the active site. Thus it is likely that the isotope effect must be described by the complex contribution of the fractionation factors of additional groups or water associated with the enzyme and that the fractionation factor of the aqueous ligand of the metal is by itself not sufficient to account for the isotope effect on I^- binding.

Conclusion

We have shown the utility of this NMR relaxation method to measure the fractionation properties of the aqueous ligands of cobalt in some specific cases. The fractionation factors of Co(II)-substituted carbonic anhydrase represent the first direct measurement of this parameter at the active site of an enzyme. In particular, the characterization of the relaxation mechanisms at 300 MHz has allowed the detection of the fractionation of two populations of ligand sites associated with cobalt, one having a significant contribution of the inner-shell aqueous ligand of the metal and the other apparently dominated by outer-shell water. The fractionation factor for the inner-shell ligand can differ significantly from that for solvent water. The value of the fractionation factor measured directly from NMR represents a reactant state fractionation factor that can be used to interpret solvent hydrogen isotope effects associated with the enzyme. The inability of the fractionation factor for the aqueous ligand of cobalt in Co(II)-substituted carbonic anhydrase to completely account for the solvent hydrogen isotope effect on the binding of iodide ion is not surprising, but simply shows that there must be other hydrogenic positions whose isotope preference changes on going from the reactant to product states. To account for the solvent hydrogen isotope effects on a rate constant is even more complex because knowledge of the fractionation factor values for the transition state is required. This work represents an important first step in the accurate interpretation of the solvent hydrogen isotope effects in the catalysis by carbonic anhydrase.

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