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(20) CD spectra from 200 to 250 nm were performed on a Jasco J-20 spectrophotometer using 0.1-mm cells containing $9.0 \times 10^{-5}$ enzyme in water pools where [AOT] = 0.11 M and $R = 25$. Spectra from 250 to 300 nm were carried out with the same solutions but with 5-mm cells. We thank Professor A. J. Sophianopoulas for his assistance with the CD experiment.
(21) Note Added in Proof: We call attention to the related work of Professor P. L. Luisi in Zurich which will be published (*Biochem. Biophys. Res. Commun.*).

# Rate of Exchange of Water from the Active Site of Human Carbonic Anhydrase C

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**Abstract:** The rate constant for the exchange of $^{18}O$ between $CO_2$ and water and the rate constant for the exchange of $^{18}O$ between $^{12}C$- and $^{13}C$-containing species of $CO_2$ have been measured in the range of pH 6 to 8 in the presence of human carbonic anhydrase C. Solving the kinetic equations for the distribution of isotopes, we express these data as the rates of two independent steps in the catalysis: $R_1$, the rate at equilibrium of the catalytic interconversion of $CO_2$ and $HCO_3^-$, and $R_{H_2O}$, the rate of exchange from the enzyme of water containing the oxygen abstracted from bicarbonate. $R_1$ is generally independent of buffer, whereas $R_{H_2O}$ increases with the concentration of the buffer imidazole or 2,4-lutidine, then levels off near 10 mM buffer. In the presence of 50 mM imidazole or 2,4-lutidine, $R_{H_2O}$ when plotted against pH is bell-shaped with a maximum at pH 6.8. These results are consistent with an internal proton transfer between two ionizing groups of the enzyme $E(^{18}OH)BH \rightleftharpoons E(^{18}OH_2)B$, which becomes rate limiting for $R_{H_2O}$ at larger buffer concentrations. The data suggest a $pK_a$ near 6.8 for each ionizing group with a rate constant for proton transfer of $3.5 \times 10^6\ s^{-1}$. In the absence of external buffers, the $^{18}O$ content of E(OH)B will be greater than that of E(OH)BH because of the possibility of internal proton transfer converting E(OH)BH to $E(OH_2)B$ from which exchange of water can occur. Hence, the presence of external buffer enhances $^{18}O$ exchange by causing rapid interconversion between E(OH)B and E(OH)BH.

The zinc-containing metalloenzyme carbonic anhydrase catalyzes the hydration of $CO_2$ to produce bicarbonate and a proton.[1]

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad (1)$$

The turnover number for the catalyzed hydration of $CO_2$ by bovine and human C forms of carbonic anhydrase is very large (about $10^6\ s^{-1}$) so that the catalysis can be limited in rate by the transfer of the proton between the enzyme and external buffers in solution, a transfer which is necessary to regenerate the form of the enzyme active in hydration:

$$EH + B \rightleftharpoons E^- + BH^+ \quad (2)$$

The proton transfer of eq 2, in which B indicates a buffer that is not part of the enzyme, becomes rate limiting in the catalyzed reaction at buffer concentrations less than about 5 mM, as shown by initial velocity studies[2] and measurements of the rate of $^{18}O$ exchange between $CO_2$ and $H_2O$ at chemical equilibrium.[3]

At high concentrations of external buffers the proton transfer of eq 2 is not rate limiting; however, measurement of the solvent isotope effects in $H_2O$ and $D_2O$ on maximal steady-state turnover numbers for bovine carbonic anhydrase[4] and human carbonic anhydrase C[5] suggests that an intramolecular proton transfer is rate limiting. It has been proposed that the intramolecular transfer involves a proton transfer group near the active site and the catalytic group.[5] Measuring the line widths of the $^{13}C$ resonances of a $CO_2$–$HCO_3^-$ equilibrium mixture in the presence of human carbonic anhydrase C, Simonsson et al.[6] have determined that this intramolecular proton transfer is not involved directly in the catalytic conversion of $CO_2$ to $HCO_3^-$. Studies of the inhibition of esterase activity of human carbonic anhydrase C by equilibrium mixtures of $CO_2$ and $HCO_3^-$ have also suggested the presence of a rate-limiting intramolecular proton transfer.[7]

The $^{18}O$ exchange between $CO_2$ and $H_2O$ catalyzed by carbonic anhydrase is a complement to these studies. It has

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been shown that the catalyzed dehydration of $HCOO^{18}O^-$ can label the active site with $^{18}O$ and that the rate of dissociation of $^{18}O$ from the active site is dependent on the presence of buffers.[3] That is, the catalyzed rate of exchange of $^{18}O$ between $CO_2$ and $H_2O$ contains information on both the interconversion of $CO_2$ and $HCO_3^-$ and the release of water containing substrate oxygen from the enzyme. We report here the pH dependence of the rate constants for $^{18}O$ exchange in the presence of human carbonic anhydrase C and in the presence and absence of buffers. This allows us to elucidate the rate-determining intramolecular proton transfer process and the kinetics of the release of oxygen during catalysis.

## Experimental Section

**Materials and Enzyme.** Oxygen-18 labeled bicarbonate was prepared by dissolving $KHCO_3$ in enriched water (up to 90 atom % $^{18}O$ enrichment). This solution was allowed to come to isotopic equilibrium overnight, after which water was removed by vacuum distillation. Carbon-13 labeled bicarbonate was prepared in the following manner. $^{13}CO_2$ was generated by adding phosphoric acid to enriched barium carbonate (90 atom % $^{13}C$ enrichment). The $^{13}CO_2$ generated was then absorbed into an equimolar amount of KOH in solution within a vacuum system. Imidazole was purified by recrystallization from benzene.

Carbonic anhydrase was obtained from human erythrocytes of outdated blood. The erythrocytes were washed three times with isotonic saline and then lysed by addition of a volume of water equal to the volume of packed cells. The separation of membranes was achieved by centrifugation, and human carbonic anhydrase C was prepared from the supernatant by the affinity chromatography procedure of Khalifah et al.[8] The resulting enzyme was determined to be greater than 95% pure as determined by polyacrylamide gel electrophoresis, which showed one band. The enzyme was dialyzed for 10 days at 2 °C against 9 changes of a large volume of water.

**Experimental Procedure.** The isotopic content of $CO_2$ in solution was measured with a mass spectrometer (Finnigan 3000) using a $CO_2$ inlet vessel devised by Hoch and Kok[9] and described earlier.[10] Solutions containing labeled $CO_2$ were placed in the inlet vessel, the bottom of which was a membrane (Membrane Kit 5937, Yellow Springs Instrument Company) permeable to $CO_2$ and supported by a porous stainless steel disk. Gas passing across the membrane had water vapor removed in a dry ice–acetone trap and then entered the mass spectrometer. This apparatus provided continuous monitoring of the isotopic content of $CO_2$ in solution. The time lag from the vessel to the mass spectrometer was less than 3 s, measured by sudden injection of $^{13}CO_2$ into the vessel. The half-times for exchanges of $^{18}O$ between $CO_2$ and $H_2O$ in this study were greater than 45 s. The inlet vessel was fitted with a water-circulation jacket to maintain constant temperature, and a Teflon plunger in the inlet vessel prevented contact of the contents of the vessel with atmospheric $CO_2$.

Isotope exchange experiments were performed in the range of pH 6 to 8 with pH adjustments made using NaOH and $H_2SO_4$. At values of pH above 8, the $^{18}O$ exchange is too slow and the concentration of $CO_2$ too small to allow accurate measurements with the inlet vessel. In all experiments the total ionic strength of the solutions was maintained constant at 0.2 by adding the appropriate amount of $Na_2SO_4$. Before each experiment, the concentration of human carbonic anhydrase C was determined by both a changing pH assay[11] and the molar extinction coefficient of $5.6 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm.

Each experiment was started by placing into the inlet vessel 8.0 mL of a solution in which $^{18}O$- and $^{13}C$-labeled bicarbonate had been dissolved. A period as long as several minutes elapsed to allow approach to chemical equilibrium; then measurements of isotopic content of $CO_2$ were taken. Enzyme was added in a volume less than 0.1 mL and at least 10 s was allowed for mixing before measurements were taken. During an experiment the pH remained constant to within 0.02 pH unit. All data presented here were obtained at 25 °C.

**Analysis of Kinetic Data.** One measured variable in this technique is $\alpha$, the atom fraction of $^{18}O$ in all $CO_2$:

$$\alpha = \frac{(46) + (47) + 2(48) + 2(49)}{2[(44) + (45) + (46) + (47) + (48) + (49)]}$$

where (44), (45), (46), ... represent the heights of the mass peaks of $CO_2$, $^{13}CO_2$, $CO^{18}O$, .... As shown by Mills and Urey,[12] the exchange of $^{18}O$ between $CO_2$ and $H_2O$ at chemical equilibrium is a first-order kinetic process; hence, the slope of a plot of ln $(\alpha - \alpha_\infty)$ vs. time yields the rate constant $\theta$ for $^{18}O$ exchange: $\theta = \theta_{uncat} + \theta_{cat}$. The atom fraction measured at infinite time $\alpha_\infty$ is close to the natural abundance of $^{18}O$, which is 0.002.

Two other variables measured in this technique are $^{(12)}\alpha$ and $^{(13)}\alpha$, the atom fraction of $^{18}O$ in $^{12}C$- and $^{13}C$-containing $CO_2$, respectively:

$$^{(12)}\alpha = \frac{(46) + 2(48)}{2[(44) + (46) + (48)]}$$

$$^{(13)}\alpha = \frac{(47) + 2(49)}{2[(45) + (47) + (49)]}$$

As shown in a previous publication,[10] the change in both $^{(12)}\alpha$ and $^{(13)}\alpha$ as exchange proceeds is biphasic and can be described by eq 3 and 4. These equations pertain to an experiment in which $^{18}O$-containing bicarbonate not enriched in $^{13}C$ is mixed with $^{13}C$-containing bicarbonate not enriched in $^{18}O$. The rate constant $\phi$ is characteristic of the exchange of $^{18}O$ between $^{12}C$- and $^{13}C$-containing species of $CO_2$, an exchange which occurs slowly in the absence of enzyme, and is catalyzed by carbonic anhydrase:[13] $\phi = \phi_{uncat} + \phi_{cat}$. Reference 10 describes how $\phi$ is obtained from experiments.

$$^{(12)}\alpha = b_1 e^{-\theta t} + b_2 e^{-(\theta+\phi)t} \quad (3)$$

$$^{(13)}\alpha = c_1 e^{-\theta t} + c_2 e^{-(\theta+\phi)t} \quad (4)$$

## Results

Figure 1 presents the variation with pH of $\theta_{cat}$, the rate constant for the catalyzed exchange of $^{18}O$ between $CO_2$ and water, and $\phi_{cat}$, the rate constant of the catalyzed exchange of $^{18}O$ between $^{12}C$- and $^{13}C$-containing species of $CO_2$. Figure 1a gives those data obtained in the absence of buffer, although the substrate itself provides a very small buffer capacity in the pH range of these data (see Discussion). These data are bell-shaped as pH is varied from 6 to 8, a feature which was also obtained using bovine carbonic anhydrase in the absence of buffer but at larger total substrate concentrations.[10] The data in ref 10 that extend into the region of pH 8 to 9 were obtained by rapid acidification of samples of the reaction solution. This procedure was not performed in the experiments reported here.

Comparing Figure 1b to 1a demonstrates the increase in $\theta_{cat}$ and decrease in $\phi_{cat}$ above pH 6.25 when buffer, in this case 50 mM imidazole, is present. This change in the rate for $^{18}O$ exchange upon adding many different buffers has been attributed to proton transfer between the buffer and the enzyme,[3] the same proton transfer that functions to maintain equilibrium distributions between the ionization states of the enzyme, described in eq. 2. Experiments at pH 7.0 in which the concentration of imidazole was varied show that $\theta_{cat}$ increases upon addition of imidazole reaching a plateau at about 5 mM imidazole; $\phi_{cat}$ decreases also leveling off at 5 mM imidazole.[3]

Table I gives the variation of $\theta_{cat}$ and $\phi_{cat}$ as a function of total substrate concentration in the presence of 25 mM imidazole and in the absence of buffer: $\theta_{cat}$ decreases as total concentration of all species of $CO_2$ increases, whereas the variation in $\phi_{cat}$ is small. Our experiments show that $\theta_{cat}$ and $\phi_{cat}$ are independent of the fraction of all $CO_2$ species containing $^{13}C$. The experimental error observed in $\theta_{cat}$ and $\phi_{cat}$ precludes the measurement of the small kinetic isotope effects. There is a range of ±10% in repeated measurements of catalyzed rates of $^{18}O$ exchange when using different enzyme solutions, each diluted to the same apparent enzyme concentration. The source of this variation is not known, but we believe it to be related to the necessity of diluting enzyme to very low concentration (near $10^{-9}$ M). This is not a problem when many experiments are performed using a single enzyme solution, but is a problem in comparing the results of different series of experiments. This is responsible for part of the observed scatter in Figure 1. It should also be noted that the presence of sulfate ion, used in

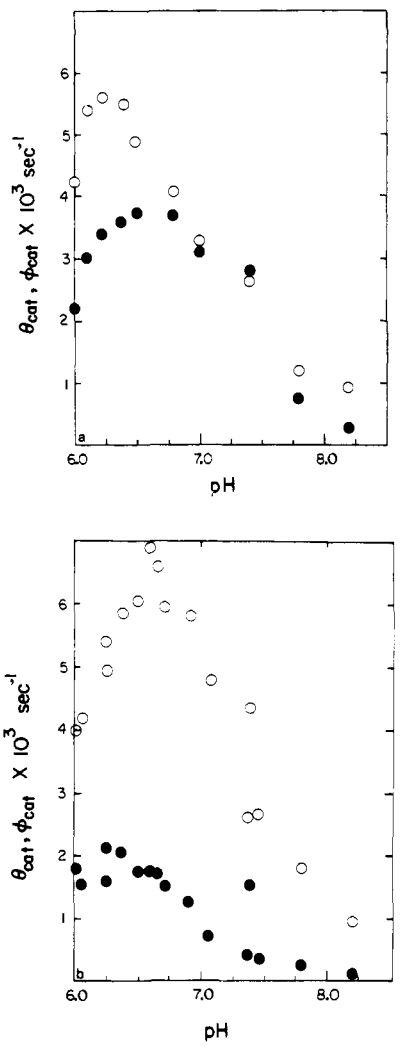



Figure 1. (a, top) The rate constant $\theta_{cat}$ (○) for the catalyzed exchange of $^{18}O$ between $CO_2$ and water, and $\phi_{cat}$ (●) for the catalyzed exchange of $^{18}O$ between $^{12}C$- and $^{13}C$-containing species of $CO_2$ at 25 °C. This experiment was performed in the absence of buffers. The total concentration of all species of $CO_2$, $[S_{tot}]$, was 15 mM and human carbonic anhydrase C was $1.6 \times 10^{-9}$ M. The fraction of all $CO_2$ species containing $^{13}C$ was 0.45. (b, bottom) The same as Figure 1a, except 50 mM of the buffer imidazole was present.

these experiments to maintain final ionic strength of solutions at 0.2 M, has been demonstrated to shift to higher values the p$K$ of the group that is responsible for solvent proton relaxation in solutions of cobalt-substituted bovine carbonic anhydrase.[14,15] However, this effect is greatest at low concentrations of sulfate and should not introduce another experimental variable in the measurements reported here.

Since this is a slow kinetic technique, the concentration of enzyme used in these experiments is low, in the range of $10^{-9}$ M. Hence, the rate constants for catalyzed $^{18}O$ exchange are the same order of magnitude as the rate constants for uncatalyzed $^{18}O$ exchange. Values of $\theta_{uncat}$ may be calculated from hydration rate constants as described in ref 10; under the conditions of these experiments at pH 7.5 the value of $\theta_{uncat}$ is $7.6 \times 10^{-4}$ $s^{-1}$. Values of $\phi_{uncat}$, also given in ref 10, do not exceed $2 \times 10^{-4}$ $s^{-1}$ in the pH range of these studies.

Changing the solvent from $H_2O$ to 98% $D_2O$ has the effect of decreasing $\theta_{cat}$ and increasing $\phi_{cat}$ as shown in Table II. The pH of 7.4 reported in these experiments refers to the uncorrected pH meter readings. The p$K_a$ value characterizing the pH-rate profile for carbonic anhydrase C is about 0.5 unit larger in $D_2O$ than in $H_2O$.[5] This shift is approximately equal

Table I. Variation of $\theta_{cat}$ and $\phi_{cat}$ with Total Concentration of All Species of $CO_2$ at pH 7.4 and 25 °C[a]

| $[CO_2] + [HCO_3^-]$ $\times 10^3$ M | no buffer added, $\times 10^3$ $s^{-1}$ | | imidazole at 0.025 M, $\times 10^3$ $s^{-1}$ | |
|---|---|---|---|---|
| | $\theta_{cat}$ | $\phi_{cat}$ | $\theta_{cat}$ | $\phi_{cat}$ |
| 2.5 | 3.2 | 0.6 | 3.5 | 0.3 |
| 5.0 | 2.7 | 1.1 | 3.2 | 0.4 |
| 15 | 1.3 | 1.2 | 2.1 | 0.5 |
| 25 | 1.3 | 1.6 | 2.2 | 0.8 |
| 30 | 0.9 | 1.7 | 1.9 | 0.8 |
| 50 | 0.4 | 1.2 | 1.2 | 0.8 |

[a] Human carbonic anhydrase C was present at $1.2 \times 10^{-9}$ M with the fraction of all species of $CO_2$ containing $^{13}C$ at 0.45.

Table II. Rate Constants for $^{18}O$ Exchange and $R_1$, $R_{H_2O}$ Obtained in $H_2O$ and $D_2O$[a]

| | solvent $H_2O$ | | solvent 98% $D_2O$ | |
|---|---|---|---|---|
| | without imidazole | 50 mM imidazole | without imidazole | 50 mM imidazole |
| $\theta_{cat}$ ($\times 10^3$ $s^{-1}$) | 3.9 | 4.5 | 1.7 | 2.3 |
| $\phi_{cat}$ ($\times 10^3$ $s^{-1}$) | 1.8 | 0.67 | 3.2 | 2.2 |
| $R_1$ ($\times 10^4$ M $s^{-1}$) | 2.5 | 2.3 | 2.2 | 2.0 |
| $R_{H_2O}$ ($\times 10^4$ M $s^{-1}$) | 5.7 | 15.0 | 1.1 | 2.1 |

[a] Human carbonic anhydrase C was present at $2.4 \times 10^{-9}$ M with $[CO_2] + [HCO_3^-]$ equal to 15 mM. Uncorrected pH meter reading was 7.4 in each case.

to the factor that must be added to the reading of a pH meter, calibrated in aqueous solution, to obtain the value of pD.

## Discussion

Human carbonic anhydrase C cannot reach maximal activity in initial velocity experiments, which corresponds to a turnover number of about $10^6$ $s^{-1}$, when only species of water are available to act as proton acceptors and donors.[16-18] This accounts for the increase as buffer concentration increases of the initial rate of the catalyzed hydration of $CO_2$[2] and of $\theta_{cat}$, the rate constant for catalyzed exchange of $^{18}O$ between $CO_2$ and $H_2O$.[3] The buffer provides a concentration of proton donor and proton acceptor that is large relative to the concentration of $H_3O^+$ and $OH^-$. Further information about the catalysis is provided by the $^{18}O$ exchange experiment; $\phi_{cat}$ describes the catalyzed exchange of $^{18}O$ between $^{12}C$- and $^{13}C$-containing species of $CO_2$.[10] This rate constant, $\phi_{cat}$, arises because $^{18}O$ labels the active site of carbonic anhydrase during the dehydration of labeled bicarbonate. When the residence time of the label is sufficiently long in the active site, it is incorporated into $^{13}C$-containing species by the catalytic hydration of $^{13}CO_2$.[10] These kinetic parameters provide information about the rate of interconversion of $CO_2$ and $HCO_3^-$, the lifetime of oxygen at the active site, and the ionization state of the groups that affect the exchange of oxygen with solvent.

**Kinetic Theory.** A relation between $\theta_{cat}$ and the rate of the chemical reactions causing $^{18}O$ exchange between $CO_2$ and water has been derived previously.[3,12] A similar relation for $\phi_{cat}$ has been derived by Silverman and Tu,[10] but this derivation specified neither the form of $CO_2$ (i.e., $CO_2$ or $HCO_3^-$) from which $^{18}O$ exchange occurs nor the stoichiometry of oxygen in the exchange. We refine this older derivation by assuming a two-part, general model for the catalyzed exchange; we then examine the experimental results to test whether the model is compatible with the data. Oxygen-18 exchange is assumed to occur in a two-step process: step 1 is the dehydration of $^{18}O$-labeled bicarbonate giving $CO_2$ and leaving $^{18}O$ in the active site; step $H_2O$ is the release of labeled oxygen to solvent water, a step which can occur independently

**Scheme I**

$$\begin{array}{l} HCOO^{18}O^- + E(OH_2) \\ \quad\downarrow \text{step 1} \\ E(^{18}OH)^- + CO_2 + H_2O \xrightarrow[+H^+]{\text{step } H_2O} E(OH_2) + CO_2 + H_2{}^{18}O \\ \quad\downarrow +{}^{13}CO_2 \\ E(OH_2) + H^{13}COO^{18}O^- \end{array}$$

of step 1. The step in which $^{13}CO_2$ reacts with labeled active site to give $H^{13}COO^{18}O^-$ is a hydration step; the chemical reaction causing this exchange has the same rate as step 1 at equilibrium. This model is general, encompassing many proposed mechanisms; however, in writing Scheme I below we are more specific, utilizing the feature of ionization of water at the active site. This choice is defended later in the Discussion. In this scheme, $E(OH_2)$ and $E(OH)^-$ represent the form of enzyme at the active site at low pH and at high pH, respectively; these are possibly zinc-bound water and hydroxide.[1]

Five differential equations describe the exchange of oxygen in this model. We define $^{(12)}\gamma$ and $^{(13)}\gamma$ as the atom fraction of $^{18}O$ in $^{12}C$- and $^{13}C$-containing bicarbonate, respectively; $\delta$ is the atom fraction of all oxygens at the enzyme's active site which are $^{18}O$; and $r$ is the fraction of all $CO_2$ species containing $^{13}C$. By accounting for oxygen atoms in the manner of Mills and Urey,[12] we obtain:

$$\frac{-d^{(12)}\alpha}{dt} = \frac{R_1}{[CO_2]}\left(^{(12)}\alpha - {}^{(12)}\gamma\right) \quad (5)$$

$$\frac{-d^{(13)}\alpha}{dt} = \frac{R_1}{[CO_2]}\left(^{(13)}\alpha - {}^{(13)}\gamma\right) \quad (6)$$

$$\frac{-d^{(12)}\gamma}{dt} = \frac{R_1}{[HCO_3^-]}\left(^{(12)}\gamma - \frac{2^{(12)}\alpha + \delta}{3}\right) \quad (7)$$

$$\frac{-d^{(13)}\gamma}{dt} = \frac{R_1}{[HCO_3^-]}\left(^{(13)}\gamma - \frac{2^{(13)}\alpha + \delta}{3}\right) \quad (8)$$

$$\frac{-d\delta}{dt} = \left(\frac{R_1 + R_{H_2O}}{[E(OH)]}\right)\delta - \frac{R_1}{[E(OH)]}\left[^{(12)}\gamma(1-r) + {}^{(13)}\gamma r\right] \quad (9)$$

Here $R_1$ and $R_{H_2O}$ are the rates of the chemical reactions of step 1 and step $H_2O$ in Scheme I; $[E(OH)]$ is the concentration of the high pH form of the enzyme.

The solution of eq 5-9 is made easier by first setting $r$, $^{(13)}\alpha$, and $^{(13)}\gamma$ equal to zero (i.e., no $^{13}C$ labeling) and then solving the resulting three differential equations. The result will also be part of the solution of the more complicated case considering all five differential equations. Setting $r$, $^{(13)}\alpha$, and $^{(13)}\gamma$ equal to zero and working with eq 5, 7, and 9 gives a third-order characteristic equation. The solution of the three differential equations is of the form:

$$^{(12)}\alpha = \sum_{i=1}^{3} a_i e^{-\theta_i t} \quad (10)$$

with the $\theta_i$ being roots of the characteristic equation. In experiments with no $^{13}C$ labeling, the exchange of $^{18}O$ between $CO_2$ and $H_2O$ can be described by a single exponential.[10] That is, in the exchange experiments only one root $\theta_1$ is important, the others, $\theta_2$ and $\theta_3$, being too large to be observed. This one small root in the characteristic cubic equation $\theta^3 + A\theta^2 + B\theta + C = 0$ is approximated by $\theta_1 = -C/B$. Subtracting $\theta_{uncat}$ from $\theta_1$ yields the approximate solution:

$$\theta_{cat} = \frac{\frac{1}{3}R_1 R_{H_2O}}{(R_1 + R_{H_2O})[S_{tot}] - \frac{1}{3}R_1[CO_2]} \quad (11)$$

where $[S_{tot}] = [CO_2] + [HCO_3^-]$.

Guided by eq 3 and 4, which fit the experimental data, we expect two significant roots to the full set of eq 5-9, one root of which is given by eq 11. The characteristic equation of degree 5 obtained from eq 5-9 was divided by the characteristic equation from the case considering $^{13}C$ content to be zero, which resulted in a quadratic equation. The smallest root of this quadratic is eq 12:

$$\theta_{cat} + \phi_{cat} = \frac{R_1}{2}\left(\frac{1}{[CO_2]} + \frac{1}{[HCO_3^-]}\right) \times \left[1 - \sqrt{1 - \frac{4[CO_2][HCO_3^-]}{3([CO_2] + [HCO_3^-])^2}}\right] \quad (12)$$

In order to deal with this solution more easily, we have approximated the square root in eq 12 by:

$$\left|1 - \frac{2[CO_2][HCO_3^-]}{3([CO_2] + [HCO_3^-])^2}\right|$$

This approximation for the square root is excellent in regions of pH for which either $CO_2$ or $HCO_3^-$ is the predominant species; that is, for pH >7 and pH <5. In the region of pH between 6 and 7, this approximation introduces an error no greater than 3% of the true value of the square root in eq 12. Substituting this approximation into eq 12 gives:

$$R_1 = 3[S_{tot}](\theta_{cat} + \phi_{cat}) \quad (13)$$

Using the value of $\theta_{cat}$ from eq 11 yields:

$$R_{H_2O} = \frac{R_1\theta_{cat}}{\phi_{cat}}\left(1 - \frac{[CO_2]}{3[S_{tot}]}\right) \quad (14)$$

In the next section we determine whether experimental values of $\theta_{cat}$ and $\phi_{cat}$ are consistent with this model by comparing values of $R_1$ obtained by eq 13 with values of $R_1$ obtained by other kinetic methods performed at chemical equilibrium.

***R*$_1$.** According to eq 13, $R_1$, the catalyzed rate of interconversion of $CO_2$ and $HCO_3^-$ at chemical equilibrium, is easily obtained from measurements of $\theta_{cat}$ and $\phi_{cat}$. In Figure 2a, values of $R_1$ found from $^{18}O$ exchange experiments are presented over the range of pH 6 to 8. Measurement of $^{13}C$ NMR line widths also gives the catalytic rate of interconversion at chemical equilibrium between $CO_2$ and $HCO_3^-$. As described by Koenig et al.,[19] and utilized by Simonsson et al.,[6] this rate of interconversion $R_1$ can be expressed in the form:[20]

$$R_1 = \frac{k_{cat}^{exch}[S_{tot}][E_{tot}]}{K_{eff} + [S_{tot}]} \quad (15)$$

in which $K_{eff}$ has a complicated form but corresponds to the apparent dissociation constant between substrate and enzyme. Using values of $k_{cat}^{exch}$ and $K_{eff}$ determined for human carbonic anhydrase C by Simonsson et al.,[6] we have calculated values of $R_1$ using $[E_{tot}]$ and $[S_{tot}]$ of $1.6 \times 10^{-9}$ M and 15 mM, respectively, the concentrations used in this study. These values of $R_1$ appear in Figure 2b and are to be compared with those of Figure 2a. In Figure 2b we have also plotted values of $R_1$ calculated in another way: using $K_{eff}$ obtained by inhibiting with equilibrium mixtures of $CO_2$ and $HCO_3^-$ the catalyzed hydrolysis of *p*-nitrophenyl acetate.[7] These values of $R_1$ are calculated using the values of $k_{cat}^{exch}$ determined for carbonic anhydrase C by the NMR method.[6] Despite the scatter of data in these equilibrium techniques, there is agreement between the values of $R_1$ in Figures 2a and 2b.

Examination of Figure 2a also shows that $R_1$ is independent of buffer for pH >6.5. At pH <6.5 it appears that the value of $R_1$ found in $^{18}O$ experiments with no buffer is slightly greater than $R_1$ in the presence of 50 mM of the buffer imidazole. Simonsson et al.,[6] measuring $^{13}C$ NMR line widths, have

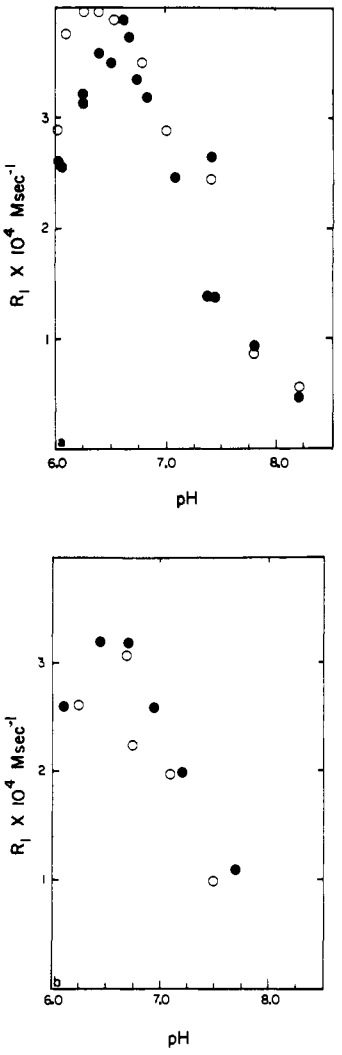



Figure 2. (a, top) Values of $R_1$ determined using eq 13 from measurements of $\theta_{cat}$ and $\phi_{cat}$ at 25 °C (○) in the absence of buffer and (●) in the presence of 50 mM imidazole. [$S_{tot}$] was 15 mM and human carbonic anhydrase C was present at $1.6 \times 10^{-9}$ M. (b, bottom) Open circles (○) are values of $R_1$ calculated from eq 15 using $K_{eff}$ and $k_{cat}^{exch}$ determined by effects of human carbonic anhydrase C on the $^{13}C$ NMR spectra of equilibrium mixtures of $^{13}CO_2$ and $NaH^{13}CO_3$ reported in ref 6. Filled circles (●) are values of $R_1$ calculated using $k_{cat}^{exch}$ from ref 6 and $K_{eff}$ from studies of the inhibition of esterase activity of the human C enzyme by equilibrium mixtures of $CO_2$ and $HCO_3^-$ reported in ref 7. In each case [$S_{tot}$] = 15 mM and [$E_{tot}$] = $1.6 \times 10^{-9}$ M.

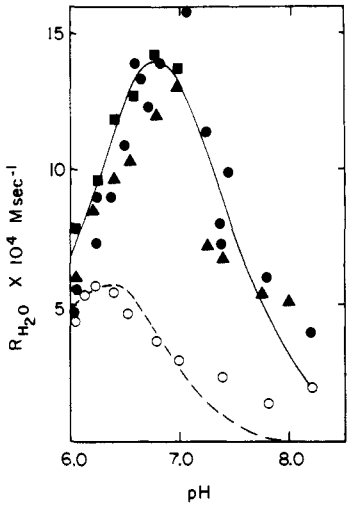



Figure 3. $R_{H_2O}$ calculated, using eq 14, from $\theta_{cat}$ and $\phi_{cat}$ measured (○) in the absence of buffer and (●) in the presence of 50 mM imidazole, (▲) 50 mM 2,4-lutidine, or (■) 50 mM 3,5-lutidine. In each case [$S_{tot}$] = 15 mM and human carbonic anhydrase C was $1.6 \times 10^{-9}$ M. The solid line is $R_{H_2O}$ calculated from eq 16 of the text with $K_1 = K_2 = 10^{-6.8}$ and $k_{-3} = 3.5 \times 10^6$ s$^{-1}$. The dashed line is calculated from eq 17 for $R_{H_2O}$ (no buffer) using a value of 6.4 for the p$K_a$ of the internal proton transfer group.

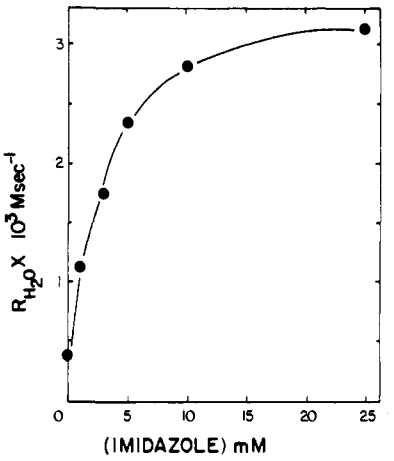



Figure 4. $R_{H_2O}$ determined from $\theta_{cat}$ and $\phi_{cat}$ at 25 °C and pH 7.0. Human carbonic anhydrase C was present at $2.8 \times 10^{-9}$ M and [$S_{tot}$] was 15 mM with $r = 0.45$.

also determined that the rate of interconversion between $CO_2$ and $HCO_3^-$ at equilibrium is independent of buffer. Table II demonstrates a further agreement between the $^{18}O$ results and the $^{13}C$ NMR work of Simonsson et al.: both techniques show $R_1$ relatively unaffected by the change of solvent from $H_2O$ to $D_2O$.

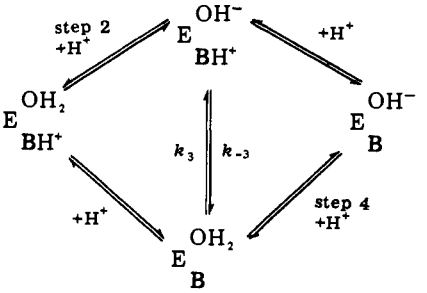
**$R_{H_2O}$ in the Presence of Buffer.** $R_{H_2O}$ is the rate at which water containing oxygen abstracted from bicarbonate is released from carbonic anhydrase or exchanged with a water molecule from the solvent. This is the second step in Scheme I. As shown in Figures 3 and 4, it is $R_{H_2O}$ that describes the buffer-dependent process in the catalysis. The maximal value of $R_{H_2O}$ caused by the addition of the buffer imidazole (p$K_a$ ≃ 7.15) at pH 7.5 is $R_{H_2O} = 3.4 \times 10^{-3}$ M s$^{-1}$, obtained by linearization in the manner of Eadie and Hofstee of the data in Figure 4. Increasing the concentration of imidazole beyond 25 mM produces relatively small increases in $R_{H_2O}$. Hence, the values of $R_{H_2O}$ measured with 50 mM imidazole and given in Figure 3 are taken to be in a region of excess buffer concentration such that the rate-determining step for $R_{H_2O}$ is not buffer dependent. This was confirmed by repeating the experiments of Figure 3 using 25 mM imidazole; the results were identical within experimental error with the data in Figure 3 for 50 mM imidazole. Changing the buffer to 2,4-lutidine (p$K_a$ ≃ 6.8) or 3,5-lutidine (p$K_a$ ≃ 6.2) also gave similar results for $R_{H_2O}$ as shown in Figure 3.

An important interpretation from these studies is that the exchange rate $R_{H_2O}$ when measured in the presence of excess buffer depends on more than one ionization in the enzyme, as demonstrated in Figure 3. We consider in Scheme II two ionizations within the enzyme itself that may be considered to influence the pH dependence of $R_{H_2O}$.

In this scheme protonation step $EOH^- \rightleftharpoons EOH_2$ is considered to involve the catalytic group, possibly zinc-bound water. The ionization $EB \rightleftharpoons EBH^+$ is considered to involve a proton transfer group which is part of the enzyme, possibly His-64 or Thr-199 hydrogen bonded to Glu-106.[21] The catalytic interconversion of $CO_2$ and $HCO_3^-$ is assumed to be independent of the ionization state of the proton transfer group.

The data of Figure 3 demonstrate that the processes described by $R_{H_2O}$ are not determined solely by the sum of the

Scheme II




concentrations of $E(OH)_2)B$ and $E(OH_2)BH$ because the pH dependence of Figure 3 is not a simple titration curve corresponding to the ionization of the catalytic group. Hence, $R_{H_2O}$ is not limited in rate solely by the dissociation of $H_2O$ from the active site. And by similar arguments, $R_{H_2O}$ is not limited in rate by dissociation of $OH^-$ from the active site. Furthermore, zinc-bound hydroxide would be unlikely to exchange rapidly. Also, it is unlikely that the oxygen would exchange only from the form $E(OH_2)B$ and not from $E(OH_2)BH$. Substrate binding to human C enzyme is rather weak as demonstrated by the large values (as large as 0.5 M) for $K_{eff}$ of eq 15 found by $^{13}C$ and inhibition studies.[6,7] We conclude that the fraction of total enzyme involved in an enzyme-substrate complex is small (because substrate concentration is 0.015 M), and we assume that any $^{18}O$ exchange that might occur from the enzyme-substrate complex is not a significant fraction of experimentally observed $^{18}O$ exchange.

We prefer to interpret Figure 3 in a manner consistent with the data of Table II and the observations of others: in the presence of excess external buffer the release of oxygen from the active site to solvent as described by $R_{H_2O}$ is limited in rate by an intramolecular proton transfer between the catalytic group and a proton transfer group, specifically, step 3 of Scheme II. This situation occurs only if protonation of EOH, steps 2 and 4 in Scheme II, is not mediated by external buffers; this condition is described in the proposal of Steiner et al.[5] Evidence from the work of others for a rate-limiting, intramolecular proton transfer in the catalytic pathway comes from measurements of the maximal turnover numbers for the hydration and dehydration reactions catalyzed by the human C enzyme[5] and bovine enzyme[4] in $H_2O$ and $D_2O$. Further work measuring $^{13}C$ magnetic resonance line widths determined that this rate-limiting proton transfer is not in the catalytic steps involved in the interconversion of $CO_2$ and $HCO_3^-$; rather, it is in the steps by which the high pH form of the catalytic group is regenerated from the low pH form.[6] These $^{18}O$ studies support that interpretation, since Figure 3 is consistent with the limiting rate:

$$k_{-3}[E(OH)BH] = k_{-3}[E_{tot}] \frac{[H^+]}{[H^+] + K_2} \frac{K_1}{K_1 + [H^+]} \quad (16)$$

As shown by the solid line of Figure 3, this equation fits the data when $K_1$, the ionization constant of the catalytic group, is $10^{-6.8}$ and $K_2$, the ionization constant of the proton transfer group, is also $10^{-6.8}$. The rate constant for the intramolecular proton transfer which makes eq 16 fit the data is $k_{-3} = 3.5 \times 10^6\ s^{-1}$. These values are to be compared to the approximate rate and ionization constants obtained by Steiner et al.,[7] by fitting the steady-state rate equation to kinetic data: $K_1 = 10^{-6.9}$, $K_2 = 10^{-7.5}$, $k_{-3} = 7 \times 10^5\ s^{-1}$.

Further support of this interpretation of Figure 3 appears in Table II, which demonstrates that $R_{H_2O}$ is significantly decreased in changing solvent from $H_2O$ to predominantly $D_2O$, both in the presence and absence of buffer. From this we conclude that a step involving proton transfer is at least partially rate determining even in the presence of buffer. It is worthy of note in Table II that $R_1$ is relatively unaffected by the change of solvent from $H_2O$ to $D_2O$, in agreement with the $^{13}C$ magnetic relaxation studies.[6]

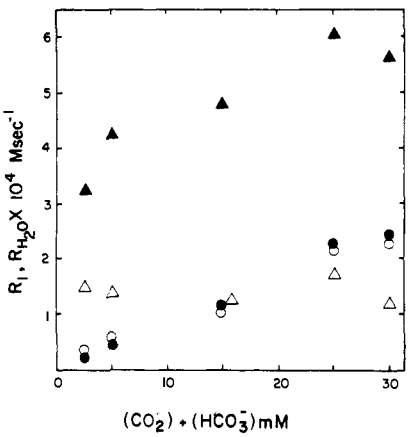



Figure 5. $R_1$ and $R_{H_2O}$ calculated using eq 13 and 14 and the data in Table I: (○) $R_1$ in the absence of buffer; (●) $R_1$ in the presence of 25 mM imidazole; (△) $R_{H_2O}$ in the absence of buffer; (▲) $R_{H_2O}$ in the presence of 25 mM imidazole.

**$R_{H_2O}$ in the Absence of Buffer.** $R_{H_2O}$ in the absence of buffer is smaller than $R_{H_2O}$ in the presence of excess buffer but is still appreciable in magnitude as shown in Figure 3. It is unlikely that the substrate itself acts as buffer in the range of pH studied here, a range far from the $pK_a$ of the $HCO_3^-$–$CO_3^{2-}$ buffer system which is 10.3, and far from the $HCO_3^-$–$H_2CO_3$ system, which is 3.8. Thus, the presence of $CO_3^{2-}$ provides a concentration of buffer near $10^{-4}$ M at pH 8, and the presence of $H_2CO_3$ provides a similar concentration of buffer near pH 6. These concentrations are below the millimolar range in which buffers such as imidazole and 3,5-lutidine cause enhancement of activity.[2,3] Another indication that species of $CO_2$ are not acting as buffer at pH 7.4 is given in Figure 5. All of the buffer dependence of $^{18}O$ exchange rates is accounted for by $R_{H_2O}$ at pH 7.4. Yet Figure 5 shows that $R_{H_2O}$ measured in the absence of external buffers does not increase as the total concentration of $CO_2$ species increases.

Since $R_{H_2O}$ does not decrease to zero in the absence of buffer, we infer that there is an internal buffer present to contribute a proton to $E^{18}OH$ and to allow $^{18}O$ exchange with solvent. Moreover, the data of Figure 3 suggest that in the low pH range (pH <6.25) the fraction of the internal buffer that is protonated is sufficiently large that $R_{H_2O}$ is near its maximal value (that is, $R_{H_2O}$ at pH <6.25 is not enhanced greatly by addition of external buffer). This requires that in Scheme I in the absence of buffer step $H_2O$ occurs mainly in that fraction of total enzyme that has the internal transfer group protonated. That is, the forms E(OH)B and E(OH)BH will be at their equilibrium concentrations but will have different $^{18}O$ contents in the absence of buffer since they are not rapidly convertible.

A rather complex set of rate equations results from these considerations; however, we can approximate this situation by assuming that only the fraction of total enzyme having a protonated internal transfer group BH will participate in the exchange with water (the proton exchange reactions involving $H_3O^+$ directly are neglected). On this premise, the following relationship represents $R_{H_2O}$ in the absence of buffer:

$$R_{H_2O\ (no\ buffer)} = R_{H_2O\ (excess\ buffer)} \times \left[\frac{[E(OH)BH]}{[E(OH)BH] + [E(OH)B]}\right] \quad (17)$$

Using this relationship gives the dashed line in Figure 3 when $R_{H_2O\ (excess\ buffer)}$ is taken as the solid line in Figure 3 and the

ionization constant of the internal proton transfer groups is $10^{-6.4}$. Taking the ionization constant to be $10^{-6.8}$ results in a poorer fit of $R_{H_2O}$. However, considering the experimental errors and the approximations in attempting to fit these data to models for $^{18}O$ exchange, we believe that the $pK_a$ of the internal buffer detected in the absence of external buffer is indistinguishable from the $pK_a$ detected in the presence of 50 mM imidazole. Figure 5 shows the linear relationship at pH 7.4 between $R_1$ and $[S_{tot}]$ for values of $[S_{tot}]$ 30 mM or less. This behavior is expected from consideration of eq 15, since the value of $K_{eff}$ at this pH is 0.45 M (see ref 6) and is larger than the total substrate concentration. For measurements in the absence of buffer, $R_1$ and $R_{H_2O}$ are not greatly different in magnitude, also shown in Figure 5. Under these conditions, the catalytic rate of oxygen exchange between $CO_2$ and water, $R_1R_{H_2O}/(R_1 + R_{H_2O})$, depends on both $R_1$ and $R_{H_2O}$. In the presence of 25 mM imidazole, $R_{H_2O}$ is much larger than $R_1$ and the overall catalytic rate of exchange of $^{18}O$ between $CO_2$ and water depends more on $R_1$ over the range of total substrate concentrations shown in Figure 5.

**Implications and Errors.** Qualitatively, there are striking similarities between the data and the curves obtained from eq 16 and 17 based on incorporating Scheme II into Scheme I. The pH profile of $R_{H_2O}$ and its response to buffer are similar in theory and experiment and $R_1$ obtained using equations derived from Scheme I is in good agreement with $R_1$ obtained by a different kinetic technique. However, the quantitative fit of the experimental data of Figure 3 by curves derived from the model of Scheme I is not as good, with disagreements especially for $R_{H_2O}$ in the absence of buffer and for $R_1$ at pH 6, which decreases by about 20% in the presence of 50 mM imidazole.

The source of the disagreements is uncertain. A clue could be hidden in the value of $k_{-3} = 3.5 \times 10^6\ s^{-1}$ obtained from the observed value of $R_{H_2O}$ with buffer (Figure 3), which is about 5 times larger than $k_{-3}$ from stopped-flow kinetic studies.[7] The decrease in $R_1$ caused by imidazole at pH <6.5 shown in Figure 2a is perhaps another clue and emphasizes the point that the role of buffers in the catalysis is not well understood. For example, the unusual effect of histidine as buffer has prompted the suggestion that histidine may bind near the active site of carbonic anhydrase;[22] the buffer studies of Jonsson et al.[2] also contain postulates about bound buffers in some cases. The buffer (and other ions in solution[14]) probably has more complicated effects than considered in the model mechanisms discussed so far. With the present data it is difficult or impossible to say what the buffers are doing other than acting as proton transfer agents.

These $^{18}O$ exchange results make several implications concerning the catalytic mechanism. First, the appreciable values of $\theta_{cat}$ and $\phi_{cat}$ even in the absence of buffer imply that the enzyme has a rapid path for $H_2O$ exchange from the active site that can proceed without protonation from the solvent. We have favored here the interpretation that this exchange of $H_2O$ proceeds by intramolecular proton transfer. Second, there is no doubt that the rate constants $\theta_{cat}$ and $\phi_{cat}$ measure events occurring at the active site and that these events are dependent on proton transfer near neutral pH. It is for this reason that we prefer to interpret our data in terms of a mechanism that incorporates an ionizable group into the active site, such as the mechanism of Scheme I. It should be pointed out that alternative mechanisms have been proposed in which zinc-bound water is considered active in both the hydration and dehydration reactions;[23] such a mechanism does not rely on an ionizable group to account for the pH dependence of catalytic activity.

Since the zinc-bound water mechanism is based largely on the results of proton relaxation studies,[24] it is appropriate to compare these with the $^{18}O$ results. The two techniques agree in assigning a value greater than $10^5\ s^{-1}$ for the rate constant for the exchange of water from the active site. The following $^{18}O$ results, however, are different from the proton exchange characteristics obtained from proton relaxation rates: the $^{18}O$ studies suggest that $H_2O$ containing oxygen from substrate does not exchange as rapidly at high pH as it does near pH 7, and the data indicate that $R_{H_2O}$ is relatively rapid at values of pH near 6; moreover, the buffer has a large effect on this water exchange rate. The large values of $\theta_{cat}$ and $\phi_{cat}$ measured at pH 6 and shown in Figure 1 indicate that there is a water molecule at the active site at low pH. The lack of agreement on these points between the $^{18}O$ exchange and proton relaxation data suggests several possibilities: (1) there is a difference in the exchange properties of water at the active sites of the human zinc and cobalt-substituted bovine enzymes; (2) the water containing the oxygen abstracted from bicarbonate is not exchanging from the inner coordination sphere of zinc in the human enzyme; (3) the proton exchange with solvent observed by proton relaxation in the presence of cobalt-substituted bovine carbonic anhydrase is not involved in the catalysis.

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