Carbonic Anhydrase Catalyzed Hydration Studied by ^{13}C and ^{18}O Labeling of Carbon Dioxide

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Abstract: The catalysis by bovine carbonic anhydrase of the exchange of oxygen-18 between species of CO_2 and water at chemical equilibrium was measured in the range of pH 6 to 9. Near pH 6, in the presence of excess substrate CO_2, the rate of catalyzed hydration of CO_2 determined by ^{18}O exchange is less than the known, maximum, initial velocity of hydration in nonequilibrium experiments. To explain this observation, it is hypothesized that ^{18}O removed from bicarbonate during a catalyzed dehydration step does not rapidly dissociate from the enzyme at low pH. With a long residence time in the active site, it is able to participate in a catalytic hydration step, yielding ^{18}O-labeled bicarbonate once again. In these steps, although catalytic hydration and dehydration are occurring, ^{18}O is not lost to solvent water. Bovine carbonic anhydrase also catalyzes the exchange of oxygen between species of CO_2, a process which is measured as an exchange of ^{18}O between ^{12}C- and ^{13}C-containing molecules and which occurs in the absence of enzyme. This observation is compatible with two explanations: catalysis of the exchange observed in the absence of enzyme and labeling of the active site by ^{18}O causing the transfer of this label between ^{12}C- and ^{13}C-containing species. The latter explanation is consistent with the hypothesis to account for low values of the catalyzed exchange with water. These results suggest that, in regions of low pH, the oxygen abstracted from bicarbonate in a catalytic dehydration step has a long lifetime in the active site compared to the turnover time for one catalytic cycle.

Catalysis by the zinc metalloenzyme carbonic anhydrase (EC 4.2.1.1) of the hydration–dehydration reaction of CO_2 depends on the ionization of a group in the enzyme with a $pK_a \sim 7$ for bovine carbonic anhydrase and human carbonic anhydrase C. The low pH or acid form is active in the dehydration, and the basic form is active in the hydration reaction. Although its identity has not been conclusively determined, there is strong evidence that the group which controls the pH dependence of catalytic activity is near or liganded to the zinc. For example, the visible absorption spectrum of cobalt(II) carbonic anhydrase[1,2] and the nuclear relaxation rates of water protons in the presence of Co(II) or Mn(II) carbonic anhydrase[3,4] show a pH dependency similar to the pH dependency of the enzymatic activity. Furthermore, the pH dependency of the binding of anions and sulfonamides, inhibitors of the hydration–dehydration catalysis, can be accounted for by the ionization of the same activity-controlling group.[5] Since x-ray diffraction patterns indicate a water molecule liganded to Zn, it is a reasonable hypothesis that ionization of Zn-coordinated water, directly or through intervening water bridges, is responsible for the pH dependence of activity.[6,7] However, it is also possible that the pH-dependent activity is related to the ionization of an amino acid side chain near or liganded to the metal.[8-10]

Carbonic anhydrase also catalyzes the exchange of oxygen-18 between labeled bicarbonate and water.[11,12] This process involves the abstraction of oxygen-18 from bicarbonate at the active site and its subsequent dilution in the solvent. The purpose of the work reported here is to determine the properties of the catalyzed isotope exchange process and interpret them in such a way as to reflect the nature of the activity-controlling group in the enzyme.

Experimental Section

Materials. Oxygen-18 labeled bicarbonate was prepared by dissolving potassium bicarbonate in enriched water (normalized, up to 95 atom % ^{18}O enrichment). The resulting solution was allowed to come to isotopic equilibrium overnight, after which the water was removed by vacuum distillation. Carbon-13 enriched bicarbonate was prepared in the following way. $^{13}CO_2$ was generated by adding 4 N phosphoric acid to enriched barium carbonate (90 atom % ^{13}C enrichment). The CO_2 generated was then absorbed into an equimolar amount of KOH in solution within a vacuum system.

Bovine carbonic anhydrase (BCA) was obtained in lyophilized form from Worthington Biochemical Corp. and purified chromatographically with Sephadex DEAE A-25 preequilibrated at pH 8.7 with 0.05 M Tris sulfate. The Tris buffer was then separated from the purified carbonic anhydrase by dialysis, and the lyophilized enzyme was stored at −15 °C. Enzymatic activity was determined by measuring the BCA-catalyzed rate of hydrolysis of *p*-nitrophenyl acetate, following the rate of appearance of *p*-nitrophenolate ion at 400 nm, as described by Pocker and Stone.[13] Comparison of this activity with the concentration of BCA determined from measurements of the optical density at 280 nm, using a molar absorptivity for BCA of $5.6 \times 10^4\ M^{-1}\ cm^{-1}$,[2] showed the enzyme to be greater than 96% active.

Kinetic Methods. Oxygen-18 Exchange with Water. Oxygen-18 enriched bicarbonate and CO_2 in aqueous solution undergo exchange of oxygen with water as a result of the hydration–dehydration reaction.[11,12,14] Carbonic anhydrase catalyzes the hydration–dehydration reaction; hence, it also catalyzes the depletion of ^{18}O from bicarbonate and CO_2.

The kinetic equations which describe the rate of change of ^{18}O content of bicarbonate and CO_2 in aqueous solution have been derived and solved.[11,14] These equations consider the two possible mechanisms for the uncatalyzed hydration–dehydration reaction[15] and take into account bicarbonate and CO_2 singly and multiply labeled with ^{18}O. Neglecting kinetic isotope effects, the rates of the catalyzed and uncatalyzed exchange of ^{18}O between species of CO_2 and H_2O are independent of enrichment in ^{13}C. Consequently, we modify the previously used notation[14,16] to include the atom fraction of ^{18}O in species containing both ^{12}C and ^{13}C. The atom fraction of ^{18}O in CO_2 is α:

$$\alpha = \frac{[^{12}C^{16}O^{18}O] + [^{13}C^{16}O^{18}O] + 2[^{12}C^{18}O^{18}O] + 2[^{13}C^{18}O^{18}O]}{2[CO_2]}$$

An analogous expression can be written for γ, the atom fraction of ^{18}O in bicarbonate. The kinetics of ^{18}O exchange between species of CO_2 and water is then measured by observing the decrease in enrichment $d(\alpha - \alpha_\infty)/dt$ and $d(\gamma - \gamma_\infty)/dt$, where α_∞ and γ_∞ are the atom fractions at infinite time and are close to the natural abundance of ^{18}O which is 0.002.

The exchange of ^{18}O between CO_2 and water at chemical equilibrium is a simple, first-order process described by

$$-d(\alpha - \alpha_\infty)/dt = \theta_1 t \quad (1)$$

$$\theta_1 = \frac{R}{3[CO_2]}\left[\frac{[CO_2]}{[CO_2] + [HCO_3^-] + [CO_3^{2-}]}\right] = \frac{f_{CO_2}R}{3[CO_2]} \quad (2)$$

R is the rate of the hydration of CO_2 at chemical equilibrium which is equal to the rate of dehydration of bicarbonate at equilibrium, and f_{CO_2} is the fraction of all species of CO_2 in solution existing as carbon dioxide. The exchange of ^{18}O between bicarbonate and water yields the same rate constant, $-d(\gamma - \gamma_\infty)/dt = \theta_1 t$, a result of the fact that the rate of hydration equals the rate of dehydration at equilibrium. Consequently, the rate constant θ_1 can be written in terms of the bicarbonate concentration, and eq 2 is equivalent to eq 8 of ref 16. The rate constant for exchange, θ_1, can be separated into its uncatalyzed and catalyzed parts:

$$\theta_1 = \theta_{cat} + \theta_{uncat} = \frac{f_{CO_2}}{3[CO_2]}(R_{cat} + R_{uncat}) \quad (3)$$

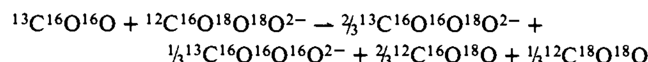
Boyer[17] provides a discussion of the kinetics of catalyzed isotope exchange at equilibrium.

Oxygen-18 Exchange between Species of CO_2. The exchange of oxygen between species of CO_2 is a process which occurs in aqueous solution in the absence of enzyme and is catalyzed by bovine carbonic anhydrase[18] and also by human carbonic anhydrase B.[19] This exchange is detected by placing in solution ^{18}O-enriched bicarbonate not enriched in ^{13}C and ^{13}C-enriched bicarbonate not enriched in ^{18}O. The experimental observation is that ^{13}C-containing species become enriched in ^{18}O and that ^{18}O exchanges with water in a way described in the previous section. To quantitate these observations, we define these additional atom fractions:

$$^{(12)}\alpha = \frac{[^{12}C^{16}O^{18}O] + 2[^{12}C^{18}O^{18}O]}{2[^{12}CO_2]}$$

$$^{(13)}\alpha = \frac{[^{13}C^{16}O^{18}O] + 2[^{13}C^{18}O^{18}O]}{2[^{13}CO_2]}$$

The uncatalyzed exchange processes have been investigated in an alkaline pH region in which ^{18}O transfer between species of CO_2 is due to the following reaction:[18,20]



$$^{13}C^{16}O^{16}O + {}^{12}C^{16}O^{18}O^{18}O^{2-} \rightarrow \tfrac{2}{3}\,{}^{13}C^{16}O^{16}O^{18}O^{2-} + \tfrac{1}{3}\,{}^{13}C^{16}O^{16}O^{16}O^{2-} + \tfrac{2}{3}\,{}^{12}C^{16}O^{18}O + \tfrac{1}{3}\,{}^{12}C^{18}O^{18}O$$

The rate of exchange between species of CO_2 can be obtained by considering the case of generalized isotopic exchange at equilibrium as treated by Duffield and Calvin.[21]

$$BX^* + AX \rightarrow AX^* + BX$$

Here BX represents ^{12}C-containing species and AX, ^{13}C-containing species, with X* representing oxygen-18. Then, following Duffield and Calvin, we can express the rate of change of $^{(12)}\alpha$ due only to exchange between species of CO_2 as:

$$-\frac{d^{(12)}\alpha}{dt} = \frac{R'}{\Sigma(^{12}C)}(^{(12)}\alpha - {}^{(13)}\alpha)$$

Here, $\Sigma(^{12}C)$ is the sum of the concentrations of all ^{12}C-containing species in solution. R' is the rate of exchange of oxygen between species of CO_2. A more detailed description of the uncatalyzed exchange which occurs in alkaline pH has been made elsewhere.[20]

Including the exchange with water, two simultaneous differential equations describe the rate of distribution of ^{18}O [$^{(12)}\alpha_\infty$ and $^{(13)}\alpha_\infty$ are omitted]:

$$-\frac{d^{(12)}\alpha}{dt} = \frac{R'}{\Sigma(^{12}C)}(^{(12)}\alpha - {}^{(13)}\alpha) + \theta_1{}^{(12)}\alpha$$

$$-\frac{d^{(13)}\alpha}{dt} = \frac{R'}{\Sigma(^{13}C)}(^{(13)}\alpha - {}^{(12)}\alpha) + \theta_1{}^{(13)}\alpha$$

Solutions are of the form

$$^{(12)}\alpha - \alpha_\infty = a_1 e^{-\theta_1 t} + a_2 e^{-(\theta_1 + \phi)t} \quad (4)$$

with

$$\phi = R'\left[\frac{1}{\Sigma(^{12}C)} + \frac{1}{\Sigma(^{13}C)}\right]$$

a measure of the exchange of oxygen between species of CO_2. The expression for $^{(13)}\alpha$ has the same form as eq 4 with different coefficients, a_3 and a_4.

With these considerations, data obtained from the double-labeling experiments are treated in the following manner. The slope of a plot of ln $(\alpha - \alpha_\infty)$ vs. time yields as slope $-\theta_1$, the rate constant for the exchange with water as given in eq 1. Using eq 1 and 4, a plot of ln $(^{(12)}\alpha - \alpha)$ vs. time yields as slope $-\theta_1 - \phi$. Also, a plot of ln $(\alpha - {}^{(13)}\alpha)$ vs. time yields as slope the same rate constant $-\theta_1 - \phi$.

Procedure. Isotope exchange experiments were performed over a range of pH from 6.0 to 9.0. The apparatus used to measure the kinetics of the exchange was different for the low and high pH regions. This arrangement was necessary since at high pH the isotope exchange reactions are slow and the concentration of CO_2 small; whereas, at neutral pH the exchange reactions are rapid and the concentration of CO_2 large.

Preparation of the carbonic anhydrase solutions was identical throughout these experiments. The enzyme was diluted to the appropriate activity and divided into four to six portions; each was used in a different experiment within a 3-h period. Prior to each experiment, carbonic anhydrase activity was measured by the changing pH method of Maren[22] and was determined to be constant during the course of each series of experiments.

High pH Range. Experiments at pH $\geq$8.0 were performed using a gas-tight syringe fitted with a water jacket for temperature control (25.0 $\pm$ 0.1 °C). A solution (2.5 ml) containing ^{13}C-enriched bicarbonate was adjusted to the required pH with 1 N H_2SO_4 or NaOH and placed in this syringe. Enzyme was added followed by addition of solution (2.5 ml) containing ^{18}O-enriched bicarbonate which had been preadjusted to the required pH. The resulting 5-ml solution in the syringe was either 30 or 50 mM in total concentration of all CO_2 species and 1.6×10^{-9} M in BCA with ionic strength maintained at 0.20 with the noninhibitory sodium sulfate. Carbon-13 enrichments varied between 43 and 47%. At the end of each exchange experiment, the pH of the contents of the syringe was measured. This reading agreed, within 0.03 pH units, with the reading taken prior to the start of the experiment.

A period of time exceeding six half-times for the chemical reaction was allowed to elapse in order for the solution to approach chemical equilibrium. Then aliquots (0.3 ml) from the syringe were injected at periodic intervals through a serum stopper into evacuated vessels containing 9 M sulfuric acid. This rapidly stopped the exchange reactions by liberating CO_2. The CO_2 was passed through a trap immersed in a dry ice–acetone bath to remove water vapor and collected in vials. Each CO_2 sample was then analyzed for isotopic content using a Finnigan 3000 mass spectrometer at an ionizing voltage of 70 eV. The ^{18}O enrichment in the CO_2 samples was determined by the following formulas:

$$\tau = \frac{\tfrac{1}{2}(46) + \tfrac{1}{2}(47) + (48) + (49)}{(44) + (45) + (46) + (47) + (48) + (49)}$$

$$^{(12)}\tau = \frac{\tfrac{1}{2}(46) + (48)}{(44) + (46) + (48)}$$

$$^{(13)}\tau = \frac{\tfrac{1}{2}(47) + (49)}{(45) + (47) + (49)}$$

where (44), (45), (46) ... are the heights of the corresponding mass peaks. The standard deviation obtained from 4 to 10 measurements of τ was 1.5 to 2.0%. At alkaline pH there is a negligibly small concentration of CO_2 in the reaction solution (for pH >8, percent CO_2 is 2% or less). As a result, the atom fraction of ^{18}O in the CO_2 liberated by acid is taken as equal to the atom fraction of ^{18}O in bicarbonate of the reaction solution ($\gamma = \tau$, $^{(12)}\gamma = {}^{(12)}\tau$, $^{(13)}\gamma = {}^{(13)}\tau$).

Low pH Range. To determine the rapid rate of isotope exchange in the range of pH <8.0, an apparatus for continuous measurement of the isotopic enrichment of CO_2 dissolved in aqueous solution was constructed.[23] The apparatus is a glass cylindrical vessel, with a water jacket for thermoregulation, which contains the isotopically enriched solutions. A Teflon plunger is used to eliminate air space into which CO_2 could diffuse and a pH electrode is embedded in the plunger. The bottom of the vessel is a membrane (5×10^{-4} in. thick, Membrane Kit 5937, Yellow Springs Instrument Co., Yellow Springs, Ohio) which allows CO_2 to pass from solution into the mass spectrometer. The membrane is 250 mm^2 in area and

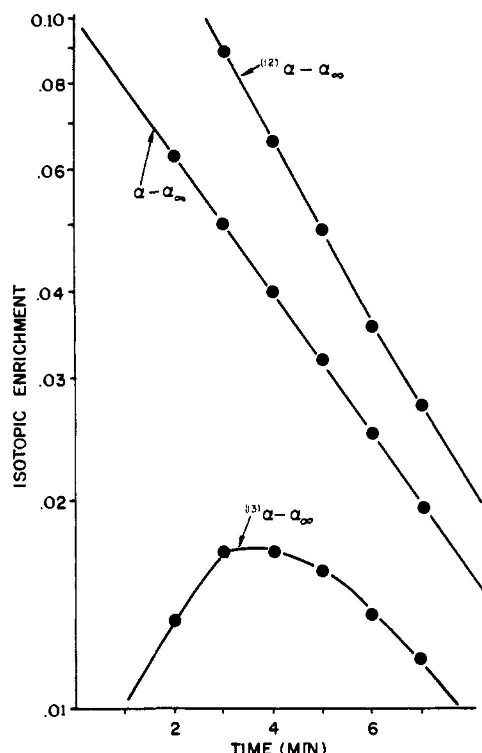


Figure 1. The decrease of isotopic enrichments in CO_2 at pH 6.45 and 25 °C with a 50 mM concentration of total CO_2 species. The concentration of bovine carbonic anhydrase was 1.6×10^{-9} M. The atom fractions ($^{(12)}\alpha - \alpha_\infty$), ($^{(13)}\alpha - \alpha_\infty$), and ($\alpha - \alpha_\infty$) are defined in the text. They represent the enrichments of ^{18}O in ^{12}C-containing CO_2, ^{13}C-containing CO_2, and all CO, respectively. The ^{13}C enrichment was 45%.

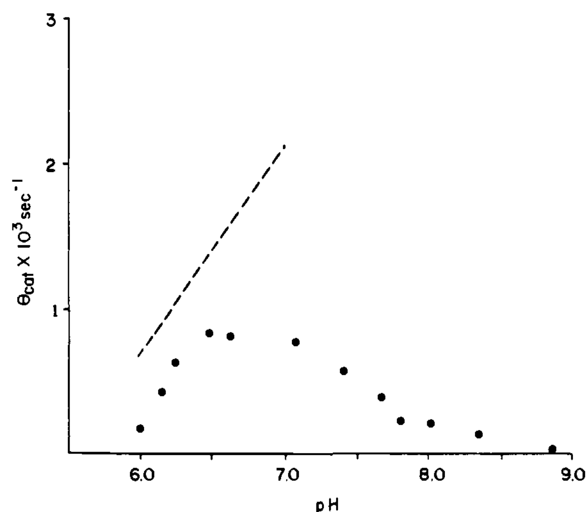


Figure 2. The points are experimentally determined values of θ_{cat}, the first-order rate constant for the catalyzed exchange of ^{18}O between CO_2 and H_2O at chemical equilibrium and 25 °C. The concentration of bovine carbonic anhydrase was 1.6×10^{-9} M, and the total concentration of CO_2 species was 50 mM. The dashed line represents the values of θ_{cat} calculated using eq 3 of the text and the kinetic constants of the Michaelis equation as determined from the stopped-flow studies of BCA-catalyzed hydration and dehydration (ref 5 and 25).

is supported by a porous, stainless steel disk (1/16 in. thick, filtration grade 40 μm, Mott Metallurgical Corp., Farmington, Conn.). The contents of the vessel is stirred and the solution in the vessel is depleted of CO_2 to a negligibly small extent, as judged by the CO_2 intensity on the mass spectrometer and stability of the pH in unbuffered solutions containing CO_2. The lag time for CO_2 to pass from the vessel to the detector of the mass spectrometer is less than 5 s, as determined by rapid injection of $^{13}CO_2$ into the vessel. A dry ice–acetone trap between the vessel and mass spectrometer reduces the water peak when the vessel is in use to less than approximately 1.5 times the background water peak. The lower limit of detectability for this apparatus in our hands is 0.3 mM CO_2 dissolved in solution.

Consequently, in the range of pH <8.0, the kinetics of isotope exchange are measured from the enrichments of CO_2. That is, for pH <8.0

$$\alpha = \tau,\ ^{(12)}\alpha = {}^{(12)}\tau, \text{ and } ^{(13)}\alpha = {}^{(13)}\tau$$

Following the same procedure described for the high pH range, 8 to 12 ml of isotopically enriched solution was placed in the vessel described above, with the ^{13}C- and ^{18}O-containing solutions added separately and mixed in the vessel. The total concentration of all CO_2 species was either 30 or 50 mM with BCA at 1.6×10^{-9} M, and ionic strength was held constant at 0.20 with Na_2SO_4. In all experiments, the pH of the contents of the reaction vessel measured at the start of an experiment agreed to within 0.05 pH units with the reading taken at the termination of the experiment. Experiments were carried out in reaction vessels with and without a hydrophobic surface coating (Desicote, Beckman) with no detectable differences in the kinetics of isotope exchange. Although isotope enrichments were monitored continuously, kinetic constants were determined from data taken at a time exceeding 6 half-times for the chemical reaction.

Results

The ^{18}O enrichments $\alpha - \alpha_\infty$, $^{(12)}\alpha - \alpha_\infty$, and $^{(13)}\alpha - \alpha_\infty$ vary during a typical carbonic anhydrase catalyzed exchange at pH 6.45 and 25 °C as shown in Figure 1. Such data, obtained at chemical equilibrium, indicate the presence of at least two exchange processes. First, the exchange with water which leads to the depletion of ^{18}O in both ^{12}C- and ^{13}C-containing CO_2. The slope of the ln ($\alpha - \alpha_\infty$) plot yields the rate constant for this exchange with water which is independent of the ^{13}C enrichment, neglecting small kinetic isotope effects. Second, at least one exchange process exists between species of CO_2 in solution leading to the initial enhancement of $^{(13)}\alpha$ and the initial rapid decrease in $^{(12)}\alpha$. Figure 4 demonstrates the separation of rate constants; the plots of ln ($^{(12)}\alpha - \alpha$) and ln ($\alpha - {}^{(13)}\alpha$) vs. time yield the same slopes $-\theta_1 - \phi$, as mentioned previously in connection with eq 4.

Rate constants for the uncatalyzed exchange of ^{18}O between species of CO_2 and water (θ_{uncat}) were determined at 25 °C for the range of pH 6–9. The resulting experimental values of θ_{uncat} were compared with the values calculated using the hydration rate constants given by Gibbons and Edsall:[24] $k'_{uncat} = k'_{31} + k_{OH}(OH^-) = 0.0375 + 8500(OH^-)\ s^{-1}$. A value of θ_{uncat} can be calculated using $k'_{uncat}[CO_2] = R_{uncat}$ in eq 3. The experimental and calculated values of θ_{uncat} agree to within 5% over the pH range 6–9. This demonstrates that the kinetic apparatus and techniques used in this work give accurate and continuous values of θ over the range of pH 6–9. Furthermore, values of θ in the presence of carbonic anhydrase were kept within the general range of values found in these uncatalyzed experiments ($\theta_1 < 10^{-2}\ s^{-1}$) by employing low concentrations of enzyme, generally 1.6×10^{-9} M BCA.

The points in Figures 2 and 5 represent values of $\theta_{cat} = \theta_1 - \theta_{uncat}$ obtained at 25 °C and 1.6×10^{-9} M BCA using a constant, total concentration of all CO_2 species, either 30 or 50 mM. These solutions were buffered only to the extent that the substrates and enzyme themselves act as buffers. Initial enrichment in ^{18}O was as high as 40% so that the exchange processes could be followed with good precision for many half-times. The presence of 10^{-7} M ethoxzolamide, a potent carbonic anhydrase inhibitor ($K_1 = 10^{-9}$ M), abolished θ_{cat}, leaving an exchange with the same rate constant, θ_{uncat}, observed in the absence of enzyme.

In the low pH region of Figures 2 and 5, θ_{cat} can be com-

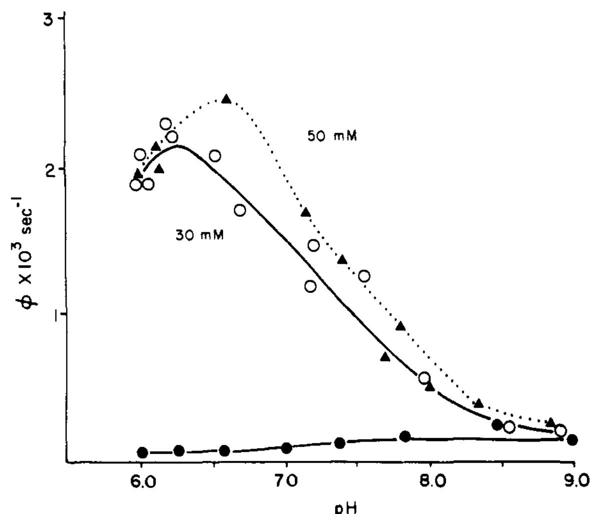


Figure 3. The first-order rate constant describing the exchange of ^{18}O between ^{12}C-containing and ^{13}C-containing species of CO_2 at 25 °C. The points represent data for the (●) uncatalyzed exchange at 30 mM total CO_2 species, (○) catalyzed exchange at 30 mM total CO_2 and 1.6 × 10^{-9} M carbonic anhydrase, and (▲) catalyzed exchange at 50 mM total CO_2 and 1.6 × 10^{-9} M carbonic anhydrase. The ^{13}C enrichment was 44–46% in a final volume of 8 ml.

pared with values of θ_{cat} calculated from the turnover numbers for bovine carbonic anhydrase. This comparison is based on the following relationship between the catalytic component of the equilibrium rate, R_{cat} in eq 3, when measured in the presence of excess substrate and the maximum initial velocities for the catalyzed hydration and dehydration reactions, V_h and V_d (see eq 12 of ref 17):

$$R_{cat} = \left(\frac{1}{V_h} + \frac{1}{V_d}\right)^{-1} \quad (5)$$

This relation can only be applied to the low pH region of these exchange experiments in which the concentration of CO_2 is greater than the Michaelis constant for hydration, which is about 12 mM for bovine carbonic anhydrase.[25] At pH 6 about 70% of all CO_2 species in solution exists as carbon dioxide at equilibrium. In the high pH region, the Michaelis constant for dehydration is greater than 100 mM and eq 5 cannot be used.

From the known pH dependence of the catalytic activity in regions of acidic pH, the maximum initial dehydration velocity V_d is greater than the magnitude of V_h for a given enzyme concentration. However, this is not true near neutral pH, and both V_d and V_h are important in eq 5. The dashed lines in Figures 2 and 5 represent values of θ_{cat} calculated from eq 3 using R_{cat} obtained in the following way. Values for $V_h = k_{cat}^{CO_2}E$ were determined from the turnover number given by Kernohan for BCA-catalyzed hydration of CO_2 in the presence of buffers and 80 mM chloride ($k_{cat}^{CO_2}$ is given in Figure 4 of ref 25). For dehydration, $V_d = k_{cat}^{HCO_3^-}E$ was determined using $k_{cat}^{HCO_3^-} = 4 \times 10^5$ s^{-1} (see Table VIII of ref 5), assuming this value to be invariant in the region of pH 6.0–7.0. R_{cat} was obtained using eq 5.

The result is that θ_{cat} calculated from Kernohan's data is greater than the value of θ_{cat} determined by ^{18}O exchange. This conclusion is most firm for Figure 2 at pH 6.0 since these particular data were taken at the largest CO_2 concentration. Kernohan's data[25] were obtained using 80 mM chloride, a weak inhibitor of bovine carbonic anhydrase ($K_I \sim 200$ mM); as a consequence, the calculated curves for θ_{cat} are too low.

Rate constants for the exchange of ^{18}O between ^{12}C- and

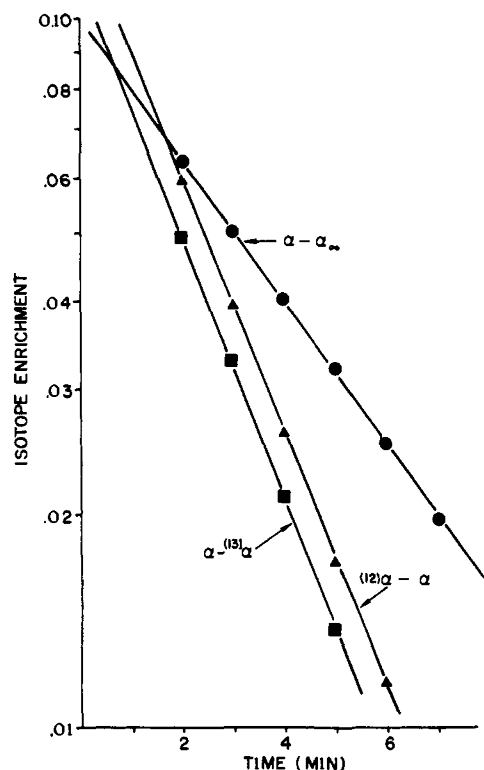


Figure 4. A graphical treatment of the data in Figure 1 showing the separation of at least two exchange rates. The decrease of $(\alpha - \alpha_\infty)$ represents the exchange of ^{18}O between CO_2 and water. The decrease of $({}^{(12)}\alpha - \alpha)$ and $(\alpha - {}^{(13)}\alpha)$, which exhibit identical slopes, represent the exchange of ^{18}O between ^{13}C-containing and ^{12}C-containing species of CO_2 as well as ^{18}O exchange between species of CO_2 and water.

^{13}C-containing species were determined from the slope of plots of ln $({}^{(12)}\alpha - \alpha)$ vs. time, as discussed in the Experimental Section. In all experiments using double labels, the value of ϕ computed in this way agreed within 5% with the value of ϕ from a plot of ln $(\alpha - {}^{(13)}\alpha)$ vs. time.

Figure 3 presents data for ϕ obtained in solutions 30 mM in total CO_2 species but not containing carbonic anhydrase. The rate constant for the uncatalyzed exchange between species of CO_2 in solution has been investigated for the pH region above 7.5[18,20] but is too small to be observed clearly under the conditions of Figure 3. Also shown in Figure 3 are values for ϕ in solutions containing 1.6 × 10^{-9} M BCA and either 30 or 50 mM in total CO_2 species. The values of ϕ in the presence of carbonic anhydrase are reduced to the values of ϕ without enzyme for the pH range shown in Figure 3 by the addition of ethoxzolamide at 10^{-7} M. Furthermore, no catalyzed exchange of ^{18}O between species of CO_2 was observed in the presence of 2 × 10^{-9} M apoenzyme derived from BCA.

Discussion

There are three main advantages to observing ^{18}O exchange at chemical equilibrium as a means of investigating the hydration–dehydration reaction of CO_2 and its catalysis by carbonic anhydrase: the rates of the reactions of CO_2 can be accurately measured in very low or very high pH regions; this is an equilibrium technique and the hydration–dehydration reaction can be observed in the absence of buffers; and finally, the fate of oxygen during the reaction can be traced. The first two of these advantages have been utilized in previous ^{18}O studies,[11,12,16] and it is the last which is applied here. The main disadvantage of the technique as applied in this work arises from the relatively slow

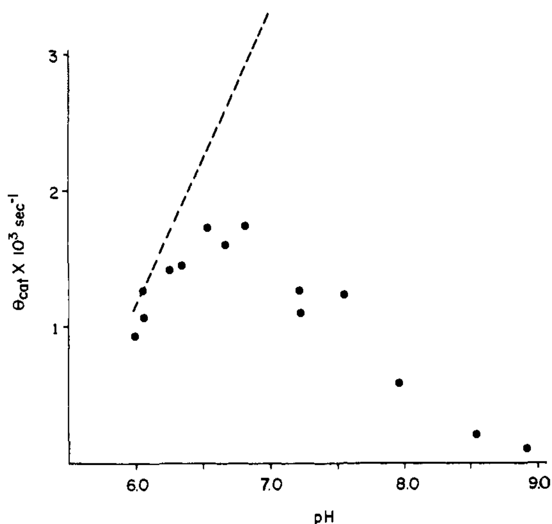


Figure 5. The same as in Figure 2, except the total concentration of CO_2 species in solution was 30 mM.

processes of mixing reactants and repeated sweeping of the mass range 44–49. In order to observe ^{18}O depletion from species of CO_2 before isotopic equilibrium is reached, low concentrations of enzyme must be used. Consequently, for the low pH region in which the uncatalyzed exchange with water is rapid, the catalyzed rate of exchange is not greater than the uncatalyzed rate.

Oxygen-18 Exchange with Water. The first-order rate constant for the catalyzed portion of the ^{18}O exchange between CO_2 species and water, θ_{cat}, is presented in Figures 2 and 5. In these experiments the total concentration of CO_2 species is held constant; however, the fraction of all CO_2 species existing as carbon dioxide, f_{CO_2}, varies in a sigmoidal fashion, reaching a maximal value at low pH. The rate constant for catalyzed hydration of CO_2, $R_{cat}/[CO_2]$, depends on the ionization state of the enzyme and will follow a titration curve, but with a maximum value at alkaline pH. Consequently, it is expected that the pH dependency of θ_{cat} be bell-shaped since, as shown in eq 3, $\theta_{cat} = f_{CO_2}R_{cat}/3[CO_2]$.

The rate constants for the *uncatalyzed* exchange of ^{18}O between species of CO_2 and water are simply related to the rate constants for the dehydration of bicarbonate since each uncatalyzed dehydration step involves the release of one oxygen atom, in the form of H_2O or OH^-, to the solvent.[11,14] To determine if such a simple relationship exists for the catalyzed exchange, θ_{cat} is compared with values of θ_{cat} calculated from known rate constants for the catalyzed chemical reaction. It is apparent in Figures 2 and 5 that the experimentally determined values of θ_{cat} are less than the values for this parameter calculated using the turnover number obtained by stopped-flow techniques[25] and represented by the dashed lines in Figures 2 and 5. Near pH 6 for the solutions 50 mM in total species of CO_2, ^{18}O exchange with H_2O in the solvent is nearly reduced to the uncatalyzed rate. Yet the enzyme is catalytically active at pH 6 and above.[7] Furthermore, each of the solutions in the low pH region, when alkalinized and tested in the neutral pH region, gave a value of θ_{cat} identical with those shown on the experimental curves of Figures 2 and 5. That is, in the low pH region, the enzyme was not irreversibly denatured. This is also shown in these experiments by the catalysis of the exchange of ^{18}O between species of CO_2 in the same low pH region using the same solutions for which θ_{cat} was determined.

The enzyme is not denatured or inhibited but will not catalyze ^{18}O depletion from species of CO_2 with the rate constant predicted using nonequilibrium, initial, catalyzed rates of hydration and dehydration. The following hypothesis explains this observation. The catalytic hydration and dehydration are proceeding but one crucial step necessary for ^{18}O exchange with water, the loss of $H_2{}^{18}O$ to solvent water, is not occurring at a rate faster than the rate of the catalyzed hydration–dehydration. That is, ^{18}O removed from bicarbonate during a catalyzed dehydration step does not rapidly dissociate from the active site at low pH—its residence time is sufficiently long that it is able to participate in the catalytic hydration of CO_2 yielding, once again, ^{18}O-labeled bicarbonate. Hence, although hydration and dehydration steps occur in this low pH region, some ^{18}O is not lost to the solvent.

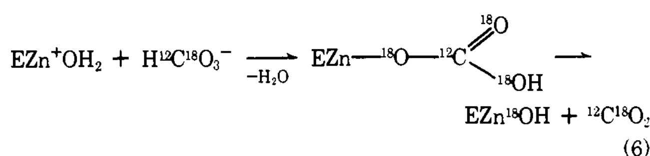
The difference between the observed rate constants θ_{cat} and those calculated from Kernohan's data[25] can be explained by assuming a rate of replacement of ^{18}O in the active site which is comparable to or less than the equilibrium catalytic rate for pH near or below neutral. It is pertinent to compare this assumption with magnetic resonance relaxation studies providing evidence that the residence lifetime of protons on metal-bound H_2O or OH^- is a function of pH in carbonic anhydrase. This evidence is a decrease in the relaxivity of water protons with decreasing pH in the presence of Co(II) or Mn(II) bovine carbonic anhydrase.[3,4] The relaxivity displays a pH dependency which can be fitted to a titration curve with a pK near 7. This is the pK_a of the activity-controlling group in the BCA-catalyzed CO_2 hydration–dehydration reaction suggesting, along with much additional evidence,[5] that zinc-bound water or hydroxide is the catalytic entity for carbonic anhydrase. Magnetic resonance studies of water coordinated in simple transition metal complexes show identical exchange rates for ^{17}O and ^1H,[27,28] indicating that proton exchange of coordinated water occurs by replacement of the entire water molecule. There is no evidence to show that proton exchange with bulk solvent occurs by metal–oxygen cleavage in carbonic anhydrase and the ^1H NMR data could be consistent with cleavage or no cleavage. If cleavage occurs, the lower limit of 0.2 ms for the residence time of a proton on water in Co(II)BCA in the low pH region, reported by Koenig and Brown,[26] is also a measure of the residence time of oxygen bound to the metal. This corresponds to an upper limit for the frequency of oxygen exchange of 5×10^3 s^{-1}. For pH 6, the pseudo-first-order rate constant for the catalytic reaction (velocity/enzyme concentration), calculated from the dotted line in Figure 2, is about 10^5 s^{-1}. Consequently, if the NMR data reflect the lifetime of oxygen on zinc, this comparison is consistent with the hypothesis that, at low pH, ^{18}O has a slow rate of replacement with oxygen from solvent water compared with the rate of the catalyzed reaction. These considerations establish a possible experimental connection between the NMR results for protons and the behavior of oxygen during the catalysis.

Comparison of Figures 2 and 5, showing data taken at the same enzyme concentration but different (30 and 50 mM) total concentration of CO_2 species, demonstrates that the first-order rate constant for exchange decreases as substrate concentration increases, in agreement with eq 2. Another consideration to account for this observation is that the higher turnover per enzyme molecule at the higher substrate concentration provides less time for ^{18}O to be replaced in the active site.

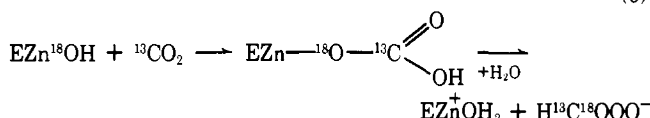
Oxygen-18 Exchange between Species of CO_2. The exchange of ^{18}O between species of CO_2, an effect first observed by Gerster et al.,[18] occurs in the absence of enzyme and is enhanced by the presence of bovine carbonic anhydrase. Figure 3 shows the values of ϕ, a rate constant for ^{18}O exchange between ^{12}C- and ^{13}C-containing species, for

the uncatalyzed exchange at 30 mM total concentration of CO_2 species and the catalyzed exchange at 30 and 50 mM total concentration of CO_2 species. The solutions in which these catalyzed exchanges were performed correspond to the same solutions used in measuring the ^{18}O exchange with water shown in Figures 2 and 5. In an alkaline pH region, the uncatalyzed process has been attributed to an exchange of oxygen between CO_2 and CO_3^{2-};[18,20] but for the region of pH below 8, the uncatalyzed exchange has not been analyzed. The catalyzed exchange of ^{18}O between species of CO_2 was abolished by the carbonic anhydrase inhibitor ethoxzolamide at 10^{-7} M and was not detected when the apoenzyme was used in place of the active form of the enzyme.

Catalysis of the exchange of ^{18}O between species of CO_2 could arise by two different processes. The first is the catalysis by carbonic anhydrase of the exchange process observed in the absence of enzyme, that is, a process not involving the hydration–dehydration reaction in which two molecules of CO_2 or its hydrated forms exchange oxygen at the active site. In fact, the presence of two binding sites for bicarbonate in the active site of BCA has been detected by NMR.[29] The second explanation of the catalysis of ^{18}O exchange between species of CO_2 is the hypothesis made from considerations of θ_{cat}: in lower pH regions, ^{18}O abstracted from labeled bicarbonate has a small rate of replacement in the active site compared with the rate of the enzyme-catalyzed reaction. Following our previous reasoning, we assume that ^{18}O lingers in the enzyme active site following a dehydration step at low pH. As a result, ^{18}O is available to be incorporated into bicarbonate with the hydration of $^{13}CO_2$, according to several possible schemes; the one shown below involves bicarbonate binding directly to zinc.



(6)



Both of the schemes described here can account for the catalyzed exchange between ^{12}C- and ^{13}C-containing species of CO_2. However, if the hypothesis to explain the difference between the calculated and observed values of θ_{cat} is correct, that is, if ^{18}O lingers in the active site at low pH long enough to react with CO_2, then a portion of ϕ in the presence of BCA must be attributed to a scheme such as shown in eq 6. Furthermore, the difference between the observed and calculated values of θ_{cat} is substantial, as great as $1 \times 10^{-3}\ s^{-1}$; the catalyzed portion of ϕ is larger but has roughly the same magnitude. In the proposed scheme of eq 6, those catalyzed processes which do not contribute to the rate of exchange with water, θ_{cat}, contribute to the exchange between species of CO_2, ϕ. Consequently, the similarity of the difference between observed and calculated values of θ_{cat}, and the catalyzed portion of ϕ, is consistent with the hypothesis that a sizable portion of the rate constant ϕ is due to a mechanism such as shown in eq 6. These indications of the long lifetime of oxygen in the active site at low pH are less compatible with the interpretation of the proton relaxation data which states that there is no water bound to zinc at low pH than with the interpretation which states that water bound to zinc must exchange slowly at low pH.[4,26]

It is pertinent to note that a scheme such as presented above, in which the active site is labeled with ^{18}O, was used as a possible explanation of the buffer dependence of the ^{18}O exchange rate during the catalyzed hydration–dehydration of CO_2.[16,30] All results reported here were obtained in the absence of added buffer. If θ_{cat} increases as buffer is added in low pH regions as it increases in alkaline and neutral pH regions, then the discrepancy between the observed and calculated values of θ_{cat} should be reduced in the presence of buffer.

It is not possible from the ^{18}O exchange data to determine whether the labeled oxygen is bound directly to the zinc. The lifetime of water molecules at other positions in the active site must be considered. Glasel[31] has determined, by NMR techniques, that the water solvation structure around certain homopolymers (polyadenylic acid, poly-L-glutamic acid) is remarkably stable, the lifetime of the hydration structure being 10^{-4} to 10^{-3} s. If such lifetimes pertain for the hydration structure of amino acid side chains in the active site of carbonic anhydrase, it is quite possible that the ^{18}O which lingers in the active site lingers at a position other than an inner coordination site of the metal. The possibility of water bridges extending between zinc bound H_2O or OH^- and CO_2 has been suggested.[7,32]

A conclusion based on these isotope exchange data arises from the following considerations. It is a dehydration step involving ^{18}O-labeled bicarbonate which is postulated to leave an ^{18}O atom in the active site. This ^{18}O atom lingers in the active site long enough to react with $^{13}CO_2$, implying that the basic form of the activity-controlling group exchanges this particular oxygen relatively slowly at low pH. That is, the basic form is not of itself a species which rapidly exchanges oxygen. Therefore, it is likely that exchange of oxygen at high pH occurs because of a competition for the $^{18}OH^-$ or $H_2{}^{18}O$ binding site by an amino acid side chain or hydroxide from solution which is not prevalent at low pH. Such arguments have been made to explain the pH dependence of the nuclear quadrupole resonance of ^{35}Cl ions in solution containing bovine carbonic anhydrase.[26,33]
pendence of the nuclear quadrupole resonance of ^{35}Cl ions in solution containing bovine carbonic anhydrase.[26,33]

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Effects of Freezing an Internal Rotation on Intramolecular Catalysis by an Imidazolyl Group. Synthesis and Hydrolysis of 4,5-[1′(4′)-Acetoxymethyltetramethylene]imidazoles and 4,5-[1′(5′)-Acetoxymethylpentamethylene]imidazole

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Abstract: As models for acetyl-α-chymotrypsin, 4,5-[1′(4′)-acetoxymethyltetramethylene]imidazole (**2**), its 1′(4′)-methyl-substituted derivative (**3**), and 4,5-[1′(5′)-acetoxymethylpentamethylene]imidazole (**4**) were synthesized via 6-bromo-2-ethoxycarbonylcyclohexanones and 7-bromo-2-ethoxycarbonylcycloheptanone in moderate yields. Their hydrolytic reactivities were determined in water at 50 °C in comparison with their open-chain analogue, 4(5)-(2′-acetoxyethyl)imidazole (**1**). The rate constants (k_1) for intramolecular general base catalysis by an imidazolyl group for **2** and **4** are 2.6 and 11.5 times larger than k_1 for **1**, respectively, but k_1 for **3** is 0.83 times smaller. Thus the 1′(4′)-methyl group in **3** has a retarding effect on k_1, while one methylene unit in the carbocyclic chain of **4** has a large accelerating effect on k_1. The solvent deuterium isotope effects for **2** and **4** are in accord with general base catalysis by the imidazolyl group. The enhancement of 2.6 in k_1 for **2** can be rationalized from the entropy effect of freezing an internal rotation, and the discrepancy from the estimated rate factor of 6 to 11 is mainly attributable to the loose transition state in general base-catalyzed hydrolysis. The large enhancement of 11.5 in k_1 for **4** can be explained in terms of a preferred favorable conformation of the second internal rotation and a basicity correction for the imidazolyl group. ^{1}H and ^{13}C NMR spectra for those models were discussed for the elucidation of their structures. Comparison of k_1 for **2** and **4** with that of acetyl-α-chymotrypsin suggests that, to mimic the enzymic activity, a rate factor of about 500 must be provided by some modifications other than the freezing of remaining internal rotations in the acetoxymethyl group.

Model studies of the deacylation step of α-chymotrypsin using simple model compounds have been carried out rather extensively.[1-6] The information obtained from them is particularly useful because the simplicity of their structures enables analysis of the results more clearly, and the deacylation step is the microscopic reverse of the acylation.[7]

However, most of the models used hitherto are methyl or phenyl esters of carboxylic acid derivatives with an imidazolyl group attached to the α, β, or γ position of the acid carbon skeleton[2,4] or phenyl esters in which an imidazolyl group is bound to the phenyl ring.[1,3,5,6]

Only one model, 4(5)-(2′-acetoxyethyl)imidazole (**1**), with a primary acyloxy group and a 4(5)-imidazolyl group like the active site of acyl-α-chymotrypsin was synthesized, and its hydrolytic reactivity was determined.[2,3] Although the acetate **1** is considered to be hydrolyzed by intramolecular general base catalysis like the enzyme,[5,8] the deacylation rate constant is far below that of the enzyme. This deficiency has been suggested due to the lack of rigidity of the model.[9]

For rate enhancement, ample examples indicate the importance of rigid structures with functional groups at appropriate positions.[7] No model has ever appeared to investigate the rate enhancement caused by freezing an internal rotation in the intramolecular catalysis of ester hydrolysis by an imidazolyl group, although many recent works have concentrated on steric control and steric acceleration in cyclization reactions.[10-13] Therefore, we have synthesized three new models, 4,5-[1′(4′)-acetoxymethyltetramethylene]imidazole (**2**), its 1′(4′)-methyl-substituted derivative (**3**), and 4,5-[1′(5′)-acetoxymethylpentamethylene]imidazole (**4**), and compared their hydrolytic reactivities with the reactivity of **1**. The three new models (**2–4**) apparently have