Bicarbonate as a Proton Donor in Catalysis by Zn(II)- and Co(II)-Containing Carbonic Anhydrases

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Abstract: Catalysis of ^{18}O exchange between CO_2 and water catalyzed by a Co(II)-substituted mutant of human carbonic anhydrase II is analyzed to show the rate of release of $H_2{}^{18}O$ from the active site. This rate, measured by mass spectrometry, is dependent on proton transfer to the metal-bound ^{18}O-labeled hydroxide, and was observed in a site-specific mutant of carbonic anhydrase II in which a prominent proton shuttle residue His64 was replaced by alanine, which does not support proton transport. Upon increasing the concentration of bicarbonate, the rate of release of $H_2{}^{18}O$ increased in a saturable manner to a maximum of 4×10^5 s^{-1}, consistent with proton transfer from bicarbonate to the Co(II)-bound hydroxide. The same mutant of carbonic anhydrase containing Zn(II) had the rate of release of $H_2{}^{18}O$ smaller by 10-fold, but rate of interconversion of CO_2 and HCO_3^- about the same as the Co(II)-containing enzyme. These data as well as solvent hydrogen isotope effects suggest that the bicarbonate transferring the proton is bound to the cobalt in the enzyme. The enhancement of ^{18}O exchange caused by increasing bicarbonate concentration during catalysis by the Zn(II)-containing carbonic anhydrase from the archaeon *Methanosarcina thermophila* suggests that a very similar mechanism for proton donation by bicarbonate occurs with this wild-type enzyme.

Introduction

The carbonic anhydrases are zinc metalloenzymes that catalyze the hydration of CO_2 to produce bicarbonate and a proton. Catalysis proceeds by two processes: one is the reaction of CO_2 with the zinc-bound hydroxide of the enzyme resulting in the production of bicarbonate and in the formation of zinc-bound water (eq 1), and the second is the regeneration of the zinc-bound hydroxide by proton transfer (eq 2).[1−3]

$$CO_2 + EZnOH^- \rightleftarrows EZnHCO_3^- \xrightleftharpoons{[H_2O]} EZnH_2O + HCO_3^- \quad (1)$$

$$EZnH_2O + B \rightleftarrows EZnOH^- + BH^+ \quad (2)$$

Here B indicates exogenous proton acceptors in solution or a residue of the enzyme itself. This reaction is involved in many physiological functions,[4,5] and to date three classes of carbonic anhydrase, designated, α, *β*, and *γ*, have been discovered.[6] These are examples of convergent evolution with no structural homology between classes, although each is a zinc-containing enzyme and in two cases (α and *γ*) three ligands coordinating the metal are histidines.[7,8]

Extensive studies have examined the proton-transfer reaction, eq 2, in catalysis by the α class of carbonic anhydrases.[1−3] Histidine 64, which has its side chain extending into the active-site cavity of human carbonic anhydrase II (HCA II),[7,9] acts as a shuttle residue transporting protons at a maximal rate of 10^6 s^{-1} between the zinc-bound water at the active site and solution. In mutants in which His 64 has been replaced by a residue that does not support proton transfer such as alanine, the maximal turnover for catalysis k_{cat} is decreased about 10-fold with very little effect on k_{cat}/K_m.[10] The activity of the mutant of human carbonic anhydrase II (HCA II) in which His 64 has been replaced with Ala (H64A HCA II) can be enhanced to a level nearly equivalent to the wild type by millimolar levels of exogenous proton donors/acceptors such as imidazole. Proton transport between the active site and solution is also required to explain catalysis by carbonic anhydrase from *Methanosarcina thermophila* (Cam) which has a maximal k_{cat} for hydration of CO_2 at 6×10^4 s^{-1}.[11] Kinetic studies of site-specific mutants of Cam by Tripp and Ferry[12] reveal a pattern of catalytic properties that strongly indicate Glu 84 in Cam acts as a proton shuttle in a manner similar to His 64 in HCA II. The crystal structure of Cam showed the side chain of Glu84 extended into

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the active-site cavity with three distinct side-chain conformations.[13]

We report here experiments which indicate that bicarbonate itself can function as a proton donor in catalysis. We measured two rates in catalysis by carbonic anhydrase that are limited by proton transfer. The first is the exchange of ^{18}O between CO_2 and water measured by mass spectrometry from which we determined the rate of release from the enzyme of ^{18}O-labeled water at chemical equilibrium.[14,15] The second method is stopped-flow spectrophotometry from which we measured the maximal turnover rate for CO_2 hydration at steady state.[16,17]

Methods

Enzymes. Wild-type HCA II, wild-type Cam, and site-specific protein mutants were expressed in *E. coli* as described in previous publications.[10,12,18] All mutations were confirmed by sequencing the DNA of the entire coding region for carbonic anhydrase in the expression vector. Human carbonic anhydrases were purified by affinity chromatography;[19] wild-type Cam was purified by ion exchange followed by hydrophobic interaction chromatography.[12] Concentrations of human carbonic anhydrases were determined from the molar absorptivity at 280 nm (5.5×10^4 M^{-1} cm^{-1}); the concentration of each purified enzyme sample was also determined by titration with the tight-binding inhibitor ethoxzolamide.[20] Concentrations of wild-type Cam and mutant were determined from the molar absorptivity (1.6×10^4 M^{-1} cm^{-1}).

Oxygen-18 Exchange. This method is described fully elsewhere.[14,15,21] It is based on the measurement by membrane-inlet mass spectrometry of the exchange of ^{18}O between CO_2 and water at chemical equilibrium (eqs 3 and 4).

$$HCOO^{18}O^- + EZnH_2O \rightleftarrows EZnHCOO^{18}O^- \rightleftarrows COO + EZnO^{18}H^- \quad (3)$$

$$EZnO^{18}H^- + BH^+ \rightleftarrows EZnH_2{}^{18}O + B \xrightarrow{[H_2O]} EZnH_2O + H_2{}^{18}O + B \quad (4)$$

Here $EZnH_2O$ represents water bound as a ligand of zinc in carbonic anhydrase; cobalt at this site also supports catalysis;[22] BH^+ represents a proton donor, either an exogenous donor or a residue of the enzyme.

Measurement of the isotopic content of CO_2 was made with an Extrel EXM-200 mass spectrometer with a membrane-inlet probe.[14] Solutions were maintained at a minimum ionic strength of 0.2 M by addition of the appropriate amounts of Na_2SO_4; in cases where the ionic strength of substrate alone exceeded 0.2 M, no additional Na_2SO_4 was used. For determination of solvent hydrogen isotope effects, all pD measurements are presented as uncorrected pH meter readings.

The kinetic equations for the redistribution of ^{18}O from the $CO_2-HCO_3^-$ system to water were solved to obtain two rates for the ^{18}O exchange catalyzed by carbonic anhydrase.[14] The first is R_1, the rate of exchange of CO_2 and HCO_3^- at chemical equilibrium, as shown in eq 5.[23] Here k_{cat}^{ex} is a rate constant for maximal interconversion of

$$R_1/[E] = k_{cat}^{ex}[S]/(K_{eff}^{s} + [S]) \quad (5)$$

substrate and product, K_{eff}^{s} is an apparent binding constant for substrate to enzyme, and [S] is the concentration of substrate.

A second rate determined by the ^{18}O exchange method is R_{H_2O}, the proton transfer dependent rate of release from the enzyme of water bearing substrate oxygen (eq 4). This is the component of the ^{18}O exchange that is enhanced by exogenous proton donors.[14] The metal-bound ^{18}O-labeled hydroxide as an anion is expected to bind tightly to the metal. In such enhancements, the exogenous donor acts as a second substrate in the catalysis providing a proton (eq 4), and the resulting effect on ^{18}O exchange is described by eq 6 below. This expression describes the approach of $R_{H_2O}/[E]$ to saturation as dependent on the formation of a bound complex between the exogenous donor and the enzyme.

$$R_{H_2O}/[E] = k_B^{obs}[B]/(K_{eff}^{B} + [B]) + R_{H_2O}{}^0/[E] \quad (6)$$

Here k_B^{obs} is the observed maximal rate constant for the release of $H_2{}^{18}O$ to bulk water caused by the addition of the buffer. K_{eff}^{B} is an apparent binding constant of the buffer to the enzyme, [E] and [B] are the concentrations of total enzyme and total buffer, and $R_{H_2O}{}^0$ is the rate of release of $H_2{}^{18}O$ into solvent water in the absence of buffer and represents the contribution to proton transfer from other sites on the enzyme or possibly solvent water itself.

The pH dependence of k_B^{obs} is often bell shaped, consistent with the transfer of a proton from a single predominant donor to the zinc-bound hydroxide. In these cases the pH profile is adequately fit by eq 7, in which k_B is a maximal, pH-independent rate constant for proton transfer and the values of K_a represent noninteracting ionization constants of the proton donor and acceptor.

$$k_B^{obs} = k_B/\{(1 + K_{a(donor)}/[H^+])(1 + [H^+]/K_{a(ZnH2O)})\} \quad (7)$$

Stopped-Flow Spectrophotometry. Initial rates of CO_2 hydration were measured by the changing pH indicator method of Khalifah[24] using an Applied Photophysics SX.18MV or a KinTek Stopped-Flow SF-2001. Saturated solutions of CO_2 were prepared by bubbling CO_2 into water at 25 °C. Concentrations of CO_2 (0.5 to 17 mM) were made by diluting this saturated solution using syringes with gastight seals. Various concentrations of the buffer 4-methylimidazole were used with the indicator phenol red (2.0×10^{-5} M) measured at 557 nm. The total ionic strength of solution was maintained at 0.2 M by addition of the appropriate amount of Na_2SO_4. The mean of four to eight reaction traces of the first 5 to 10% of the reaction was used to determine initial rates. The uncatalyzed rates were subtracted, and the steady-state constants k_{cat}/K_m and k_{cat} were determined by a nonlinear least-squares method (Enzfitter, Elsevier−Biosoft).

pH Measurements. All measurements are uncorrected pH meter readings. This was done to allow a partial cancellation of two factors: the correction required of a pH meter to account for the solvent D_2O (pD = meter reading +0.4)[25] and the change in pK_a for almost all acids in the region of pK_a from 3 and 10 ($pK_{a(D_2O)} - pK_{a(H_2O)} \approx 0.5$).[26]

Results

Human Carbonic Anhydrase II (HCA II). $R_{H_2O}/[E]$ catalyzed by H64A Co(II)-HCA II has a hyperbolic dependence on total substrate concentration (Figure 1, top). This is in contrast to catalysis by H64A Zn(II)-HCA II in which $R_{H_2O}/[E]$ appears rather independent of total substrate concentration, although there is a small dependence at total substrate less than 25 mM (Figure 1, top). $R_1/[E]$, a rate constant for interconversion of

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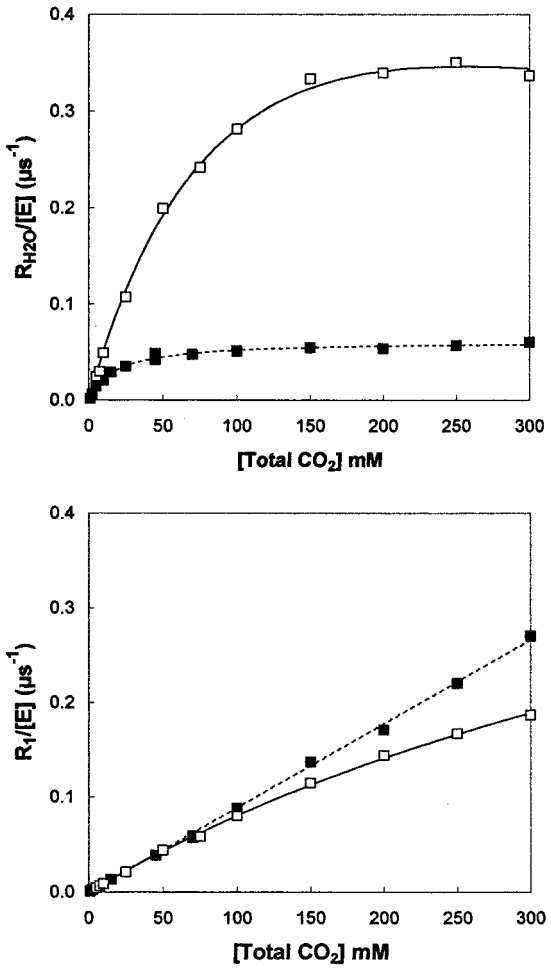

Figure 1. The dependence on the total concentration of all CO_2 species ($[CO_2] + [HCO_3^-] + [CO_3^{2-}]$) of (top) R_{H_2O}/[E], a rate constant for release of $H_2{}^{18}O$ from the enzyme, and (bottom) R_1/[E], a rate constant for the interconversion of CO_2 and HCO_3^- catalyzed by (■) H64A Zn(II)-HCA II and by (□) H64A Co(II)-HCA II. Data were obtained at pH 8.5 and 25 °C with a total ionic strength of solution maintained at a minimum of 0.2 M with Na_2SO_4. [E] is the total enzyme concentration. No buffers were added. The solid line for R_{H_2O}/[E] catalyzed by H64A Co(II)-HCA II is a least-squares fit of eq 6 with $K_{eff}{}^{B} = 64 \pm 9$ mM.

CO_2 and bicarbonate, shows a nearly linear dependence on total concentration of CO_2 species over this range of concentrations and is close to identical for the Co(II)- and the Zn(II)-containing H64A HCA II (Figure 1, bottom). The nearly linear dependence of R_1/[E] up to 300 mM total substrate indicates that for both enzymes the value of $K_{eff}{}^{S}$ of eq 5 is greater than about 200 mM.

The pH dependence of R_{H_2O}/[E] for H64A Co(II)-HCA II, measured at a total concentration of all CO_2 species of 150 mM (in the plateau region of Figure 1), was bell shaped with a maximum at pH between 7.8 and 8.5 (Figure 2). These data could be fit to eq 7 describing proton donation by one predominant group, giving the values of the pK_a of the donor and acceptor groups, but it is not possible from the data of Figure 2 to assign them specifically. Thus, a rate constant for proton transfer, k_B (eq 7), near 9×10^5 s^{-1} is obtained when the pK_a of 7.8 determined from the data is assigned to the zinc-bound water and the pK_a of 8.4 is assigned to the donor group. If these assignments are reversed, the value of k_B is 3.5×10^6 s^{-1}.

R_{H_2O}/[E] and R_1/[E] for catalysis by H64A Co(II)-HCA II were linear functions of the atom fraction of deuterium in solvent

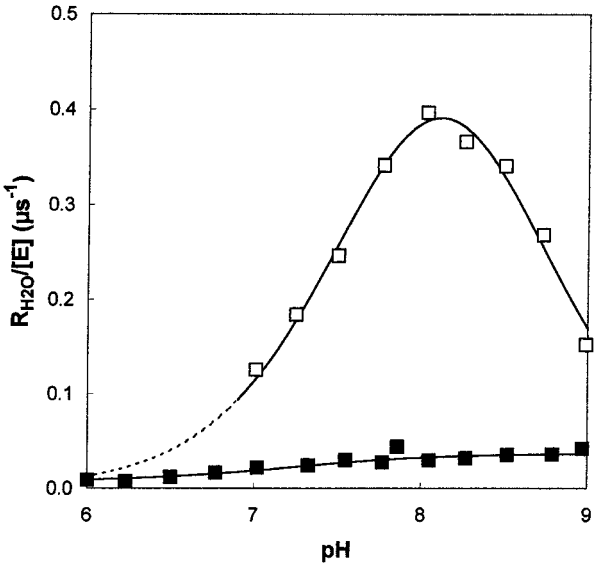

Figure 2. The pH profile for R_{H_2O}/[E], the rate constant for proton transfer dependent release of $H_2{}^{18}O$ from the enzyme as in eq 4, catalyzed by (□) H64A Co(II)-HCA II at a total concentration of all CO_2 species at 150 mM and by (■) H64A Zn(II)-HCA II with a total concentration of all CO_2 species at 25 mM. Data were obtained at 25 °C with a total ionic strength of solution maintained at a minimum of 0.2 M with Na_2SO_4. [E] is the total enzyme concentration. No buffers were added. The solid line for R_{H_2O}/[E] catalyzed by H64A Co(II)-HCA II is a least-squares fit of eq 7 resulting in two values of pK_a at 7.8 ± 0.1 and 8.4 ± 0.1 with $k_B = (8.8 \pm 1.8) \times 10^5$ s^{-1} when the pK_a of the zinc-bound water is taken as 7.8. When the assignments of pK_a are reversed, then $k_B = (3.5 \pm 1.3) \times 10^6$ s^{-1}. No measurements for the Co(II)-containing enzyme were taken at pH <7 because the solubility of CO_2 was exceeded.

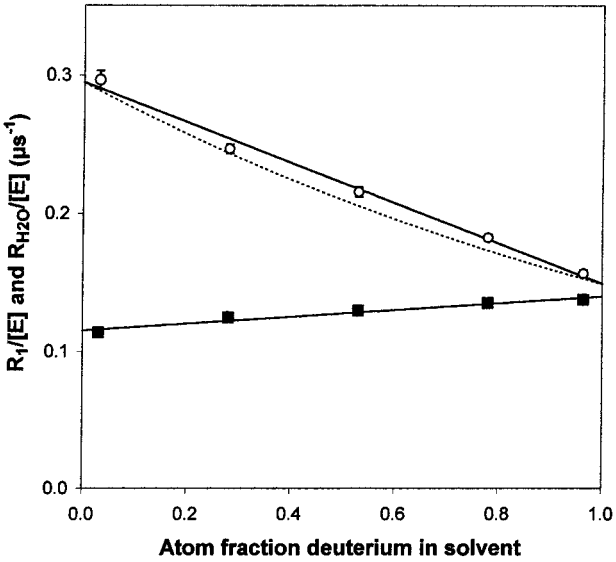

Figure 3. The variation with the atom fraction of deuterium in solvent of (°) R_{H_2O}/[E] and (■) R_1/[E] catalyzed by H64A Co(II)-HCA II. The data are the mean and standard deviations of three or four experiments. The uncorrected pH meter reading was 8.5, no buffers were used, the total concentration of all species of CO_2 was 150 mM, and the temperature was 25 °C. The solid and dotted lines represent fits of the Gross−Butler equation to the data assuming one proton and two or more protons in motion, as described in the text.

(Figure 3; uncorrected pH meter reading of 8.5). These data were obtained near the maximum in the bell-shaped pH profile of the rate constant R_{H_2O} (Figure 2). For the catalysis by H64A Co(II)-HCA II shown in Figure 3, the solvent hydrogen isotope

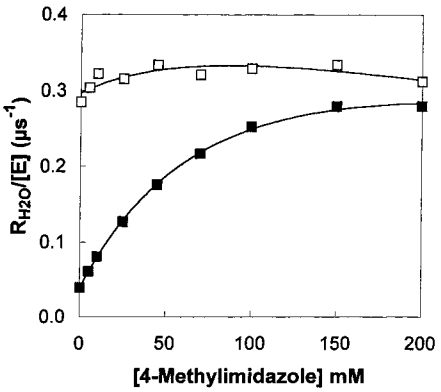

Figure 4. The dependence on the concentration of 4-methylimidazole of R_{H_2O}/[E] catalyzed by (■) H64A Zn(II)-HCA II and by (□) H64A Co(II)-HCA II. Data were obtained at pH 7.8 and 25 °C with solutions containing a 150 mM concentration of all species of CO_2 and with the total ionic strength of solution maintained at a minimum of 0.2 M with Na_2SO_4.

effect (SHIE) for the maximal value of R_{H_2O}/[E] was 2.0 ± 0.1. The SHIE for R_1/[E] catalyzed by this Co(II)-substituted variant was 0.83 ± 0.05.

Figure 4 demonstrates the effect of the exogenous proton donor 4-methylimidazole on R_{H_2O}/[E] for the Zn(II)- and Co(II)-containing mutants at pH 7.8 and in the presence of 150 mM total concentration of species of CO_2. H64A Zn(II)-HCA II showed considerable enhancement upon increasing the concentration of 4-methylimidazole, very typical of the chemical rescue effect observed with mutants of carbonic anhydrase lacking the proton shuttle residue His 64.[10] In contrast, R_{H_2O}/[E] catalyzed by H64A Co(II)-HCA II was greater than that for the Zn(II)-containing enzyme and showed no significant increase upon increasing concentration of 4-methylimidazole. There was no enhancement of R_1/[E] for H64A Co(II)-HCA II upon increasing concentrations of 4-methylimidazole, but there was a small decrease or inhibition with an apparent inhibition constant near 150 mM (data not shown).

In contrast to these results on catalyzed ^{18}O exchange at chemical equilibrium, the response in the catalysis of CO_2 hydration under initial velocity conditions to the exogenous proton shuttle 4-methylimidazole was similar for both Zn(II)- and Co(II)-containing H64A HCAII (Figure 5). The enhancement of k_{cat} for hydration in these cases is about 2- to 4-fold measuring from 5 mM 4-methylimidazole to approaching saturation. However, it is apparent from the enhancements that the overall rescue effect would be greater if we had a value for k_{cat} at very low buffer concentration. This effect of 4-methylimidazole on k_{cat} which causes a similar activation for catalysis by both the Co(II)- and Zn(II)-containing enzymes is a reflection of the initial velocity conditions in the hydration direction before the accumulation of appreciable product bicarbonate.

Carbonic Anhydrase from M. thermophila (Cam). Figure 6 shows the substrate dependence of R_{H_2O} and R_1 determined from the catalysis of ^{18}O exchange by Cam at pH 8.5. The rate constant R_{H_2O}/[E] is enhanced appreciably with increasing concentrations of total substrate and in this aspect resembles catalysis of ^{18}O exchange by H64A Co(II)-HCA II. The pH profile of R_{H_2O}/[E] at high substrate concentration during catalysis by Cam is bell-shaped indicating the presence of one or more prominent proton donors of pK_a either 6.8 or 8.9, as described in the legend to Figure 7. There was no increase in R_{H_2O} catalyzed by Cam upon increasing the concentration of 4-methylimidazole up to 200 mM. However, k_{cat} for CO_2 hydration catalyzed by Cam was increased about 4-fold to a value near 8×10^4 s^{-1} by addition of 4-methylimidazole from 2.5 mM reaching a plateau at about 25 mM of this donor (data not shown).

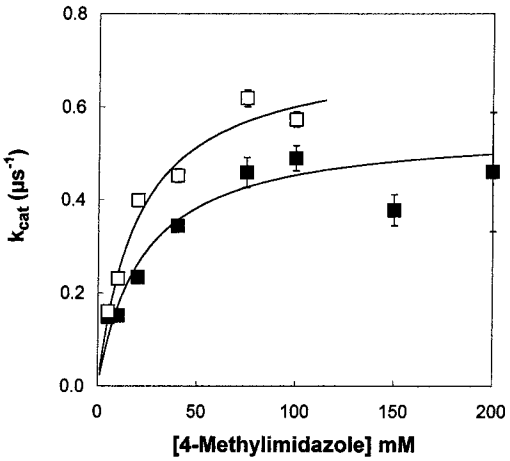

Figure 5. The steady-state turnover number k_{cat} in catalysis of the hydration of CO_2 by (■) H64A Zn(II)-HCA II and by (□) Co(II)-H64A HCA II plotted versus the concentration of the exogenous proton donor 4-methylimidazole at pH 7.7. Data were obtained by stopped-flow spectrophotometry at 25 °C and with the total ionic strength of solution maintained at a minimum of 0.2 M with Na_2SO_4.

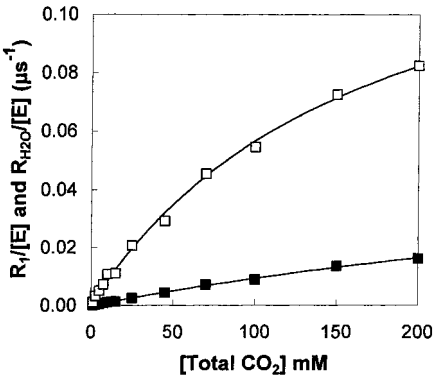

Figure 6. The dependence on the total concentration of all CO_2 species of (□) R_{H_2O}/[E] for ^{18}O exchange and (■) R_1/[E] for the interconversion of CO_2 and HCO_3^- catalyzed by Cam. Data were obtained at pH 8.5 and 25 °C with the total ionic strength of solution maintained at a minimum of 0.2 M with Na_2SO_4. [E] is the total enzyme concentration. No buffers were added.

Discussion

There is a strong similarity in the enhancement of the rate of release of labeled water from the active site of H64A Co(II)-HCA II caused by increasing concentration of CO_2 species (Figure 1) and the enhancement caused by exogenous proton donors such as imidazole and derivatives.[10] This suggests that a component of the total substrate concentration is acting as a proton donor to H64A Co(II)-HCA II to a much greater extent than to its Zn(II)-containing counterpart (Figure 1). The proton donor to which this effect is attributed is surely HCO_3^- since carbonic acid, H_2CO_3, is present in only very small amounts (about 0.1% of CO_2)[27] and is not present at sufficient concentrations to donate protons at the catalytic rates measured here. These considerations suggest that for the Co(II)-substituted enzyme, bicarbonate is a source of protons that enhances ^{18}O exchange and R_{H_2O}. Such a transfer of a proton between

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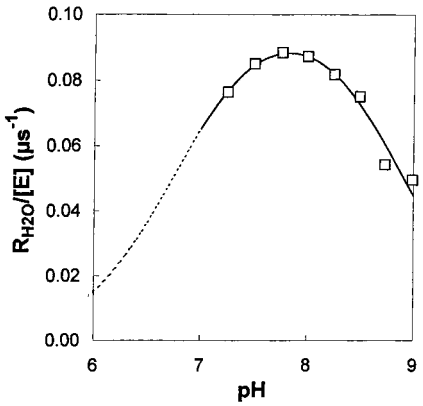


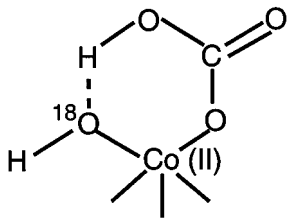
Figure 7. The pH profile for R_{H_2O}/[E], the rate constant for proton transfer dependent release of $H_2{}^{18}O$ from the Cam as in eq 4. Data were obtained at 25 °C with the total concentration of all species of CO_2 at 200 mM and the total ionic strength of solution maintained at a minimum of 0.2 M with Na_2SO_4. [E] is the total enzyme concentration. No buffers were added. The solid line is a least-squares fit of eq 7 resulting in two values of pK_a at 6.8 ± 0.1 and 8.9 ± 0.1 with k_B = (1.0 ± 0.1) × 10^5 s^{-1} when the pK_a of the zinc-bound water is taken as 6.8; when the pK_a of the zinc-bound water is taken as 8.9, the k_B = 1.4 × 10^7 s^{-1}. No measurements were taken at pH <7 because the solubility of CO_2 was exceeded.

bicarbonate and the cobalt-bound labeled hydroxide, as in eq 4, would result in the formation of $H_2{}^{18}O$ which exchanges rapidly with unlabeled bulk water.

The crystal structure of Co(II)-HCA II is nearly identical with that of the native zinc-containing enzyme; both show a coordination about the metal that is very nearly tetrahedral with three histidine ligands and a fourth aqueous ligand. The root-mean-square deviation for Cα atoms is 0.09 Å in comparing these two structures.[28,29] Hence, we anticipate no significant difference in the structures of H64A Co(II)-HCA II and H64A Zn(II)-HCA II predominating at equilibrium. This suggests that the appreciably enhanced proton shuttle mechanism in H64A Co(II)-HCA II at chemical equilibrium is related to the metal itself, and suggests a mechanism of enhancement of R_{H_2O} by proton transfer from cobalt-bound bicarbonate. It is unlikely that there is a binding site at other locations in the active-site cavity from which bicarbonate could influence R_{H_2O}, or it would also be present in H64A Zn(II)-HCA II.

The solvent hydrogen isotope effect (SHIE) on R_{H_2O}/[E] (2.0 ± 0.1) (Figure 3) provides support that the effect on R_{H_2O} of increased concentration of CO_2 species is at least in part due to proton transfer. Moreover, the proton inventory of Figure 3 shows that R_{H_2O} is consistent with a linear dependence on the atom fraction of deuterium in solvent. Such a linear dependence suggests changes in the fractionation factor of one hydrogen in the transition state of the rate-limiting step. This linear dependence is in contrast to the logarithmic dependence of such a plot for k_{cat} catalyzed by HCA II.[17,30] The dotted line of Figure 3 represents the dependence of R_{H_2O}/[E] calculated from the Gross−Butler equation[31] assuming a logarthmic dependence on the atom fraction of deuterium in solvent with the same overall SHIE on R_{H_2O}/[E]. For wild-type HCA II, the logarithmic dependence was interpreted to indicate changes in bonding to two or more hydrogens in the transition state of the second stage

Chart 1



of catalysis (eq 2), consistent with proton transport through a hydrogen-bonded water chain connecting the zinc-bound water with the imidazole ring of His 64 which is located at a distance of about 7 Å from the zinc.[30] We have sufficient precision to comment that the data for H64A Co(II)-HCA II are not adequately fit to a logarithmic dependence on deuterium content, as demonstrated by comparing the solid and dashed lines in Figure 3, but are consistent with a linear dependence suggesting the possibility of the transfer of a single proton.

In addition, whereas ^{18}O exchange catalyzed by Zn(II)-H64A HCA II is enhanced by the exogenous proton donor 4-methylimidazole, H64A Co(II)-HCA II shows no significant enhancement (Figure 4). This is presumably because H64A Co(II)-HCA II already has a maximal proton shuttle mechanism from another source. Measured at steady state, both the Zn(II)- and Co(II)-containing enzymes are activated to nearly the same extent in the CO_2 hydration direction by the external proton acceptor 4-methylimidazole (Figure 5). These data are also consistent with the suggestion that bicarbonate is a source of protons in the ^{18}O exchange experiment at chemical equilibrium, but not under initial velocity conditions in the hydration of CO_2 for which the concentration of HCO_3^- is minimal.

We therefore suggest that H64A Co(II)-HCA II is enhanced in catalysis of ^{18}O exchange by a mechanism in which HCO_3^- binds to the metal and transfers a proton directly to the cobalt-bound hydroxide, as in Chart 1. The data suggest the presence of a proton transfer between ligands of the metal in the enzyme: $M(HCO_3^-)(^{18}OH^-) \rightleftarrows M(CO_3^{2-})(H_2{}^{18}O)$, where M is cobalt. A mechanism similar to Chart 1 in which cobalt-bound water donates a proton to cobalt-bound hydroxide provides an explanation for the enhanced relaxation rate of the proton magnetic resonance of water in the presence of Co(II)-containing carbonic anhydrase.[32] Sträter et al.[33] have shown that bicarbonate bound to an arginine residue of leucine aminopeptidase acts as a general base in catalysis facilitating proton transfer from a zinc-bridging water to product.

In Chart 1, we rely on the well-known property of the carbonic anhydrases in the α class to expand their coordination number about the zinc or cobalt to accommodate additional ligands.[34,35] The visible and near-infrared spectra of the Co(II)-carbonic anhydrases are particularly rich in evidence of the variation of coordination geometries about the cobalt.[35] These spectra show a range of absorbance that reflects four- and five-coordination as well as intermediate states upon anion binding. The crystal structure of the SCN^- complex with Zn(II)-HCA II shows that the coordination geometry about the metal has expanded to include both a thiocyanate and a water molecule in an approximately square pyramidal coordination.[36] The crystal structure of the bicarbonate complex with Co(II)-

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substituted HCA II shows the coordination of two oxygens of the bicarbonate and the oxygen of a water molecule, all found within 2.3 to 2.5 Å from the cobalt;[29] moreover, the structure of T200H Zn(II)-HCA II also shows what the authors describe as "pseudo bidentate" coordination of bicarbonate to the metal with oxygen−metal distances of 2.2 and 2.5 Å from the zinc.[37] However, we suggest proton transfer in Chart 1 as the species that pertains in the ^{18}O-exchange experiments, and that bicarbonate bound to the metal is monodentate allowing proton transfer to the metal-bound hydroxide. It is likely that proton transfer from bicarbonate is more efficient for the Co-bound than the Zn-bound mutant because expansion of coordination about the cobalt favors the structure of Chart 1 to a greater extent than for zinc.

We assume from the data of Figure 2 that the pK_a of the cobalt-bound water is 7.8 and that of the cobalt-bound bicarbonate is 8.4. For unbound bicarbonate in solution the pK_a is 9.8.[27] The proton transfer appears from Figure 2 to be efficient, with a value of the rate constant k_B from eq 7 for proton transfer from bound bicarbonate to the zinc-bound hydroxide (pK_a of conjugate acid 7.8) of $(8.8 \pm 1.8) \times 10^5$ s^{-1}. This is an efficient proton transfer comparable to the rate constant near 10^6 s^{-1} estimated for the contribution of His 64 to proton transfer in wild-type HCA II.[1,16,17] We point out that we cannot firmly assign the pK_a of 7.8 to the cobalt-bound water of Chart 1; if this is the pK_a of the bound bicarbonate and 8.4 is the pK_a of the cobalt-bound water, then k_B is $(3.5 \pm 1.3) \times 10^6$ s^{-1}. The pK_a of the cobalt-bound water in the absence of bicarbonate is near 6.9.[38] Using eq 6, an apparent second-order rate constant k_B^{obs}/K_{eff}^B of 7×10^6 M^{-1} s^{-1} describes the bicarbonate activation of R_{H_2O}/[E] for H64A Co(II)-HCA II in Figure 1. This is somewhat less than the diffusion-controlled value near 10^9 M^{-1} s^{-1}, but still indicates a very efficient proton transfer. In general, dianions do not bind at the zinc in carbonic anhydrase, although oxalate has been found to bind very weakly.[39] The metal-bound carbonate, a dianion resulting from the proton transfer of Chart 1, might not need reprotonation to dissociate readily from the active site, or it might become protonated and dissociate as bicarbonate or proceed in catalysis in the dehydration direction. The enhancement of R_{H_2O} by total substrate for H64A Co(II)-HCA II at pH 8.5 shows an approach to saturation with a very weak binding constant for bicarbonate that is estimated from Figure 1 to be near 64 mM.

Carbonic Anhydrase from M. thermophila (Cam). *C*atalysis by Cam has aspects similar to those observed for Co(II)-substituted H64A HCA II, specifically the enhancement of R_{H_2O} upon increasing substrate concentration (Figure 6). This also suggests enhancement of catalysis by bicarbonate binding to Cam, possibly to the zinc of Cam. This explanation is consistent with the coordination geometry about the zinc in Cam which is pentacoordinate, with two solvent ligands of the metal,[13] because it suggests that hydroxide and bicarbonate can bind simultaneously at the metal, as in Chart 1. In addition, the crystal structure shows that in binding to Cam bicarbonate displaces two water ligands and forms a bidentate complex with the zinc.[13] It is possible therefore that ^{18}O is exchanged from the active site to solution by a mechanism equivalent to Chart 1 but with zinc. These data also provide an explanation for the exchange of ^{18}O catalyzed by Cam as first reported by Alber et al.;[11] a substantial contribution of R_{H_2O} in that case is due to activation by bicarbonate.

Also like Co(II)-substituted H64A HCA II, there was no activation of R_{H_2O} caused by increasing concentrations of the exogenous proton donor 4-methylimidazole (data not shown); however, there is substantial activation of k_{cat} for CO_2 hydration measured under initial velocity conditions for a number of buffers,[11,12] including 4-methylimidazole (data not shown). The pH profile for R_{H_2O}/[E] catalyzed by Cam in Figure 7 is consistent with a single predominant proton donor with a pK_a of either 6.9 or 8.9. Assuming that the pK_a of the zinc-bound water is 6.9 and the pK_a of zinc-bound bicarbonate is 8.9 results in a rate constant for proton transfer between bound bicarbonate and the zinc-bound hydroxide of 1.0×10^5 s^{-1}.

It is useful to address the question of whether in living cells bicarbonate enhances carbonic anhydrase catalysis by acting as a proton donor by the mechanism of Chart 1 and eq 2 in which BH^+ would designate bicarbonate. This is less likely in human and animal cells in which bicarbonate concentrations are near 25 mM than in archaea in which there is rapid intracellular production of carbon dioxide that could result in much higher bicarbonate concentrations.[40] In these cases, bicarbonate may contribute to overall dehydration activity by acting as a proton donor as suggested for wild-type Cam. Moreover, this effect could be a significant contributor to catalysis by homologues of Cam lacking Glu84 as a proton shuttle residue.[41] This mechanism for enhancement of catalysis by bicarbonate will have a much smaller effect on CO_2 hydration catalyzed by carbonic anhydrase because catalysis in this direction requires transfer of protons from enzyme to solution and bicarbonate is a poor proton acceptor.

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