Catalysis by Cobalt(II)-Substituted Carbonic Anhydrase II of the Exchange of Oxygen-18 between CO_2 and H_2O†

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ABSTRACT: We have measured the catalysis by Co(II)-substituted bovine carbonic anhydrase II from red cells of the exchange of ^{18}O between CO_2 and H_2O using membrane-inlet mass spectrometry. We chose Co(II)-substituted carbonic anhydrase II because the apparent equilibrium dissociation constant of HCO_3^- and enzyme at pH 7.4, $K_{eff}^{HCO_3^-} \simeq 55$ mM, was within a practicable range of substrate concentrations for the ^{18}O method. For the native, zinc-containing enzyme $K_{eff}^{HCO_3^-}$ is close to 500 mM at this pH. The rate constant for the release from the active site of water bearing substrate oxygen k_{H_2O} was dependent on the fraction of enzyme that was free, not bound by substrate HCO_3^- or anions. The pH dependence of k_{H_2O} in the pH range 6.0–9.0 can be explained entirely by a rate-limiting, intramolecular proton transfer between cobalt-bound hydroxide and a nearby group, probably His-64. The rate constant for this proton transfer was found to be 7×10^5 s^{-1} for the Co(II)-substituted enzyme and 2×10^6 s^{-1} for the native enzyme. These results are applied to models derived from proton-relaxation enhancement of water exchanging from the inner coordination shell of the cobalt in carbonic anhydrase. The anions iodide, cyanate, and thiocyanate inhibited catalysis of ^{18}O exchange by Co(II)-substituted carbonic anhydrase II in a manner competitive with total substrate (CO_2 and HCO_3^-) at chemical equilibrium and pH 7.4. These results are discussed in terms of observed steady-state inhibition patterns and suggest that there is no significant contribution of a ternary complex between substrate, inhibitor, and enzyme.

The catalysis by the zinc-containing metalloenzyme carbonic anhydrase II (or C) of ^{18}O exchange between species of CO_2 and water has been useful in explaining aspects of the catalytic pathway. When combined with the data from many other techniques, the following picture of the exchange process has emerged (Silverman & Vincent, 1983). The dehydration of ^{18}O-labeled bicarbonate, $HCOO^{18}O^-$, has a 1/3 probability of labeling the active site with ^{18}O. This step, the catalytic interconversion of HCO_3^- and CO_2, has been determined by equilibrium methods (Simonsson et al., 1979; Silverman et al., 1979) to occur without a rate-limiting proton-transfer step:

$$\text{>ZnOH}_2 + HCOO^{18}O^- \rightleftharpoons \text{>Zn}^{18}OH^- + CO_2 + H_2O \quad (1)$$

The resulting zinc-bound $^{18}OH^-$ is believed not to exchange rapidly with solvent. Loss of the ^{18}O from the active site of carbonic anhydrase II to solvent water near pH 7 has been shown to involve an intramolecular proton transfer (Tu & Silverman, 1982), a transfer that is probably the same as that identified by Steiner et al. (1975) as being the rate-limiting event for the maximal turnover for hydration of CO_2. This intramolecular proton transfer, probably involving His-64 and zinc-bound OH^-, allows release of $H_2{}^{18}O$ and returns the enzyme's active site to the ionization state that is catalytic for the next dehydration step:

$$^+\text{H-His-64 >Zn}^{18}OH^- \rightleftharpoons$$

$$\text{His-64 >Zn}^{18}OH_2 \overset{+H_2O}{\rightleftharpoons} \text{His-64 >ZnOH}_2 + H_2{}^{18}O \quad (2)$$

Histidine-64 is not a ligand of the zinc; its imidazole group is located about 6 Å from the zinc in the active site crevice (Notstrand et al., 1975).

Equilibrium studies using ^{13}C NMR of this catalysis by human carbonic anhydrase II, the most thoroughly studied of these isozymes, have established that the apparent equilibrium dissociation constant for the binding of substrate to the active site is very large: $K_{eff}^{HCO_3^-} \simeq 0.5$ M at pH 7.5 (Simonsson et al., 1979). We report that ^{18}O exchange catalyzed by Co(II)-substituted bovine carbonic anhydrase II showed substrate binding that was much tighter, $K_{eff}^{HCO_3^-} \simeq 0.05$ M. This value is within a practicable range of substrate concentration for the ^{18}O method and permits us to comment on the mode of inhibition of ^{18}O exchange by anions and also the effect of binding of HCO_3^- on the water-release steps of eq 2. We have found that the monoanions iodide, cyanate, and thiocyanate exerted an inhibition of the equilibrium interconversion of CO_2 and HCO_3^- at pH 7.4 that was competitive with respect to total substrate, CO_2 and HCO_3^-. Moreover, the release of water bearing substrate oxygen was dependent on the fraction of enzyme that was free, not bound by substrate HCO_3^- or anions. We also show that the pH dependence of the rate constant for the release of this water measured over the pH range of 6.0–9.0 can be explained entirely by the intramolecular proton transfer shown in eq 2.

Experimental Procedures

Materials and Enzyme. Oxygen-18-labeled bicarbonate was prepared by equilibrating $KHCO_3$ in ^{18}O-enriched water and ^{13}C-labeled bicarbonate by equilibrating $^{13}CO_2$ in an alkaline, aqueous solution as described earlier (Silverman et al., 1979). Water used for solution preparation and dialysis was distilled and passed through two ion-exchange resin cartridges (Cole-Parmer 1506-35). D_2O (99.8%) was stirred overnight with activated charcoal; then, the charcoal was filtered out, and the D_2O was distilled.

Bovine red cell carbonic anhydrase (BCA) was either obtained from whole blood or from Sigma Chemical Co. Purification was achieved by the affinity chromatography procedure of Khalifah et al. (1977). Apoenzyme was made by dialysis against 0.1 M pyridine-2,6-dicarboxylic acid in 0.2

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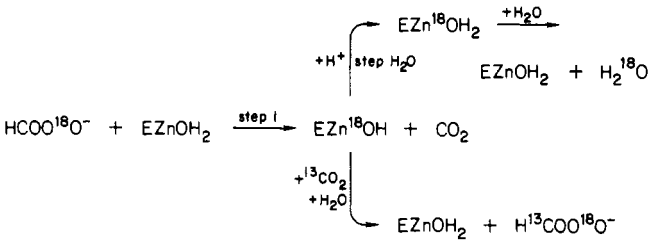
Scheme I



M phosphate buffer at pH 7.0 for 24 h at 2 °C (Hunt et al., 1977), followed by 8 changes of a 40 volume excess of water over the period of 1 week. Bovine Co(II)-substituted carbonic anhydrase was prepared by addition of 1.1 equiv of $CoCl_2$ to the apoenzyme, followed by further dialysis in some cases.

Oxygen-18 Exchange. The ^{18}O-exchange method applied to the hydration of CO_2 catalyzed by carbonic anhydrase has been described in detail in previous publications (Silverman et al., 1979; Koenig & Brown, 1981; Silverman, 1982). This method is based on the work of Mills & Urey (1940) and measures the exchange of ^{18}O between species of CO_2 and water caused by the hydration–dehydration cycle at chemical equilibrium:

$$CO^{18}O + H_2O \rightleftharpoons HCOO^{18}O^- + H^+ \rightarrow CO_2 + H_2{}^{18}O \quad (3)$$

The second step is considered irreversible since $H_2{}^{18}O$ is so substantially diluted in ^{16}O-containing solvent water. This exchange is catalyzed by carbonic anhydrase. A second exchange, discovered by Gerster et al. (1973), is the transfer of ^{18}O from ^{12}C-containing species of CO_2 to ^{13}C-containing species of CO_2. The uncatalyzed exchange is given in eq 4.

$$^{12}COO^{18}O^{2-} + {}^{13}CO_2 \rightleftharpoons {}^{13}COO^{18}O^{2-} + {}^{12}CO_2 \quad (4)$$

The exchange of ^{18}O between ^{12}C- and ^{13}C-containing species of CO_2 is catalyzed by carbonic anhydrase, but eq 4 is not the catalyzed reaction; the catalysis involves labeling the enzyme's active site with ^{18}O, as described in Scheme I.

These exchanges were measured by placing into solution ^{18}O-labeled bicarbonate not enriched in ^{13}C and ^{13}C-labeled bicarbonate not enriched in ^{18}O and waiting an appropriate time for the approach to chemical equilibrium. Then, the atom fractions α, $^{12}\alpha$, and $^{13}\alpha$ were measured as a function of time with a mass spectrometer:

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

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

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

where 44, 45, 46, ... represent the heights of the mass peaks of $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$, $^{12}C^{16}O^{18}O$, ...

These experiments were performed with a CO_2 inlet vessel attached to a mass spectrometer. The vessel had as its bottom a membrane permeable to CO_2 supported by a stainless steel, porous disk [see Silverman, (1982) for details]. This provided a continuous measurement of the ^{18}O content of CO_2. The mass spectrometer was interfaced to an IBM XT computer for data acquisition and analysis. Into this vessel was placed 8.0 mL of solution containing ^{18}O- and ^{13}C-labeled bicarbonate, Na_2SO_4 to provide an ionic strength of 0.2, and NaOH or H_2SO_4 to adjust the pH. No buffers were used. All solutions contained 1–3 μM EDTA (ethylenediaminetetraacetic acid) to sequester metal contaminants that might otherwise inhibit carbonic anhydrase (Tu et al., 1981). After a wait of as long as several minutes to allow for the approach to chemical equilibrium, α, $^{12}\alpha$, and $^{13}\alpha$ were measured as a function of time to determine the uncatalyzed ^{18}O exchange rates. Enzyme was then added in a volume less than 0.1 mL, and at least 20 s was allowed for mixing before measurements were taken. During an experiment, the pH remained constant to within 0.02 pH unit. All data were taken at 25 °C.

The analysis of the ^{18}O-exchange data has been described in detail elsewhere (Silverman et al., 1979; Koenig & Brown, 1981) and is presented here in outline. The rate of decrease of α is first order, eq 5. The rates of decrease of $^{12}\alpha$ and $^{13}\alpha$

$$\alpha - \alpha_\infty = a_1 e^{-\theta t} \quad (5)$$

are biphasic and can be described by eq 6 and 7. The rate

$$^{12}\alpha - \alpha_\infty = a_1 e^{-\theta t} + a_2 e^{-(\theta+\phi)t} \quad (6)$$

$$^{13}\alpha - \alpha_\infty = a_1 e^{-\theta t} + a_3 e^{-(\theta+\phi)t} \quad (7)$$

constant θ describes the exchange of ^{18}O between CO_2 and H_2O, a first-order process. This rate constant can be expressed as the sum of uncatalyzed and catalyzed components: $\theta = \theta_{uncat} + \theta_{cat}$. The rate constant ϕ describes the exchange of ^{18}O between ^{12}C- and ^{13}C-containing species of CO_2, an exchange that occurs slowly in the absence of carbonic anhydrase (Gerster et al., 1973): $\phi = \phi_{uncat} + \phi_{cat}$. A plot of ln $(\alpha - \alpha_\infty)$ vs. time yields as slope $-\theta$. A plot of ln $(^{12}\alpha - \alpha)$ vs. time and a plot of ln $(\alpha - {}^{13}\alpha)$ vs. time yield as slope $-\theta - \phi$.

Because of the small fraction of total substrate existing as CO_2 above pH 8.0, we used a different method for obtaining θ and ϕ in this region. It is described previously (Silverman & Tu, 1976) and involves taking small aliquots (0.3 mL) from the reaction solution by injection into evacuated vessels containing 9 M sulfuric acid. This rapidly stopped the reaction and liberated CO_2. The CO_2 was then passed through a dry ice/acetone trap to remove water vapor, and the CO_2 was analyzed for isotopic content as described above.

The rate constants for ^{18}O exchange θ_{cat} and ϕ_{cat} can be used to obtain the rates of two steps in the catalytic pathway (Silverman et al., 1979; Koenig & Brown, 1981), step 1 and step H_2O of Scheme I. Koenig & Brown (1981) have presented an interpretation based on a zinc-bound water mechanism that is very similar to that of Scheme I. Step 1 of Scheme I is the dehydration of ^{18}O-labeled bicarbonate leaving labeled oxygen at the active site. The step in which $^{13}CO_2$ reacts with labeled active site to yield $H^{13}COO^{18}O^-$ is a hydration step with the same rate as step 1 at equilibrium. A proton transfer converts zinc-bound hydroxide into zinc-bound water, which allows water to exchange rapidly with solvent, as shown in Scheme I. The rate of step 1 at chemical equilibrium is denoted R_1. Step H_2O is the release to solvent of water bearing substrate oxygen, a step that is assumed to occur independently of step 1. The rate of step H_2O is denoted R_{H_2O}.

Solution of the kinetic equations for ^{18}O exchange on the basis of Scheme I yields the following expressions for R_1 and R_{H_2O} (Silverman et al., 1979; Koenig & Brown, 1981):

$$R_1 = 3[S_{tot}](\theta_{cat} + \phi_{cat}) \quad (8)$$

$$R_{H_2O} = \frac{R_1\theta_{cat}}{\phi_{cat}}\left(1 - \frac{[CO_2]}{3[S_{tot}]}\right) \quad (9)$$

$$[S_{tot}] = [CO_2] + [HCO_3^-] \quad (10)$$

The catalytic rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium can be expressed (eq 11) in the form of

$$R_1 = \frac{k_{cat}^{exch}[E_{tot}][S]}{K_{eff}^{S} + [S]} \quad (11)$$

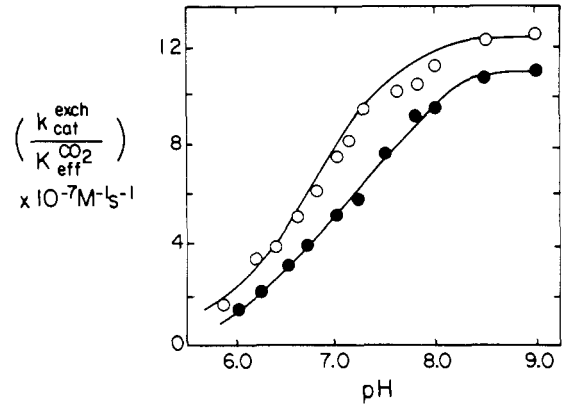


FIGURE 1: Variation with pH of $k_{cat}^{exch}/K_{eff}^{CO_2}$ determined from ^{18}O exchange in the presence of (○) bovine carbonic anhydrase II or (●) Co(II)-substituted bovine carbonic anhydrase II. Ionic strength of solutions was maintained at 0.2 with Na_2SO_4, and no buffers were added; total concentration of all CO_2 species was 15 mM or less, and temperature was 25 °C. Values of $k_{cat}^{exch}/K_{eff}^{CO_2}$ were calculated from eq 11 assuming $[CO_2] \ll K_{eff}^{CO_2}$. The solid lines were obtained from the equation in footnote 1 with the following constants: for native enzyme, $pK_{x1} = pK_{y1} = 5.1$, $pK_{x2} = pK_{y2} = 6.8$, and $K_i = 2 \times 10^{-3}$ M; for the Co(II)-substituted enzyme, $pK_{x1} = 5.7$, $pK_{y1} = 5.8$, $pK_{x2} = pK_{y2} = 7.3$, and $K_i = 5 \times 10^{-3}$ M.

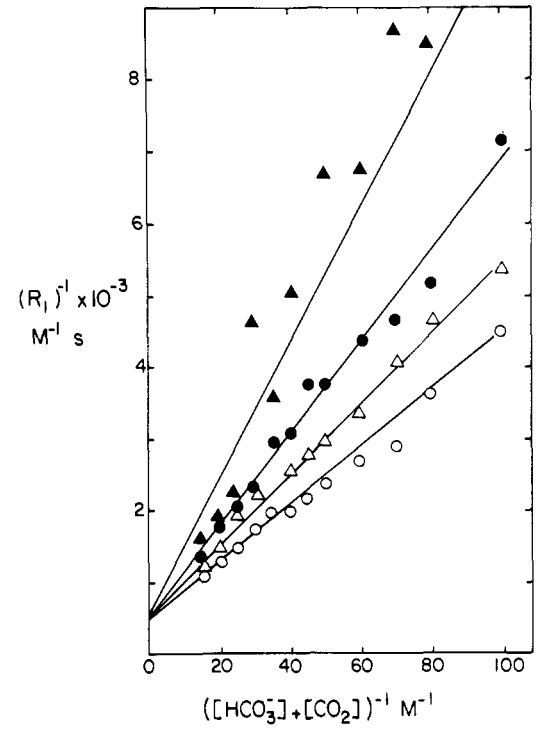


FIGURE 2: Reciprocal plot showing the variation with total substrate concentration of R_1, the rate of catalytic interconversion of CO_2 and HCO_3^- at chemical equilibrium. Co(II)-substituted bovine carbonic anhydrase was present at 6.0×10^{-9} M and NaI at the following concentrations: (○) 0, (△) 3.8, (●) 10, and (▲) 23 mM. The pH was 7.4, and no buffers were added; the total ionic strength of solution was maintained at 0.2 with Na_2SO_4. Temperature was 25 °C. The solid lines were calculated from the equation in footnote 2 with the constants given in the legend to Figures 1 and 3 and $K_I = 5$ mM, $K_p = 25$ mM, and $(k_{cat}^{exch})_{max} = 3.5 \times 10^5$ s^{-1}. K_I and K_p are equilibrium dissociation constants for the binding of I^- and HCO_3^- to the forms of enzyme with a protonated active site.

the Michaelis equation although k_{cat}^{exch} and K_{eff}^{S} are not the turnover number and Michaelis constant; rather, they are respectively the maximal rate of interconversion of CO_2 and HCO_3^- at equilibrium and the apparent equilibrium dissociation constant of enzyme-bound substrate S (Simonsson et al., 1979; Koenig et al., 1974). K_{eff}^{S} is equivalent to a true substrate dissociation constant only for the simplest mechanism. With more realistic mechanisms, K_{eff}^{S} is the apparent dissociation constant with the denominator containing the concentrations of complexes of enzyme bound with substrate and product (Simonsson et al., 1979). $K_{eff}^{HCO_3^-}$ and $K_{eff}^{CO_2}$ are related through an equilibrium constant:

$$K_{eff}^{HCO_3^-}/K_{eff}^{CO_2} = K_e/[H^+] = [HCO_3^-]/[CO_2] \quad (12)$$

The rate constant k_{H_2O} was obtained by dividing R_{H_2O} by the total enzyme concentration.

RESULTS

Values of the ratio $k_{cat}^{exch}/K_{eff}^{CO_2}$ in the pH range 6–9 for both bovine carbonic anhydrase and bovine Co(II)-substituted carbonic anhydrase are given in Figure 1 and behave roughly as a single ionization with pK_a near 7. The maximal values of $k_{cat}^{exch}/K_{eff}^{CO_2}$, observed at pH >8, are similar: 1.2×10^8 M^{-1} s^{-1} for the native and 1.1×10^8 M^{-1} s^{-1} for the cobalt-substituted enzyme. Although these ratios are similar, the individual values of k_{cat}^{exch}, $K_{eff}^{CO_2}$, and $K_{eff}^{HCO_3^-}$ are different for the two enzymes. With our apparatus we cannot measure $K_{eff}^{HCO_3^-}$ and k_{cat}^{exch} for the native bovine enzyme because K_{eff} is so much larger than the highest substrate concentration at which we get accurate results. But, we expect the values to be very close to those for the kinetically similar human carbonic anhydrase II measured by ^{13}C NMR (Simonsson et al., (1979): at pH 7.5, $K_{eff}^{CO_2} \simeq 25$ mM, $K_{eff}^{HCO_3^-} \simeq 500$ mM, and $k_{cat}^{exch} = 1.5 \times 10^6$ s^{-1}. For bovine Co(II)-substituted carbonic anhydrase at pH 7.5, $K_{eff}^{HCO_3^-} = 55 \pm 15$ mM and $k_{cat}^{exch} = (2.2 \pm 0.8) \times 10^5$ s^{-1} (mean and standard deviation from four experiments). Identical values within the given standard deviations were found by using as solvent 98% D_2O–2% H_2O at pD 7.9.

In Figure 2 we present a reciprocal plot showing the variation with total substrate of R_1, the rate at chemical equilibrium of the interconversion of CO_2 and HCO_3^- catalyzed by the cobalt-substituted enzyme. The value of $K_{eff}^{HCO_3^-} = 55 \pm 15$ mM for this process is within our substrate range, and hence, we are able to differentiate between various modes of inhibition of R_1. The inhibition by sodium iodide at pH 7.4 is clearly competitive with total substrate under these equilibrium conditions. Competitive inhibition was also observed in similar experiments with cyanate at pH 7.4 and thiocyanate at pH 7.3, with the other conditions as described in the legend to Figure 2.

The rate constant for the release from the enzyme of water bearing substrate oxygen k_{H_2O} is bell shaped for both native and Co(II)-substituted bovine carbonic anhydrase, showing a rapid decrease as pH increases above 7 (Figure 3). The maximal value of k_{H_2O} for the native enzyme occurs at pH 6.5, 6×10^5 s^{-1}, and is greater than that for the cobalt-substituted enzyme, which is 2.5×10^5 s^{-1} at pH 7.0.

The variation with substrate bicarbonate concentration of k_{H_2O} in the presence of Co(II)-substituted bovine carbonic anhydrase is shown in Figure 4. The values of k_{H_2O} decreased as substrate concentration increased, although the scatter of points appears to mask this effect at the highest concentration of the inhibitor iodide shown in Figure 4. Patterns very similar to Figure 4 were observed at pH 7.4 with cyanate as inhibitor and at pH 7.5 with iodide again. The same rate constant k_{D_2O} measured in 98% D_2O–2% H_2O at pD 7.9 also decreased as substrate increased, and we observed a solvent hydrogen isotope effect $k_{H_2O}/k_{D_2O} = 2.5 \pm 0.5$, which was independent of total

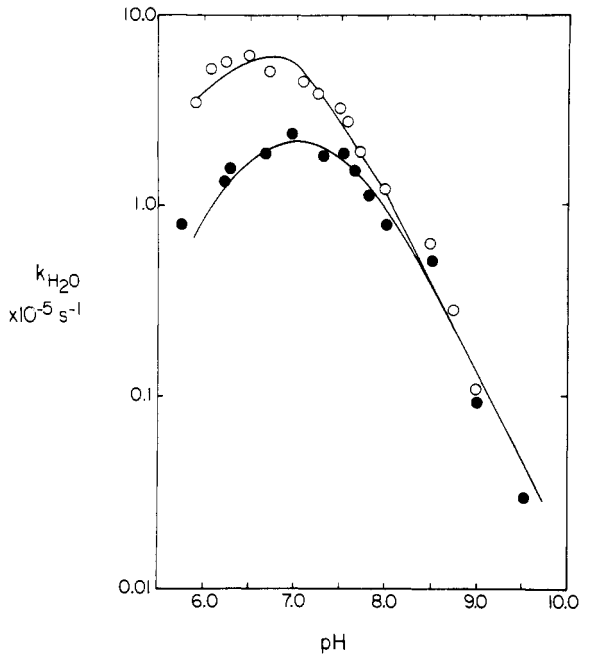


FIGURE 3: Variation with pH of k_{H_2O}, the rate constant for release from enzyme of water bearing substrate oxygen. (○) Bovine carbonic anhydrase II; (●) Co(II)-substituted bovine carbonic anhydrase II. Data were obtained at 25 °C with solutions containing 15 mM of all species of CO_2 and with total ionic strength of solution maintained at 0.2 with Na_2SO_4. No buffers were added to solutions. Solid lines were calculated from eq 13 with $k_{-3} = 1.9 \times 10^6$ s^{-1} for native enzyme and $k_{-3} = 7.0 \times 10^5$ s^{-1} for the Co(II)-substituted enzyme. Other constants used were identical with those given in the legend to Figure 1.

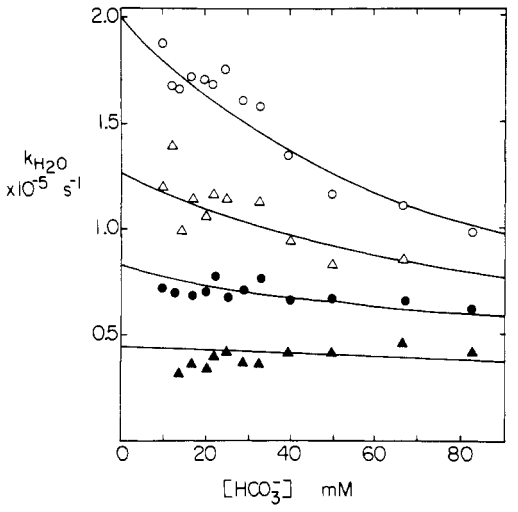
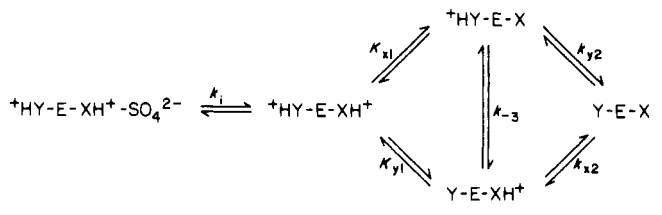


FIGURE 4: Dependence on substrate HCO_3^- concentration of k_{H_2O} at pH 7.4 in the presence of Co(II)-substituted bovine carbonic anhydrase (6×10^{-4} M) and NaI at the following concentrations: (○) 0, (△) 3.8, (●) 10, and (▲) 22.5 mM. Solutions contained no buffers and were maintained at a total ionic strength of 0.2 with Na_2SO_4. Temperature was 25 °C. The solid lines were calculated from eq 14 with $K_I = 2$ mM and $K_p = 25$ mM for the binding of I^- and HCO_3^-, respectively, to the forms of enzyme with protonated active site. Other constants were as given in the legends to Figures 1 and 3.

Scheme II



substrate concentration.

Discussion

The purpose of this study is to help explain the catalysis of the hydration of CO_2 by carbonic anhydrase with ^{18}O exchange and, in particular, to take advantage of one helpful feature of the cobalt(II)-substituted enzyme: i.e., the tight binding of substrate bicarbonate to Co(II)-substituted enzyme relative to the native, zinc-containing enzyme. The apparent equilibrium dissociation constant $K_{eff}^{HCO_3^-}$ is about 55 mM at pH 7.5 for Co(II)-BCA compared with $K_{eff}^{HCO_3^-}$ close to 500 mM at the same pH for native human carbonic anhydrase II (Simonsson et al., 1979).

First, we can comment that the ^{18}O-exchange method shows similarities in the catalysis of CO_2 hydration by the native and cobalt-substituted enzyme as have been shown by other kinetic methods. Hence, $k_{cat}^{exch}/K_{eff}^{CO_2}$ is approximately the same at its maximal value for native BCA (1.2×10^8 M^{-1} s^{-1}) as for Co(II)-BCA (1.1×10^8 M^{-1} s^{-1}) (Figure 1). As shown by several workers (Koenig et al., 1974; Simonsson et al., 1979), the ratio $k_{cat}^{exch}/K_{eff}^{CO_2}$ from equilibrium experiments is equivalent to $k_{cat}^{CO_2}/K_m^{CO_2}$ from steady-state experiments, although these constants taken individually need not be equal (i.e., $K_{eff}^{CO_2} \neq K_m^{CO_2}$). At steady state, $k_{cat}^{CO_2}/K_m^{CO_2}$ is also approximately the same at its maximal value for native BCA (6×10^7 M^{-1} s^{-1}; Pocker & Bjorkquist, 1977) as for Co(II)-BCA (7×10^7 M^{-1} s^{-1}; Lindskog, 1966). As with native human carbonic anhydrase II (Simonsson et al., 1979), Co(II)-BCA catalyzed CO_2 hydration with a solvent hydrogen isotope effect of unity on both k_{cat}^{exch} and $K_{eff}^{HCO_3^-}$ at pH near 7.4. This has been interpreted to suggest direct nucleophilic attack of metal-bound hydroxide on CO_2 (Simonsson et al., 1979). Also, for both native and cobalt-substituted carbonic anhydrase, there is a considerable isotope effect on k_{H_2O} (the rate constant for the release from the enzyme of water bearing oxygen abstracted from substrate HCO_3^-). This indicates proton transfer in the water-release step, such as would occur if the protonation of metal-bound hydroxide were a necessary and rate-contributing event in the pathway leading to the exchange of bound water for water from solvent.

The pH dependence of $k_{cat}^{exch}/K_{eff}^{CO_2}$ in Figure 1 is interpreted with the model of Simonsson & Lindskog (1982) to describe the complex activity profile of bovine carbonic anhydrase. Their kinetic data are consistent with a microscopic ionization scheme (Scheme II) involving two electrostatically interacting groups: the zinc-bound water at the active site and a second group, probably His-64. The activity in CO_2 hydration and ester hydrolysis depends on the basic form of the active site, zinc-bound hydroxide. The effect of sulfate ions, which we have used in this study to maintain ionic strength at 0.2, was found by Simonsson & Lindskog (1982) to be consistent with the binding of sulfate to the form of enzyme with both the active site and the second group protonated. In Scheme II, XH^+ indicates a protonated active site, zinc-bound water, and ^+HY indicates a protonated nearby group, probably imidazolium of His-64. Simonsson & Lindskog (1982) and Lindskog et al. (1982) measured the effect of sulfate on the pH profile of the hydrolysis of 4-nitrophenyl acetate catalyzed by native BCA and found this SO_4^{2-} inhibition constant and microscopic pK_a values: $K_i = 2$ mM; $pK_{x1} = pK_{y1} = 5.1$; $pK_{x2} = pK_{y2} = 6.8$. These values were used to draw the solid line in Figure 1 for BCA,[1] which is seen to be a good fit to our

observations. The effect of sulfate is to depress the role of the ionizations at $pK = 5.1$ and results in a profile similar to that of a single ionization curve of $pK = 6.8$. The values for the hydrolysis of 4-nitrophenyl acetate by Co(II)-BCA are (Simonsson & Lindskog, 1982; Lindskog et al., 1982) $K_i = 5$ mM, $pK_{x1} = 5.7$, $pK_{y1} = 5.8$, $pK_{x2} = 7.0$, and $pK_{y2} = 7.1$. The values found to fit our observations and shown as a solid line in Figure 1 are the same except $pK_{x2} = pK_{y2} = 7.3$. NMR studies of human carbonic anhydrase II have shown that His-64 titrates with a pK_a of 7.1 (Campbell et al., 1975). A pK_a near 7 for the zinc- or cobalt-bound water at physiologic ionic strength is consistent with a wide body of data (Bertini, 1982; Lindskog, 1982).

The rate constant for the release from the enzyme of water-bearing substrate oxygen, k_{H_2O}, can be measured because the dehydration of $HC^{18}O_3^-$ leaves ^{18}O at the active site, which is then released to bulk water (Silverman et al., 1979). For native human carbonic anhydrase II, k_{H_2O} was found to have a large solvent hydrogen isotope effect (as great as 8; Tu & Silverman, 1982) and a pH dependence consistent with a rate-limiting intramolecular proton transfer between the zinc-bound hydroxide and the second ionizable group, probably His-64 (Silverman et al., 1979). Figure 3 shows k_{H_2O} as a function of pH for native BCA and for Co(II)-BCA extended to pH 9.0–9.5. Here we point out that the kinetic analysis of the ^{18}O-exchange data obtains a water off-rate that is independent of the rate of interconversion of HCO_3^- and CO_2; the data of Figure 3 are not determined by the slow rate of dehydration at very alkaline pH regions. We would like to emphasize that values of k_{H_2O} for both enzymes over the pH range measured are very similar and can be explained by the ionizations and sulfate inhibition of Scheme II assuming that the intramolecular proton transfer described by k_{-3} is rate limiting for the release from enzyme of water bearing substrate oxygen:

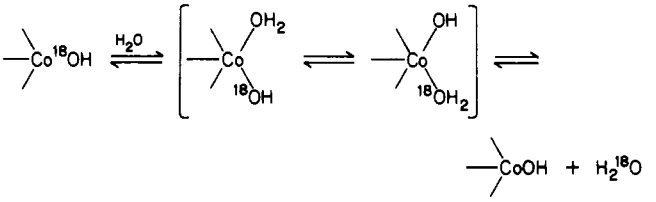
$$^{+}HY\text{-}E\text{-}^{18}OH^- \xrightarrow{k_{-3}} Y\text{-}E\text{-}^{18}OH_2 \xrightarrow{+H_2O} Y\text{-}EOH_2 + H_2{}^{18}O$$

The solid lines in Figure 3 were generated from eq 13 and the

$$k_{H_2O} = \frac{R_{H_2O}}{[E_{tot}]} = \frac{k_{-3}[^{+}HY\text{-}E\text{-}X]}{[E_{tot}]} = \frac{k_{-3}}{1 + K_{y2}/K_{x2} + K_{y2}/[H^+] + ([H^+]/K_{x1})[1 + [SO_4]/K_i]} \quad (13)$$

same values of pK_{x1}, pK_{y1}, pK_{x2}, pK_{y2}, and K_i that were found to fit the pH profiles of Figure 1. For native BCA, $k_{-3} = 1.9 \times 10^6$ s^{-1}, and for Co(II)-BCA, $k_{-3} = 7 \times 10^5$ s^{-1}, obtained from the fit to the data of Figure 3. Because of this fit, we need invoke no processes other than those of Scheme II to account for the exchange from the enzyme of water bearing substrate oxygen. These values for the rate-limiting step k_{-3} can be compared with $k_{-3} = 7 \times 10^5$ s^{-1} obtained from steady-state data for human carbonic anhydrase II (Steiner et al., 1976) and $k_{-3} = 3.5 \times 10^6$ s^{-1} obtained from ^{18}O exchange for human isozyme II in the presence of buffers (Silverman et al., 1979).

Scheme III



To account satisfactorily for the catalysis, it is required that k_{H_2O} be at least as large as the turnover rate for dehydration, $k_{cat}^{HCO_3^-}$. The data of Figure 3 meet this requirement when compared with the catalysis of dehydration of HCO_3^- by BCA (Pocker & Bjorkquist, 1977). The data of Figure 3 were measured in the presence of 15 mM total substrate, $[HCO_3^-] + [CO_2]$, and hence, the pH dependence of k_{H_2O} pertains to this total substrate concentration.

Figure 3 becomes worthy of comment with respect to the NMR studies of the proton-relaxation enhancement of water in the presence of Co(II)-substituted carbonic anhydrase II (Fabry et al., 1970). These show that the relaxivity of water protons can be fit approximately to a single ionization of pK_a near 7 when measured in the presence of sulfate (about 0.1 M), with a maximum at alkaline pH requiring a water off-rate in excess of 10^5 s^{-1}. This result has been interpreted to indicate the presence of a water molecule exchanging rapidly from the inner coordination shell of cobalt when the enzyme is in the form that predominates at high pH. This high-pH form of the enzyme must also have the metal-bound hydroxide, which is expected to exchange slowly with solvent. Koenig et al. (1983) have proposed that the form of Co(II)-BCA that predominates at high pH is able to form a pentacoordinate intermediate having both OH^- and H_2O as ligands of cobalt. Proton transfer between these ligands, as shown in Scheme III, would then account for solvent proton relaxation in alkaline regions and the ^{18}O loss from the enzyme.

Our data in Figure 3 show that water bearing substrate oxygen is released from the enzyme in the alkaline region with an off-rate well below the 10^5 s^{-1} required by the NMR relaxation data. Consequently, it is not necessary to invoke Scheme III to account for exchange from the enzyme of water bearing substrate oxygen; it can be accounted for entirely by the intramolecular proton transfer described by k_{-3} in Scheme II followed by exchange of metal-bound water for solvent water. The solvent proton relaxation enhancement observed at alkaline pH may be due to a rapidly exchanging, outer sphere water, which is hydrogen bonded to the cobalt-bound hydroxide. This would give a cobalt–hydrogen distance for this outer sphere water short enough to provide significant relaxation by the paramagnetic metal. Koenig et al. (1981) and Koenig & Brown (1983) have proposed such an outer-sphere relaxation mechanism for the proton relaxation enhancement of solvent water in the presence of fluoromethemoglobin and in other cases involving metalloproteins. These arguments suggest that the solvent proton relaxation enhancement caused by Co(II)-BCA at alkaline pH reports the coordination of the cobalt or the ionization state of the ligands of the cobalt, or both, and that the rapidly exchanging solvent

[1] The solid lines in Figure 1 were calculated by assuming that both species Y-E-X and HY-E-X of Scheme II have equal enzymatic activity in the hydration of CO_2. The fraction of total enzyme in the form with the active site unprotonated is

$$f = \frac{[HYEX] + [YEX]}{[E_{tot}]} = \frac{1 + K_{y2}/[H^+]}{1 + K_{y2}/K_{x2} + K_{y2}/[H^+] + [H^+](1 + [SO_4^{2-}]/K_i)/K_{x1}}$$

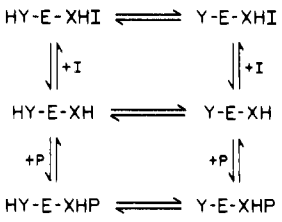
then

$$k_{cat}^{exch}/K_{eff}^{CO_2} = (k_{cat}^{exch}/K_{eff}^{CO_2})_{max} f$$

water molecule thus relaxed does not play a direct role in the catalysis itself (i.e., this is not the water containing oxygen abstracted from substrate HCO_3^-).

We now show that Figure 4 giving k_{H_2O} as a function of substrate and anionic inhibitor is consistent with a model (Scheme II) that predicts that k_{H_2O} is proportional to the concentration of free enzyme. The model is based on the observation that k_{H_2O} appears to be limited by intramolecular proton transfer (k_{-3} of Scheme II). That is, the oxygen abstracted from HCO_3^- appears in the active site as metal-bound hydroxide, which does not exchange rapidly with solvent. This form of the enzyme does not readily bind anions or bicarbonate. Exchange with solvent occurs when this OH^- has been protonated by a transfer probably from His-64.

This situation is described by the following addition to Scheme II, which contains the feature that substrate HCO_3^-, designated P, and anionic inhibitors, designated I, bind to the form of enzyme with the active site protonated:



Adding these new bound species to Scheme I results in the following expression for k_{H_2O}:

$$k_{H_2O} = k_{-3}/(1 + K_{y2}/K_{x2} + K_{y2}/[H^+] + ([H^+]/K_{x1})[1 + [SO_4^{2-}]/K_I] + [[P]/K_p + [I]/K_I][[H^+]/K_{x1} + K_{y2}/K_{x2}]) \quad (14)$$

where K_I is the equilibrium dissociation constant of I bound to enzyme with active site protonated. [We have not added the complication that K_I probably is different depending on the ionization state of group Y; see Simonsson & Lindskog (1982) and Tibell et al. (1984).] K_p is the equilibrium dissociation constant for substrate HCO_3^- and enzyme with active site protonated and is different from $K_{eff}^{HCO_3^-}$ for substrate that is strictly not a simple dissociation constant (Simonsson et al., 1979). To avoid further complications, we have neglected the binding of substrate CO_2 on the water off-rate. At pH 7.4 of Figure 4, CO_2 is about 5% of total substrate. Its binding site and whether it displaces zinc- or cobalt-bound water are not clearly understood (Lindskog, 1982). The solid lines in Figure 4 were generated with the values of k_{-3} and the pK_a's that were found to fit Figures 1 and 3. In addition, the values given in the legend to Figure 4, K_p = 25 mM and K_I = 2 mM for iodide, are reasonable. K_I for iodide was estimated at 1 mM from kinetic experiments using Co(II)-BCA (Lindskog, 1966).

The fit of eq 14 to the data of Figure 4 excludes some possibilities for the exchange of water at the metal. For example, it is unlikely that ^{18}O-labeled water remains bound when bicarbonate binds to the active site and exchanges with solvent water equally well from the free enzyme and enzyme–bicarbonate complex.

The rate of the intercoversion of CO_2 and HCO_3^-, R_1, catalyzed by Co(II)-BCA at chemical equilibrium and pH 7.4 is inhibited by the anions I^- (Figure 2), OCN^-, and SCN^- in a manner that is competitive with total substrate. This is consistent with a wide body of evidence that monoanions bind directly to the metal in carbonic anhydrase (Lindskog, 1966; Bertini 1982). The solid lines in Figure 2 are a fit to the data of an equation[2] based on $R_1 = (k_{cat}^{exch})_{max}([HY\text{-}E\text{-}XHP] + [Y\text{-}E\text{-}XHP])$ and using the same parameters that were found to fit Figures 1, 3, and 4 (see legend to Figure 2). An exception is the inhibition constant K_I for iodide, as defined above, which is 5 mM to fit the data for R_1 (Figure 2) but 2 mM to fit the data for k_{H_2O} (Figure 4). This discrepancy is not surprising considering the simplification of the binding process in our model. One possible source of this discrepancy is that we have ignored the effect of the binding of substrate CO_2; another is that we have neglected the effect on the binding of inhibitors of other ionizations near the active site.

The competitive pattern in Figure 2 suggests that there is no significant contribution of a ternary complex between substrate, inhibitor, and enzyme. This pertains at concentrations of substrate and inhibitor comparable to or greater than their respective equilibrium dissociation constants with enzyme. These comments are made with reference to the two interpretations of the uncompetitive inhibition by anions of CO_2 hydration catalyzed by native isozyme II observed at steady state and alkaline pH. One interpretation is that a ternary complex forms between enzyme, substrate, and inhibitor (Pocker & Deits, 1982). The other is that anions bind to the form of enzyme that accumulates at steady state; that is, the form of enzyme occurring before the rate-limiting step (k_{-3} of Scheme II): the enzyme with a protonated active site (Tibell et al., 1984). Figure 2, obtained under equilibrium conditions, shows no evidence of ternary complex formation. A similar result with human carbonic anhydrase I and from measurement of R_1 by ^{13}C NMR line widths has been reported by Lindskog et al. (1982). This competitive inhibition obtained under equilibrium conditions points out that inhibitor and substrate do not bind to the active site simultaneously and supports the hypothesis that the uncompetitive inhibition obtained in initial velocity experiments is due to steady-state buildup of particular enzyme species (mainly Y–E–XH).

These results support the hypothesis that the maximal velocity of the hydration of CO_2 catalyzed by carbonic anhydrase II is limited by an intramolecular proton transfer (Steiner et al., 1975), which in ^{18}O-exchange studies is manifested in the rate constant k_{H_2O} (Silverman et al., 1979). This study offers some further clues and leaves some unanswered questions concerning the catalysis. Although the pH profiles of k_{H_2O} for BCA and Co(II)-BCA are quite similar (Figure 3), the solvent hydrogen isotope effects on k_{H_2O} are different [8.0 for native and 2.5 for Co(II)-substituted enzyme]. This reinforces some questions that have been raised concerning the large value of the solvent hydrogen isotope effect in k_{H_2O} for the native enzyme; several researchers (Tibell et al., 1984; Rowlett, 1985) have suggested that processes other than the intramolecular proton transfer described by k_{-3} (Scheme II) are involved. Another problem is that the ^{18}O-exchange method appears to give values of k_{-3} larger than obtained from steady-state experiments, although experimental error in both techniques can be substantial, especially in k_{H_2O}. The ^{18}O data reported here were obtained with solutions to which no buffers were added. This is an advantage in avoiding the effects of buffers as possible inhibitors of catalysis and as activators of catalysis through proton transfer. The effect of buffers has been shown

[2] The solid lines in Figure 2 were calculated assuming that

$$R_1 = (k_{cat}^{ex})_{max}([HY\text{-}E\text{-}XHP] + [Y\text{-}E\text{-}XHP])$$

$$R_1 = [(k_{cat})_{max}[E_{tot}]([P]/K_p)[[H^+]/K_{x1} + K_{y2}/K_{x2}]]/(1 + K_{y2}/K_{x2} + K_{y2}/[H^+] + ([H^+]/K_{x1})[1 + [SO_4^{2-}]/K_I] + [[P]/K_p + [I]/K_I][[H^+]/K_{x1} + K_{y2}/K_{x2}])$$

to cause k_{H_2O} to increase at pH 7 (Silverman et al., 1979); the effect of specific buffers on the pH profile of k_{H_2O} would be helpful in understanding the role of buffer in proton transfer to solution during catalysis.

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Registry No. CO_2, 124-38-9; HCO_3^-, 71-52-3; O_2, 7782-44-7; I^-, 20461-54-5; cyanate, 661-20-1; thiocyanate, 302-04-5.

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