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# $^{13}C$ Nuclear Magnetic Resonance Studies on the Mechanism of Action of Carbonic Anhydrase

**(substrate binding/cobalt metalloenzyme/paramagnetic enhancement of longitudinal relaxation time)**

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**ABSTRACT Binding of the substrate, bicarbonate, to bovine cobalt carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) has been studied with $^{13}C$ nuclear magnetic resonance. Two binding sites for bicarbonate have been identified. One loosely binds bicarbonate, inhibits *p*-nitrophenyl acetate activity, and must be the bicarbonate substrate binding site; the other tightly binds bicarbonate, is noninhibitory, and plays another role. Spin-lattice relaxation times for the carbon atom of bicarbonate indicate that the substrate bicarbonate is bound directly to the metal center of the enzyme, while the other bicarbonate is bound in the outer coordination sphere of the metal. It is proposed that dehydration proceeds via $HCO_3^-$ coordinated directly to the metal center, while the outer sphere bicarbonate facilitates catalytically important proton transfers.**

The zinc metalloenzyme carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) catalyzes the hydration of $CO_2$ and the dehydration of $HCO_3^-$ with exceptionally high efficiency. Although much is known of the nature of this enzyme, including the structure of the crystalline human c isozyme to a resolution of 2.0 Å (for a recent review see ref. 1), little is known about the nature of its interaction with substrates. It was to increase our understanding of the latter that we undertook a study of bicarbonate binding to carbonic anhydrase using $^{13}C$ nuclear relaxation measurements.

$^{13}C$ nuclear relaxation is useful for probing structural features of enzyme–substrate interactions, although the first studies of this type have only recently appeared (2–4). In certain cases, paramagnetic enhancement of nuclear relaxation rates provides a measurement of the distance between the paramagnetic center and the bound substrate (for a recent review see ref. 5).

In this study, paramagnetic enhancement of bicarbonate $^{13}C$ relaxation in solutions of cobalt carbonic anhydrase demonstrates that the substrate is weakly bound to the metal center of the enzyme. Further analysis, combined with a comparison of dissociation constants of the enzyme–bicarbonate complex determined from nuclear magnetic resonance (NMR) experiments and from inhibition experiments, reveals a noninhibitory, tightly bound bicarbonate in the outer coordination sphere of the metal. We propose that this tightly bound bicarbonate facilitates the proton transfers that are required for the dehydration of the substrate $HCO_3^-$ and the reverse hydration of $CO_2$.

Abbreviation: NMR, nuclear magnetic resonance.

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## MATERIALS AND METHODS

Bovine carbonic anhydrase, obtained as a lyophilized material from Worthington Biochemical Corp., was purified by chromatography on Sephadex DEAE A-25 equilibrated with 0.05 M Tris–sulfate buffer, pH 8.7. Elution with the same buffer separated the mixture into three fractions. The first contained carbonic anhydrase, the second superoxide dismutase activity (6), and the third a brownish material, probably derived from hemoglobin.

Protein concentrations were determined from the absorbance at 280 nm, using a molar absorptivity of $5.7 \times 10^4$ $M^{-1}$ $cm^{-1}$ (7). Enzymatic activity was assayed using *p*-nitrophenyl acetate as substrate (8).

Apocarbonic anhydrase was prepared from the purified carbonic anhydrase by dialysis against 1,10-phenanthroline in 0.05 M sodium acetate buffer, pH 5.15 (9), to a residual activity of less than 5%. The cobalt enzyme was prepared by dialysis of this material, still containing 1,10-phenanthroline, against excess cobalt(II) chloride, followed by exhaustive dialysis against Tris–sulfate buffer, pH 7.5. The enzymatic activity of this material was comparable to that of the native zinc enzyme. Cobalt incorporation was verified by atomic absorption. The total amount of active enzyme was determined by titration with acetazolamide (10), and the amount of cobalt enzyme was determined by a concurrent spectral titration at 596 nm with acetazolamide (11). Within an estimated uncertainty of less than 10%, these analyses demonstrated that the protein was active cobalt carbonic anhydrase with one cobalt per molecule of carbonic anhydrase (9). There was no evidence of bound cobalt anywhere but in the active site, though such has been previously reported (12).

Metal ion distributions in our zinc and cobalt carbonic anhydrase preparations were examined by proton-induced x-ray emission analysis (13). The native zinc carbonic anhydrase contains a trace of copper which may be attributable to residual superoxide dismutase. The cobalt enzyme preparation contains residual zinc (usually less than 3%). No copper was detected in the cobalt carbonic anhydrase, or iron in either preparation, at limits of detectability of 0.5% relative abundance.

[$^{13}C$]Carbon dioxide, prepared by addition of sulfuric acid to 90% enriched barium [$^{13}C$]carbonate (obtained from Merck, Sharpe, and Dohme, Ltd.), was absorbed in a sodium hydroxide solution. A nearly quantitative yield of sodium bi-

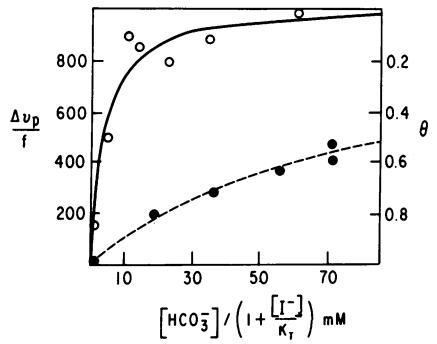


FIG. 1. Bicarbonate binding to cobalt carbonic anhydrase at pH 7.8, 25°. (○) Change in $\Delta\nu_p/f$ with addition of $I^-$ (iodide ion) to the bicarbonate–enzyme system as described. The solid line is calculated from Eq. 2 using a dissociation constant of 4 mM at pH 7.8 for bicarbonate, and an intrinsic inhibition constant of 0.9 mM for $I^-$ (32). (●) Inhibition of *p*-nitrophenyl acetate activity by bicarbonate. The relative activity, $\theta$, is plotted versus $[HCO^-]$ ($[I^-] = 0$ in the abscissa). The broken line is calculated from Eq. 1 using an inhibition constant of 100 mM at pH 7.8. The inhibition experiments were performed at pH 7.5 and corrected to pH 7.8 by: $K_i$ (pH 7.8) = $[(1 + K_H/[H^+]_a)/(1 + K_H/[H^+]_b)]\ K_i$ (pH 7.5) where $K_H$ refers to the ionization of the enzyme crucial to activity [$K_H = 4 \times 10^{-7}$ M (31)], and *a* and *b* refer to pH 7.8 and pH 7.5, respectively.

carbonate was obtained (80% [$^{13}$C]carbon by mass spectral analysis). The bicarbonate concentration was determined by titration with standard hydrochloric acid.

NMR spectra were obtained on a Bruker HFX-10 spectrometer at 22.63 MHz. Chemical shifts were measured relative to internal dioxane and calculated relative to tetramethylsilane [$\delta$ dioxane = 67.4 ppm (14)]. The longitudinal relaxation times, $T_1$, were determined by the partially relaxed Fourier transform method, and the transverse relaxation times, $T_2$, from the observed spectral linewidth (5).

## RESULTS AND DISCUSSION

*NMR Spectra.* The $^{13}$C chemical shifts of $CO_2$, $HCO_3^-$, and $CO_3^{2-}$ are 162.2 ppm, 168.8 ppm, and 125.1 ppm, respectively. The shifts are assigned on the basis of the dominant species present, as determined by solution pH, using an apparent pKa of 6.35 for the first ionization, and 10.3 for the second ionization (15). These shifts are in reasonable agreement with those reported previously (16).

Under the conditions of the experiments with enzyme present, fast exchange occurs between $HCO_3^-$ and $CO_2$, giving rise to a single average resonance (17). This is observed with both the zinc and cobalt enzyme. Addition of acetozolamide, a potent enzyme inhibitor, or removal of the metal, shifts the resonance toward a value characteristic of free $HCO_3^-$, and addition of one equivalent of $Zn^{2+}$ to the apoenzyme returns the resonance to the averaged position.

*Dissociation Constant of the Cobalt Enzyme–Bicarbonate Complex.* The carbonic-anhydrase-catalyzed hydrolysis of *p*-nitrophenyl acetate is inhibited by $HCO_3^-$. For either noncompetitive inhibition or competitive inhibition with weak substrate binding, the inhibitor constant $K_i$ can be determined from measurements of the relative activity, $\theta$, using the relation

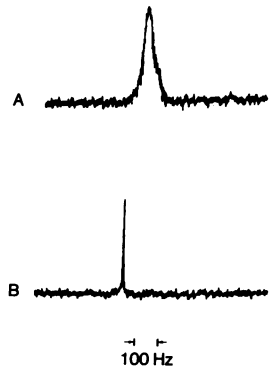


FIG. 2. Effect of inhibitors on linewidth. (A) 0.44 mM cobalt carbonic anhydrase, 15 mM $HCO_3^-$, 0.05 M Tris–$SO_4$ buffer, pH 7.7; (B) as in (A) with one equivalent of acetazolamide added.

$$\theta = \frac{R}{R_0} = \frac{K_i}{K_i + [HCO_3^-]} \qquad [1]$$

where $R$ and $R_0$ are the initial rates in the presence and absence of inhibitor, $[HCO_3^-]$ is the concentration of unbound bicarbonate, and $K_i$ is the dissociation constant of the enzyme–bicarbonate complex, and also, in the case of noncompetitive inhibition, of the enzyme–substrate–bicarbonate ternary complex (18). A plot of $\theta$ as a function of $[HCO_3^-]$ is given in Fig. 1. The value of $[HCO_3^-]$ at $\theta = 0.5$ is equal to $K_i$. A theoretical line calculated from Eq. 1, with $K_i = 100$ mM is shown for comparison. This value of $K_i$ gives a satisfactory fit of the experimental points.

The dissociation constant was also determined from NMR experiments. The linewidth is dominated by paramagnetic effects on the bound $HCO_3^-$ (compare Figs. 2 and 3). Gradual addition of the inhibitor $I^-$ (iodide ion) evidently displaces the bound bicarbonate, resulting in a decrease in linewidth. Such a titration with $I^-$ can be used to determine the equilibrium constant for the dissociation of bicarbonate, $K_d$. For a simple competition between $HCO_3^-$ and $I^-$, assuming that the $^{13}$C of the bicarbonate exists as either "bound" or "free", that exchange between the sites is rapid, and that the bound is a small fraction of the total, the net line broadening $\Delta\nu_p$, is given by

$$\Delta\nu_p = \Delta\nu - \Delta\nu_0 = \frac{f\Delta\nu_\infty[HCO_3^-]}{[HCO_3^-] + K_d(1 + [I^-]/K_I)} \qquad [2]$$

where $\Delta\nu$ is the observed linewidth, $\Delta\nu_0$ the linewidth of the free bicarbonate; $f$ is the ratio of total concentration of metalloenzyme to bicarbonate, and $f\Delta\nu_\infty$ is the net line broadening at saturation with $HCO_3^-$. A $\Delta\nu_0$ of 4 Hz was obtained from the average of 10 measurements of bicarbonate free in solution, and in the presence of the fully acetazolamide-inhibited enzyme (no significant difference was detected between the former set and the latter set of data.) The titration was carried out at constant $[HCO_3^-]$ and $f$, and in three stages; the first with a $[HCO_3^-]$ of approximately 60 mM, the second at $[HCO_3^-]$ of 20 mM, and the third at $[HCO_3^-]$ of 8 mM, adjusting $f$ in each case to give an experimentally suitable linewidth. The results are graphically displayed in Fig. 1. A theoretical line calculated from Eq. 2 taking $K_d = 4$ mM is shown for comparison. This is an unexpectedly small $K_d$; more than an order of magnitude smaller than the dissociation constant determined from the inhibition experiments. This strongly suggests that there are two binding sites for $HCO_3^-$,

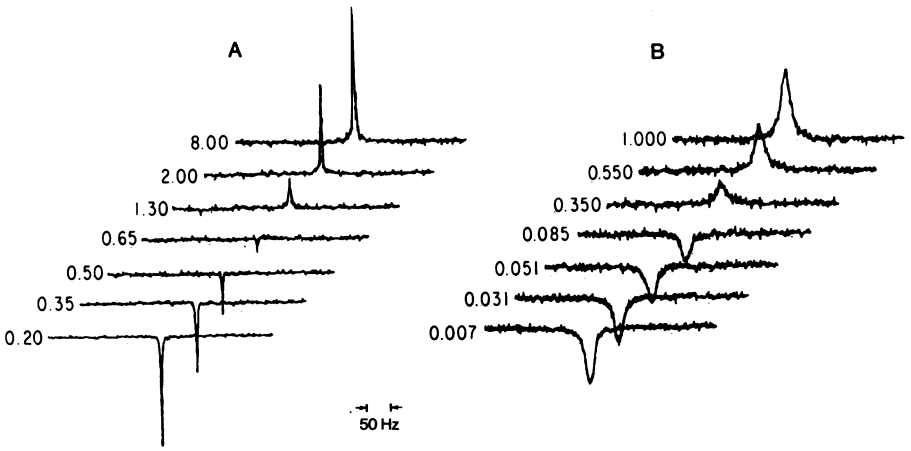


FIG. 3. Proton decoupled partially relaxed Fourier transform spectra of bicarbonate in the presence of zinc and cobalt carbonic anhydrase. (A) 0.5 mM zinc carbonic anhydrase, 30 mM $HCO_3^-$, 0.1 M Tris–$SO_4$ buffer, pH 7.8: (B) 0.5 mM cobalt carbonic anhydrase, 30 mM $HCO_3^-$, 0.1 M Tris–$SO_4$ buffer, pH 7.8. The number to the left of each spectrum indicates the delay time in sec between the 180° and the 90° pulse.

a site to which $HCO^-_3$ is bound weakly, and an additional site to which $HCO_3^-$ is bound much more strongly.

In the foregoing analysis, we do not mean to imply that the occupation of the weaker inhibitor site is without effect on the NMR linewidth. On the contrary, as will be examined in more detail in the section on relaxation rates, occupation of the weaker inhibitor site has a large effect on the linewidth. Assuming that there are two "bound" sites and a "free" site, and that exchange between the sites is rapid, the net line broadening, $\Delta\nu_p$, is more accurately given as the sum of two terms, each having the same form as the righthand side of Eq. 2, but each with its own characteristic value of $\Delta\nu_\infty$ and $K_d$. The large uncertainty in our linewidth data precludes fitting the data, quantitatively, to an equation with four unknown parameters. However, all of our linewidth measurements, both in the presence and absence of $I^-$, are qualitatively in accord with the view that there is a tight binding site for $HCO_3^-$. The inhibition experiments indicate the presence of a much weaker site for $HCO_3^-$. Taken together, the experiments indicate that near the metal center, there is a tight binding site ($K_d \leq 4$ mM) and a weak binding site ($K_d$ = 100 mM) for $HCO_3^-$.

We point out that recent NMR and inhibition experiments show that two acetate ions interact with bovine carbonic anhydrase, and that the stronger acetate binding is to a noninhibitory binding site (19). The noninhibitory sites for acetate and $HCO_3^-$ may be the same.

*Relaxation Rates.* Carbon-13 nuclear relaxation measurements of $HCO_3^-$ in cobalt carbonic anhydrase solutions are illustrated in Figs. 2 and 3. The $^{13}C$ resonance is dramatically broadened and the longitudinal relaxation rate increased in the presence of cobalt carbonic anhydrase. Such effects are not seen with zinc carbonic anhydrase, indicating strong paramagnetic effects on the longitudinal and transverse relaxation rates. Addition of stoichiometric amounts of the potent inhibitor acetazolamide eliminates the paramagnetic effects. Furthermore, significantly enhanced longitudinal and transverse relaxation rates are not observed in the presence of apocarbonic anhydrase.

The paramagnetic contributions to the longitudinal and transverse relaxation rates were calculated from

$$\frac{1}{T_{1_p}} = \frac{1}{T_1} - \frac{1}{T_1^0} \quad [3]$$

$$\frac{1}{T_{2_p}} = \frac{1}{T_2} - \frac{1}{T_2^0} \quad [4]$$

where $1/T_1$ and $1/T_2$ are the measured carbon-13 nuclear relaxation rates of $HCO_3^-$ in cobalt carbonic anhydrase solutions, and $1/T_1^0$ and $1/T_2^0$ are the corresponding relaxation rates in solutions that are similar in all respects except that they contain zinc carbonic anhydrase instead of cobalt carbonic anhydrase. The paramagnetic contributions to the relaxation rates were normalized by the factor $f$, $[\text{enzyme}]_0/[HCO_3^-]_0$, to yield $1/fT_{1_p}$ and $1/fT_{2_p}$ (20).

At binding saturation, $1/fT_{1_p}$ is equal to $1/T_{1_M}$, the longitudinal relaxation rate of the bound ligand, when chemical exchange is rapid enough. Since in these studies $1/fT_{1_p} < 1/fT_{2_p}$, the former cannot be dominated by chemical exchange, and $1/fT_{1_p} \cong 1/T_{1_M}$ is a valid approximation (21). $T_{1_M}$ can be used to calculate the distance $r$ between the paramagnetic center and the $^{13}C$ nucleus when the dipolar contribution to $T_{1_M}$ predominates.

The hyperfine coupling constant ($A/h$) of Co(II) and $^{17}O$ of coordinated water is $A/h = 1.14 \times 10^6$ Hz (29). Assuming that $A/h$ for the $^{13}C$ of $HCO_3^-$ bound to cobalt carbonic anhydrase is not much greater than this, then the hyperfine interaction cannot make a significant contribution to either $1/T_{1_M}$ or $1/T_{2_M}$. The large value of $1/fT_{2_p}$ may be due to a large change in chemical shift of the bound ligand, $\Delta\omega_m$, brought about by the unpaired electrons of Co(II) so that $1/fT_{2_p} = \tau_m\Delta\omega_m^2$ (29).

A general equation for the calculation of the distance, $r$, obtained from the dipolar term of the Solomon-Bloembergen equation (22, 23) is

$$r = C\,[T_{1_M} f(\tau_c)]^{1/6} \quad [5]$$

where $C$ is a product of physical constants whose value depends on the nature of the paramagnetic center and the relaxing nucleus. In the present work, $C$ = 563 Å $\text{sec}^{-1/6}$ (4). The

TABLE 1. *Representative longitudinal ($T_1$) and transverse ($T_2$) relaxation times for the carbon atom of bicarbonate**

| Exp. | Solution† | $T_1$ (sec) | $1/fT_{1_p}$ ($\times 10^{-2}$ $sec^{-1}$) | $1/fT_{2_p}$ ($\times 10^{-2}$ $sec^{-1}$) |
|---|---|---|---|---|
| 1 | $HCO_3^-$ | 7.0 | | |
| 2 | $HCO_3^-$ + CoCA + acetazolamide | 4.4 | | |
| 3 | $HCO_3^-$ + ZnCA | 2.0 | | |
| 4 | $HCO_3^-$ + CoCA | 0.27 | 1.9 | 41 |
| 5 | $HCO_3^-$ + CoCA | 0.60 | 1.0 | 28 |
| 6 | $HCO_3^-$ + CoCA | 0.7 | 2.6 | 190 |
| 7 | $HCO_3^-$ + CoCA + $I^-$ | | | 27 |
| 8 | $HCO_3^-$ + CoCA + $I^-$ | | | 15 |
| 9 | $HCO_3^-$ + CoCA | 0.6 | 0.9 | 28 |

* Measurements made at pH 7.8 (except Exp. 9 which was made at pH 8.8), 25°. Estimated precision: $T_1$ ± 20%, $T_2$ ± 50%.

† Abbreviations: CoCA, cobalt carbonic anhydrase; ZnCA zinc carbonic anhydrase. Concentrations (Exp. number in parentheses): (1) 100 mM $HCO_3^-$; (2) 30 mM $HCO_3^-$, 1 mM CoCA, 1 mM acetazolamide; (3) 30 mM $HCO_3^-$, 0.50 mM ZnCA; (4) 30 mM $HCO_3^-$, 0.50 mM CoCA; (5) 40 mM $HCO_3^-$, 0.49 mM CoCA; (6) 180 mM $HCO_3^-$, 0.60 mM CoCA; (7) 60 mM $HCO_3^-$, 0.8 mM CoCA, 15 mM $I^-$; (8) 20 mM $HCO_3^-$, 0.60 mM CoCA, 60 mM $I^-$. (9) 160 ,M $HCO_3^-$, 1.2 mM CoCA, pH 8.8.

$f(\tau_c)$ in Eq. **5** is defined by

$$f(\tau_c) = \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2} \quad [6]$$

where $\tau_c$ is the correlation time for dipolar interaction, and $\omega_I$ and $\omega_s$ are the nuclear and electronic precessional frequencies. Because Co(II) has a very short electron spin relaxation time [$\tau_s = 5 \times 10^{-13}$ to $10^{-11}$ sec, (5)], $\tau_c$ is determined by this correlation time. In our calculations of distance, we have used a $\tau_c$ value of $1.2 \times 10^{-11}$ sec, which is the Co(II) electron spin relaxation time determined from studies of the relaxation rate of water protons in cobalt carbonic anhydrase solutions (12).

Under conditions where a $K_d \leq 4$ mM site is saturated and a $K_d = 100$ mM site is nearly saturated (64% occupied), the measured $fT_{1_p} = 3.8$ msec (Table 1). From this we calculate that $T_{1_M} = 1.64\, fT_{1_p} = 6.2$ msec. and that (from Eq. **5**) the mean distance between the paramagnetic center and the $^{13}C$ nucleus of the bound $HCO_3^-$ ions is 4.5 Å. The distance 4.5 Å is substantially greater than the inner sphere coordination distance expected from structural studies of Co(II) complexes, which is 2.9–3.0 Å for acetate-type monodentate binding or 2.5 Å for carbonate bidentate binding (24–26). Neither is as long as the distance expected for outer sphere coordination, which is 5.1–5.7 Å (27, 28). Rather, it suggests a contribution from bound inner and outer sphere $HCO_3^-$ ions.

Data obtained at two different values of weak-site occupancy allow the equation

$$1/fT_{1_p} = {}_1P/{}_1T_{1_M} + {}_2P/{}_2T_{1_M} \quad [7]$$

to be evaluated for ${}_1T_{1_M}$ and ${}_2T_{1_M}$, where the prescripts refer to the two different sites, and ${}_iP$ the fraction occupancy of site $i$; the values of ${}_iP$ were calculated from the $HCO_3^-$ concentrations and $K_1 \leq 4$ mM and $K_2 = 100$ mM. From ${}_iT_{1_M}$, $r_i$ can be evaluated. Values of $1/fT_{1_p}$ of 145 $sec^{-1}$ and 260 $sec^{-1}$ were measured in separate experiments (Table 1, the first value is the average of Exps. 4 and 5) in which the tight site was saturated (${}_1P = 1$), and the weak site occupancy was ${}_2P = 0.26$ and 0.64, respectively. Solution of two simultaneous equations in two unknowns yielded ${}_1T_{1_M}$ and ${}_2T_{1_M}$, from which the corresponding $r_1$ and $r_2$ were evaluated using Eqs. **5** and **6**. For the tight binding site $r_1 = 5.2 \pm 0.5$ Å; for the weak site $r_2 = 4.0 \pm 0.5$ Å. The limits given are based on propagation of estimated random errors in the individual observations. The distances correspond to essentially inner and outer sphere coordination, with possible allowances for specific bicarbonate interactions with the protein. In addition, although $r_2 = 4.0 \pm 0.5$ Å is somewhat long for an ordinary inner sphere complex, some "irregular" carboxy-chelated zinc complexes have long metal–carbon distances of 3.6 Å (30).

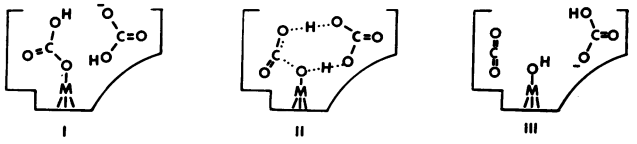

FIG. 4. Proposed mechanism.

Kernohan (10, 31) has shown for the zinc enzyme that the dissociation constant for $HCO_3^-$ as measured by the hydration of $CO_2$ and the dehydration of $HCO_3^-$ is very weak, on the order of 100 mM or greater at pH 7.8. Differences in anion dissociation constants between the cobalt and zinc enzymes are typically less than an order of magnitude (32–34). Therefore, the inner sphere weak site must also be the activity binding site and the stronger binding site must play another role.

*Catalytic Mechanism.* These studies show that dehydration likely proceeds via $HCO_3^-$ coordinated directly to the active site metal. Such direct binding has often been assumed in mechanistic proposals for carbonic anhydrase. Dehydration via $HCO_3^-$ coordinated directly to the metal requires that $CO_2$ be bound nearby (see refs. 10, 32, 35, and 36). A plausible mechanism suggests an $OH^-$ on the metal attacks the nearby $CO_2$, converting it to $HCO_3^-$ (37, 38). As shown in the mechanistic scheme (Fig. 4), an intermolecular proton transfer from the $OH^-$ to an oxygen of $CO_2$ accompanies the reaction **III** → **I**, leaving the $HCO_3^-$ coordinated through a negatively charged oxygen (36, 39). We propose that the tightly bound, noninhibitory $HCO_3^-$ facilitates this transfer. Such a facilitated proton transfer was originally suggested by Wang (40, 41); however, in his proposal, a nearby histidyl residue facilitated the transfer, and this has been rendered unlikely by the modification experiments (42–44) and the x-ray evidence. Since $H_2CO_3$ is a relatively strong acid, $pK_a = 3.8$ (45), the $HCO_3^-$ cannot function exactly in the manner proposed for a histidyl side chain; instead we suggest a concerted reaction such as shown in **II**, Fig. 4.

The rapid turnover of carbonic anhydrase poses additional problems with respect to proton transfers to and from the solvent medium. These must be unusually fast. Indeed, for any mechanism requiring a change in the state of ionization of an enzyme group with $pK_a$ near 7, direct transfer to or from water species is much too slow to keep pace with the observed turnover (46). This may not be as serious a dilemma as was

once believed because, as Lindskog and Coleman (46) and Khalifah (47) have pointed out, rapid proton exchange can occur with buffer components. Nevertheless, the tightly bound $HCO_3^-$ may facilitate these required proton transfers.

These proposals are not the only ones possible; however, we believe that they are the ones that best explain our experimental observations, and are consistent with other studies of this enzyme. In particular, we point out a recent comparison of the carbonic anhydrase-catalyzed reactions $H^+ + CH_3OCO_2^- \rightarrow CH_3OH + CO_2$ and $H^+ + HOCO_2^- \rightarrow HOH + CO_2$ (48). In the absence of enzyme, the rate constants for the two reactions are nearly the same; in contrast, although the enzyme is a good catalyst for both reactions, the turnover number for $CH_3OCO_2^-$ is a factor of $10^3$ less than the turnover number of $HOCO_3^-$, showing that substitution of the H of bicarbonate by a $CH_3$ group has important kinetic consequence in the catalyzed reaction. This observation is in accord with the mechanism proposed here.

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