Dynamic ^{13}C NMR Investigations of Substrate Interaction and Catalysis by Cobalt(II) Human Carbonic Anhydrase I†

Taffy J. Williams*,‡ and Robert W. Henkens*,§

Naval Medical Research Institute, Metabolic Research Branch, Bethesda, Maryland 20814, and Paul M. Gross Chemical Laboratory, Department of Chemistry, Duke University, Durham, North Carolina 27706

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ABSTRACT: Using ^{13}C NMR spectroscopy, we have further investigated the binding of HCO_3^- in the active site of an artificial form of human carbonic anhydrase I in which the native zinc is replaced by Co(II). The Co(II) enzyme, unlike all other metal-substituted derivatives, has functional properties closely similar to those of the native zinc enzyme. By measuring the spin–lattice relaxation rate and the line width for both the CO_2 and HCO_3^- at two field strengths, we have determined both the paramagnetic effects that reflect substrate binding and the exchange effects due to catalysis at chemical equilibrium. The following are the results at 14 °C and pH 6.3. (1) HCO_3^- is bound in the active site of the catalytically competent enzyme with the ^{13}C of the HCO_3^- located 3.22 ± 0.02 Å from the Co(II); (2) the apparent equilibrium dissociation constant for the bound HCO_3^- is 7.6 ± 1.5 mM, determined by using the paramagnetic effects on the line widths, and 10 ± 2 mM, determined by using the exchange effects; (3) the lifetime of HCO_3^- bound to the metal is $(4.4 \pm 0.4) \times 10^{-5}$ s; (4) the overall catalyzed $CO_2 \rightleftarrows HCO_3^-$ exchange rate constant of the Co(II) enzyme is $(9.6 \pm 0.4) \times 10^3$ s^{-1}; (5) the electron spin relaxation time of the Co(II), determined by using paramagnetic effects on the bound HCO_3^-, is $(1.1 \pm 0.1) \times 10^{-11}$ s. The data did not provide any direct information on the binding of CO_2. From these data we conclude that carbonic anhydrase functions as an efficient catalyst of the $CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$ reaction through a metal inner-sphere mechanism that involves direct coordination of the HCO_3^- and the metal ion in the active site. The data suggest that HCO_3^- is associated with a tetracoordinate Co(II) and that the dissociation rate constant of HCO_3^- is only about 2.5 times the overall $CO_2 \rightleftarrows HCO_3^-$ exchange rate constant k_{cat}^{exch}.

Carbonic anhydrase (EC 4.2.1.1) is a zinc metalloenzyme catalyzing the reaction

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$$

The enzyme is an extremely efficient catalyst, a fact that has intrigued many investigators of the enzyme since its discovery 50 years ago [cf. Pocker & Sarkanen (1978), Lindskog (1982), and Bertini & Luchinat (1983)]. The metal is required for catalytic activity, and it is thought to serve a direct catalytic rather than structural role. The prevalent hypothesis is that a water ligand of the metal ionizes to produce an OH^- ligand, which combines with CO_2 to produce metal-bound HCO_3^-. But there is almost no direct information on binding of HCO_3^- at the metal (Lindskog, 1982). Such information is needed to understand the role of the metal in catalysis.

Previous NMR results give an upper limit on the HCO_3^-–metal distance of Cu(II)–bovine carbonic anhydrase (Bertini et al., 1979, 1983), the HCO_3^-–metal distance of Mn(II)–human carbonic anhydrase I, and the activity of the Mn(II) enzyme, which is 7% of the normal zinc enzyme (Led et al., 1982). The relaxation NMR results reported here gave the HCO_3^-–metal distance of Co(II) human carbonic anhydrase I, the HCO_3^-–metal dissociation constant (K_d), lifetime (τ_m), and the HCO_3^- exchange parameters (k_{cat}^{exch} and K_{eff}^s).

The relaxation NMR measurements of the HCO_3^-–metal interactions in the enzyme require use of a paramagnetic probe [cf. Yeagle et al. (1975)]. Co(II) was selected rather than Mn(II) or Cu(II) because the Co(II) enzyme, unlike all other metal-substituted derivatives, has catalytic properties closely similar to those of the natural zinc enzyme. Therefore, the new information should shed additional light on the kinetics and mechanism of the normal enzyme.

Previous measurements of the paramagnetic enhancement of the ^{13}C longitudical relaxation rate of CO_2 and HCO_3^- in the presence of Co(II)–human carbonic anhydrase I (human carbonic anhydrase B) give only a weighted average substrate–metal distance because the CO_2–HCO_3^- interconversion rate is too high (Stein et al., 1977). The measurements neither distinguished between enzyme-bound CO_2 and HCO_3^- nor between catalytically productive and nonproductive binding. This study resolves this problem and allows for the conclusion that the $CO_2 \rightleftarrows HCO_3^-$ interconversion takes place by a metal inner-sphere mechanism in which the HCO_3^- is bound within the inner coordination sphere of the metal. The data also give additional information on the kinetics of the enzyme-catalyzed reaction.

Materials and Methods

^{13}C NMR measurements were made on the Varian FT-80A at 20.00 MHz or the Varian XL-200 at 50.3 MHz. The samples were thermostated at 14 °C unless the studies were performed as a function of temperature. Proton decoupling was not used. Samples generally contained 6×10^{-5}–6×10^{-7} M enzyme and 0.1–0.001 M ^{13}C-enriched (90%) sodium bicarbonate with the 4-morpholineethanesulfonic acid (MES) buffer kept constant at 0.1 M, pH 6.3, with 6% D_2O, in 10-mm tubes with vortex plugs. The pH was chosen such that $[CO_2]$

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‡ Naval Medical Research Institute.

§ Duke University.

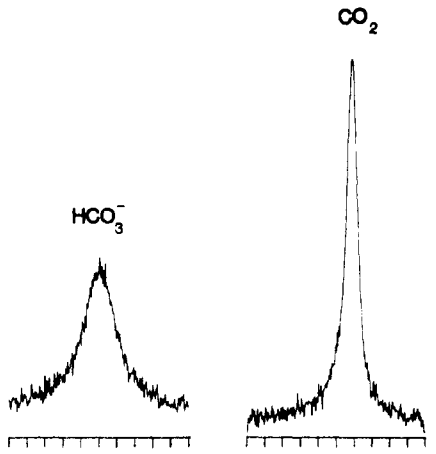

FIGURE 1: Spectrum obtained at 50.3 MHz of solution of $H^{13}CO_3^-$ and $^{13}CO_2$ at concentration of 45 mM each and ratio of $[E]_t/[S] = 1.2 \times 10^{-3}$. The pH 6.3 0.1 M MES buffer was used and the temperature held constant at 14 °C. The spectrum is the accumulation of 557 transients processed without sensitivity enhancement. The tick marks are 5 Hz apart.

$= [HCO_3^-]$ and the area of the peak for CO_2 equals the area obtained for HCO_3^-. The temperature was chosen to enhance the solubility of the CO_2. Spectra contained 16 000–32 000 data points and required a few minutes to several days to accumulate. The acquisition times used were either 2 or 4 s, depending on the resolution required. In the absence of enzyme, the CO_2 and HCO_3^- line widths were 0.5 and 0.79 Hz, respectively, and these values were subtracted from the observed line widths in the presence of the enzyme to obtain the enzyme's contribution to the line width. Samples were prepared at the highest concentrations and diluted serially to maintain a constant ratio of substrate to enzyme. Sweep widths were just wide enough to include both the CO_2 and HCO_3^- resonances at 125.1 and 162.2 ppm, respectively (Yeagle et al., 1975). The data used to determine the T_1 values were obtained by the inversion recovery method, and the analysis of the data was performed using nonlinear curve estimations.

The apoenzyme was prepared by the method of Hunt et al. (1977). Cobalt(II) chloride (10% less than 1 equiv) was added to generate the metal-substituted enzyme. The incorporation of only one cobalt in the active site of the enzyme was verified by atomic absorption spectroscopy and the enzyme assay using *p*-nitrophenyl acetate as substrate (Henkens & Sturtevant, 1968).

RESULTS

The human carbonic anhydrase I catalyzed $CO_2 \rightleftarrows HCO_3^-$ interconversion frequency is low enough that separate resonances were observed for CO_2 and HCO_3^-. Measurements of line widths of CO_2 and HCO_3^- were made as a function of substrate concentration at pH 6.3 and 14 °C at two different frequencies. At pH 6.3, the concentrations of CO_2 and HCO_3^- are equal, and the exchange contributions to the line width should be equal. Paramagnetic effects due to the binding of HCO_3^- have increased its line width. The spectrum of a typical run is shown in Figure 1, and the increase in the line widths as a function of substrate concentration (at constant molar ratio between enzyme and substrate) is shown in Figure 2A. Measurements of the increase in the line widths were also made as a function of temperature at the two different frequencies (Figure 3).

To demonstrate that the line broadening was only a result of substrate binding and the rate of catalytic conversion of the

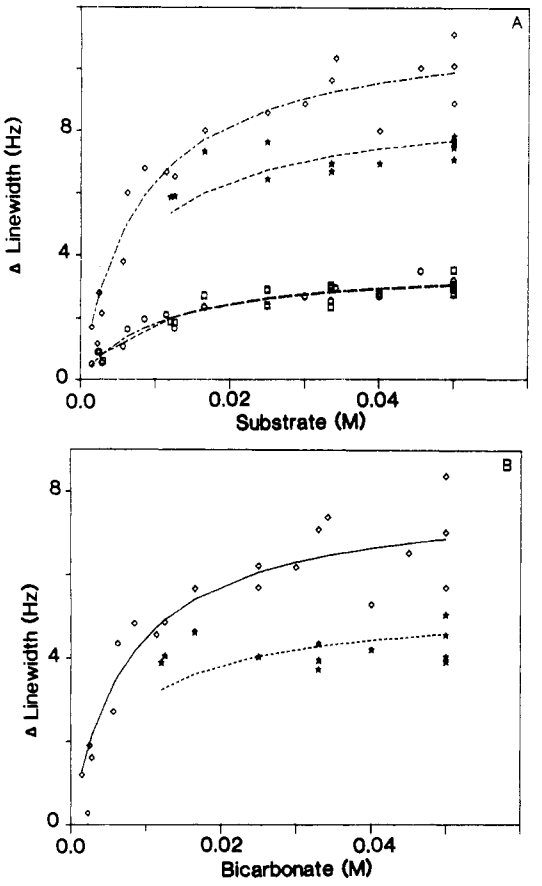

FIGURE 2: (A) Measurements were made of the differences between the line widths of $H^{13}CO_3^-$ and $^{13}CO_2$ in the absence of enzyme and in the presence of enzyme, i.e., Δline width = $\Delta\nu_{enzyme} - \Delta\nu_{no\ enzyme}$ at a constant ratio ($[E]_t/[S] = 1.2 \times 10^{-3}$), at 14 °C and pH 6.3. The increase in the line width of HCO_3^- (◇) and CO_2 (○) as a function of concentration is plotted for data obtained at 50.3 MHz. Measurements of HCO_3^- (☆) and CO_2 (□) were also obtained at 20 MHz. The lines through the data represent fits to eq 3. (B) Measurements of the difference between the line widths of $H^{13}CO_3^-$ and $^{13}CO_2$ in the presence of the enzyme were made under the conditions described in (A). Difference in line-width measurements, Δline width = $\Delta\nu_{HCO_3^-} - \Delta\nu_{CO_2}$, of HCO_3^- were made at 50.3 (◇) and 20 MHz (☆). The lines through the data represent fits to eq 5.

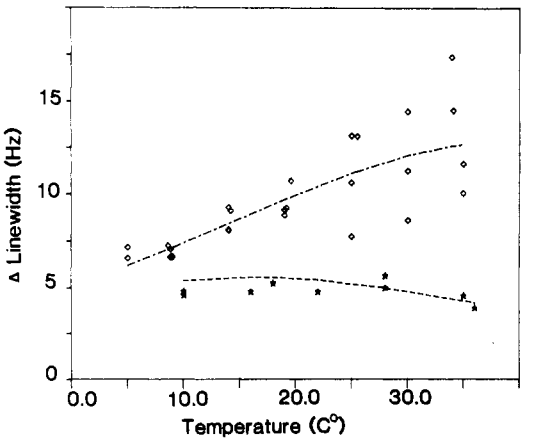

FIGURE 3: Measurements of the differences between the line widths of $H^{13}CO_3^-$ and $^{13}CO_2$ in the presence of the enzyme at a constant ratio ($[E]_t/[S] = 1.2 \times 10^{-3}$), $[CO_2] = [HCO_3^-] = 25$ mM, pH 6.3, were made as a function of temperature. The line-width measurements, Δline width = $\Delta\nu_{HCO_3^-} - \Delta\nu_{CO_2}$, were obtained at 50.3 (◇) and 20 MHz (☆). The lines through the data represent fits to eq 5 and 6.

CO_2 and HCO_3^- species, additional experiments were performed. The metal chelating agent ethylenediaminetetraacetic

acid (EDTA) had no effect on the enzyme-induced line broadening of either of the two resonances. On the other hand, enzyme inhibitor benzenesulfonamide abolished the line broadening of both resonances.

In the absence of the enzyme, cobalt(II) chloride caused line broadening and a chemical shift of the $H^{13}CO_3^-$ resonance, but not the $^{13}CO_2$ resonance. These effects were abolished by EDTA but unaffected by benzenesulfonamide.

The T_1 value of both resonances in the presence of 60 μM Co(II)–enzyme and at saturating levels of total added bicarbonate (0.1 M) was 2.95 ± 0.15 s at 50 MHz. In contrast, in the presence of a catalytically equivalent concentration of the Zn(II)–enzyme, the T_1 was 38.2 ± 1.25. The value of T_1 for both resonances in the presence of the Co(II)–enzyme was found to be field dependent with the ratio $T_1(20\ \text{MHz})/T_1(50.3\ \text{MHz}) = 0.89 \pm 0.02$.

Discussion

Under the conditions used in this study, the CO_2 and HCO_3^- resonances may be broadened by either or both of two processes. The first arises from the chemical exchange between the CO_2 and the HCO_3^-, while the second arises from paramagnetic effects due to the Co(II). In this study, all experiments were performed such that the chemical exchange of CO_2 and HCO_3^- was slow on the chemical shift time scale and two distinct resonances were always observable. Under the condition employed in these experiments, i.e., $[CO_2] = [HCO_3^-]$, the theory (Simonsson et al., 1982) predicts that both the CO_2 and the HCO_3^- resonances should be broadened by the same amount in the slow exchange limit. Identical line broadening was found for CO_2 and HCO_3^- in the presence of the Zn(II) form of the enzyme, but not in the presence of the Co(II) form of the enzyme (Figure 1). Depending on conditions, the HCO_3^- line width was found to be as much as threefold larger than the CO_2 line width. The difference between the two resonances is shown in Figure 2B.

The line width of the HCO_3^- resonance was found to be dependent on frequency, while the line width of the CO_2 resonance was found to be frequency independent. Statistical analysis performed on parameter estimates obtained from a nonlinear curve fit to the HCO_3^- line-width data at 50.3 and 20 MHz yielded a probability of greater than 99% that the two curves are different. On the other hand, similar analysis on the CO_2 line width confirmed its frequency independence.

While an undetectable frequency dependence does not rule out a paramagnetic contribution to the $^{13}CO_2$ line width, it does allow for the establishment of a limit for such a contribution. Theoretical calculations, assuming the $^{13}CO_2 \rightleftarrows E\cdot{}^{13}CO_2$ portion of the reaction to be in intermediate exchange limit on the NMR time scale, demonstrate that the ratio of the paramagnetic contributions to the $^{13}CO_2$ line width obtained at 50.3 and 20 MHz is frequency dependent. This ratio increases from a value equal to 1 in slow exchange to the square of the frequencies, or 6.3, as the rate becomes more rapid (Dwek, 1973). The resolution of the CO_2 line-width measurements is 0.5 Hz or about 17% of the signal at 50.3 and 20 MHz. This value would reduce to 0.08 Hz, or only 2.3% of the CO_2 line-width measurements obtained at 20 MHz. Although a lack of frequency dependence could be the result of the $^{13}CO_2 \rightleftarrows E\cdot{}^{13}CO_2$ portion of the reaction being in the slow exchange region on the NMR time scale, such a slow exchange model is an unlikely possibility. If the $CO_2 \rightleftarrows E\cdot CO_2$ is in fast exchange, the observed lack of detectable frequency dependence of the CO_2 line width suggests that for this resonance $\Delta\nu_p = (\pi T_{2_m})^{-1} = [(6/7)\pi T_{1_m})]^{-1}$ (Dwek, 1973). This equation suggests the maximum binding contribution to the line width could be 0.12 Hz. This contribution is less than 3% of the total measured line width of CO_2, again well within the limits of the line-width measurements. Therefore, we conclude that any paramagnetic contribution to the $^{13}CO_2$ line width is well within the limits of the actual line-width measurement. Consideration of such contributions were not found to significantly alter the results.

The data show that Co(II) human carbonic anhydrase affects the line width of both the $^{13}CO_2$ and $H^{13}CO_3^-$ resonances to different extents. From the measurements at two frequencies, we conclude from the data that the line broadening of the CO_2 resonance is due to the CO_2 and HCO_3^- exchange only, while the line broadening of the HCO_3^- resonance is the sum of two effects, i.e., of the $CO_2 \rightleftarrows HCO_3^-$ exchange and of a paramagnetic contribution due to binding. Thus, the data provide information on both substrate binding and substrate turnover (i.e., substrate exchange according to eq 1).

Under the slow $CO_2 \rightleftarrows HCO_3^-$ exchange conditions of our measurements the exchange contribution to the line width ($\Delta\nu$) is determined by the lifetime (τ) of the substrate species (Swift & Connick, 1962)

$$\Delta\nu = 1/(\pi\tau) \tag{1}$$

Consequently, the line broadening of the $^{13}CO_2$ resonance can be used to calculate the exchange rate, ν_{exch}, from the equation (Simonsson et al., 1982)

$$\nu_{exch} = \pi\Delta\nu_{CO_2}[CO_2] = \pi\Delta\nu_{HCO_3^-}[HCO_3^-] \tag{2}$$

At equal concentrations of CO_2 and HCO_3^- as used in these experiments, the exchange contributions to line broadening are the same for both resonances.

The enzyme kinetic parameters k_{cat} and K_{eff} were calculated from eq 3 (Simonsson et al., 1979) by a nonlinear least-squares

$$\Delta\nu_s = f\frac{k_{cat}^{exch}}{\pi}\frac{[S]}{K_{eff}^s + [S]} \tag{3}$$

fit of the line width of the CO_2 obtained at a constant enzyme-to-substrate ratio, $[E]_t/[S] = f$. We obtained the values $k_{cat}^{exch} = (9.6 \pm 0.4) \times 10^3\ s^{-1}$ and $K_{eff} = 10 \pm 2$ mM.

While the line broadening of the CO_2 resonance only contains contributions due to exchange, the HCO_3^- resonance contains the sum of the contributions of exchange and binding. Thus, the difference in line widths of the $^{13}CO_2$ and $H^{13}CO_3^-$ resonances results from paramagnetic enhancement of T_{2p} due to binding of the $H^{13}CO_3^-$ to the enzyme:

$$1/T_{2p} = \pi(\Delta\nu_{HCO_3^-} - \Delta\nu_{CO_2}) \tag{4}$$

The paramagnetic contributions, T_{2p}, are given by (Swift and Connick, 1962)

$$\frac{1}{T_{2p}} = \frac{f}{\tau_m}\left[\frac{(1/T_{2m})(1/T_{2m} + 1/\tau_m) + \Delta\omega_m^2}{(1/\tau_m + 1/T_{2m})^2 + \Delta\omega_m^2}\right]\left(\frac{[S]}{K_d + [S]}\right) \tag{5}$$

where K_d is the apparent equilibrium dissociation constant for the enzyme bicarbonate complex, τ_m is the apparent lifetime of the HCO_3^-–enzyme complex, T_{2m} is the transverse relaxation time of the bound substrate, and $\Delta\omega_m$ is the difference in frequency between the free and bound bicarbonate.

The value of K_d, $\Delta\omega_m$, and τ_m were estimated from nonlinear least-squares fits of pooled data to eq 5. Paramagnetic contributions to the bicarbonate line width as a function of con-

centration at both 50.3 and 20 MHz were used as the pooled sets of data. The value of T_{2m} calculated from T_{1m} was fixed in the analysis, and the values $\Delta\omega_m = (7 \pm 1) \times 10^4$ Hz, $\tau_m = (4.4 \pm 0.4) \times 10^{-5}$ s, and $K_d = 7.6 \pm 1.5$ mM were obtained as estimates. The chemical shift of the bound form ($\Delta\omega_m$) is the value for resonance at 50 MHz. That $K_d = K_{eff}$ within the experimental error indicates that K_d measures the saturation of the binding site responsible for the $CO_2 \rightleftarrows HCO_3^-$ conversion, so the NMR measurements characterize productive binding of HCO_3^-. The exchange kinetics can be represented by the simple model

$$E^- + CO_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^-\cdot CO_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EH\cdot HCO_3^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EH + HCO_3^-$$

The lifetime of the $EH\cdot HCO_3^-$ complex, $\tau_m = 0.44 \times 10^{-4}$ s, is about half the exchange time $1/k_{cat}^{exch} = 1.0 \times 10^{-4}$ s, showing that the lifetime of this complex is a major factor in determining the overall exchange time. In terms of the model, $1/k_{cat}^{exch} = (1/k_{-1})(1 + k_2/k_{-2}) + (1/k_3)(1 + k_{-2}/k_2) + 1/k_2 + 1/k_{-2}$. If the dissociation of a small neutral CO_2 molecule is rapid, so that k_{-1} is very large (including $k_{-1} \gg k_2$), and if $k_2 \gg k_{-2}$, then the approximate relation is $1/k_{cat}^{exch} \simeq 1/k_{-2} + 1/k_3$, in accord with the view that the exchange time is determined largely by rate constants involving the reaction steps

$$EH\cdot HCO_3 \xrightarrow{k_3} EH + HCO_3^- \qquad EH\cdot HCO_3^- \xrightarrow{k_{-2}} E\cdot CO_2$$

The temperature dependence on the line widths of the CO_2 and HCO_3 resonances was also examined by using eq 6.

$$\frac{1}{\tau_m} = \frac{kT}{h}\left[\exp\left(-\frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R}\right)\right] \qquad (6)$$

However, two assumptions had to be made in order to perform the analysis; first, that the fraction of bound substrate was constant throughout the experiment and, second, that τ_m as a function of temperature can be described by eq 6. k is the Boltzmann constant, T the temperature, and h is Plank's constant. The fit of the model to the data (Figure 3) yielded values of $\Delta H^\ddagger = 5.8 \pm 1.2$ kcal, $\Delta S^\ddagger = -18.0 \pm 4$ eu, and $\Delta\omega_m = (7.4 \pm 0.5) \times 10^4$ Hz. The calculated value for τ_m at 14 °C was 4.08×10^{-5} s and is in excellent agreement with the estimated value obtained from the concentration dependence of the line width. The shape of the curves, as well as the values of $\Delta\omega$ and τ_m, demonstrate the shift toward intermediate exchange at 50.3 MHz and toward fast exchange at 20 MHz as the temperature is increased.. These results demonstrate that the contributions of enthalpy and entropy to the free energy barrier are nearly equal. However, no further interpretation can be given at this time.

As expected, the value of T_1 obtained for CO_2 and HCO_3^- in the presence of the enzyme was the same. This is a result of the rate of conversion of CO_2 and HCO_3^- being in fast exchange on the T_1 time scale. The measured T_1 must be that of the HCO_3^-, since the HCO_3^- resonance was found to be the most significantly affected with respect to T_{2p}.

Since the hyperfine coupling constant does not contribute significantly to T_{1m} (Yeagle et al., 1975), the distance between the metal and the carbon of the bound HCO_3^- can be estimated from

$$r = C[T_{1m} f(\tau_c)]^{1/6} \qquad (7)$$

where C is the product of constants equal to 460 Å $S^{-1/3}$ (Stein et al., 1977) and $f(\tau_c)$ is a function of the correlation time for the dipolar interaction. The value of τ_c, determined for this system to be $(1.1 \pm 0.1) \times 10^{-11}$, was calculated from the measurement of the ratio of T_1 values at two field strengths and an expression relating τ_c to that ratio. The distance calculated from this equation is 3.22 Å, which indicates HCO_3^- binding within the inner coordination sphere of the metal. Also, the correlation time is consistent with HCO_3^- being bound to a tetracoordinate (Koening et al., 1983) cobalt.

The value of K_d for HCO_3^- binding is equal, within experimental error, to K_{eff} for the enzyme-catalyzed turnover of the substrate, indicating that the HCO_3^- located within the inner coordination sphere of the metal is productively bound for catalyzed turnover. Thus, taken together, the NMR experiments provide for the first time strong, direct evidence that Co(II) human carbonic anhydrase I functions as a catalyst by direct coordination of the HCO_3^- to the metal and also provide additional information on the kinetics of the reactions at the metal site.

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Registry No. HCO_3, 71-52-3.

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