The Catalytic Mechanism of Carbonic Anhydrase: Implications of a Rate-Limiting Protolysis of Water

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The reaction catalyzed by the zinc metalloenzyme carbonic anhydrase (EC 4.2.1.1) is usually written in biochemistry texts as

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

This is a very simple enzymic reaction involving only six substrate atoms and, at least formally, the transfer of an OH^- moiety from H_2O to CO_2. Extensive kinetic studies in our laboratories have led to a model for the mechanism of carbonic anhydrase catalyzed CO_2 hydration, implying that the rate-limiting,[1] elementary step is, in effect, the protolysis of water

$$H_2O \rightleftharpoons OH^- + H^+ \quad (2)$$

rather than the formation of a new carbon–oxygen bond in HCO_3^-. In this Account we summarize the experimental evidence supporting this rather unconventional model and describe some unorthodox consequences of the mechanism.

Carbonic anhydrase is a ubiquitous enzyme involved in the transport of CO_2 between metabolizing tissues and the lungs, in many secretory processes such as the formation of ocular fluid, in the calcification of shells of birds, and in photosynthesis. Three genetically and immunologically distinct, but structurally homologous, cytosolic isozymes of carbonic anhydrase are known to occur in higher vertebrates. They have different kinetic and ligand-binding properties. As shown in Table I, isozyme II is an extraordinarily efficient catalyst which seems to fulfill the criteria for a "perfectly evolved" enzyme.[2] Isozyme I, which occurs together with isozyme II in red blood cells, is less efficient, and the muscle-specific isozyme III has an even lower activity. Nevertheless, the basic features of the catalytic mechanism appear to be common to all three forms.

Carbonic Anhydrase II: The Zinc Hydroxide Mechanism

The catalytic zinc ion is located near the center of the enzyme molecule close to the bottom of a 15-Å-deep active site cavity (Figure 1). One part of the cavity is dominated by hydrophobic amino acid side chains, whereas another part has mostly a hydrophilic character. The zinc ion is coordinated to three imidazole groups from His-94, His-96, and His-119. A fourth ligand, completing a nearly symmetrical, tetrahedral coordination geometry, has been postulated to be a water molecule. As discussed in more detail below, this is thought to be the water molecule participating in the catalytic reaction.

Table I.
Maximal Values of the Steady-State Constants for the Hydration of CO_2 Catalyzed by Isozymes of Carbonic Anhydrase

	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}	ref
isozyme I (human)	2×10^5	5×10^7	50
isozyme II (human)	1.4×10^6	1.5×10^8	50
isozyme III (feline)	1×10^4	3×10^5	42

For the hydration of CO_2 catalyzed by carbonic anhydrase II, both k_{cat} and k_{cat}/K_m[3] have pH profiles approximated by a simple titration curve with a pK_a near 7 and a maximal activity at high pH, whereas K_m is independent of pH (Figure 2). Early studies showed that the pH dependence of the CO_2 hydration activity parallels the interconversion between an acidic and a basic form of the visible absorption spectrum of the catalytically active, Co^{2+}-substituted enzyme.[4] While the chemical nature of this activity-linked and metal-linked ionizable group was long a subject of controversy,[5,6] the number of experiments suggesting metal-bound H_2O/OH^- (as well as the lack of plausible alternatives) is so persuasive that this interpretation is

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(1) We define rate-limiting step according to W. J. Ray (*Biochemistry* **1983**, *22*, 4625) as that forward step in the reaction sequence for which a change in its rate constant produces the largest effect on the overall rate. This review describes the rate-limiting step for the hydration of CO_2 catalyzed by carbonic anhydrase II under conditions of maximum velocity. For this maximum velocity at pH greater than 7, the "rate-limiting step", the "most sensitive step", and the "least conductive step" as defined by Ray are the same, an intramolecular proton transfer. This is also the forward step with the smallest rate constant.

(2) Knowles, J. R.; Albery, W. J. *Acc. Chem. Res.* **1977**, *10*, 105.

(3) The catalysis of the hydration of CO_2 by carbonic anhydrase II has been found to obey simple Michaelis–Menten kinetics with the initial velocity of the catalyzed hydration given by $v = k_{cat}[S](E_0)/(K_m + [S])$. Here S is the substrate which is CO_2 for the hydration reaction. The maximal velocity is $V_{max} = k_{cat}(E_0)$ in which k_{cat} is the turnover number. The ratio k_{cat}/K_m is a lower limit for the second-order rate constant for the combination of CO_2 and enzyme in catalysis, and it also describes the catalysis at CO_2 concentrations small compared with K_m.

(4) Lindskog, S. *Biochemistry* **1966**, *5*, 2641.

(5) Lindskog, S.; Coleman, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2505.

(6) Lindskog, S. *Adv. Inorg. Biochem.* **1982**, *4*, 115.

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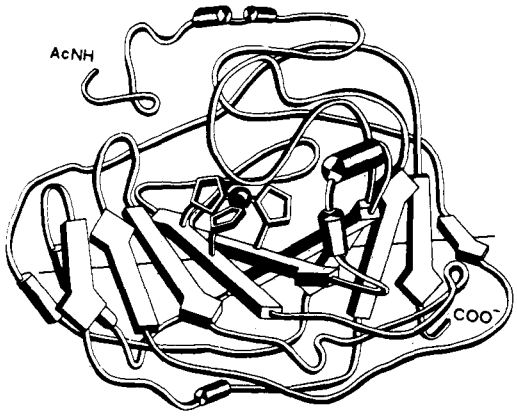

Figure 1. A simplified drawing of the backbone structure of the 259 amino acid residues in human carbonic anhydrase II showing the zinc ion coordinated to the imidazole side chains of three histidine residues. The arrows represent pleated sheet strands pointing in the direction from the amino to the carboxyl end, and the cylinders represent helical regions. Reprinted with permission from ref 51. Copyright 1972 Macmillan Journals Limited.

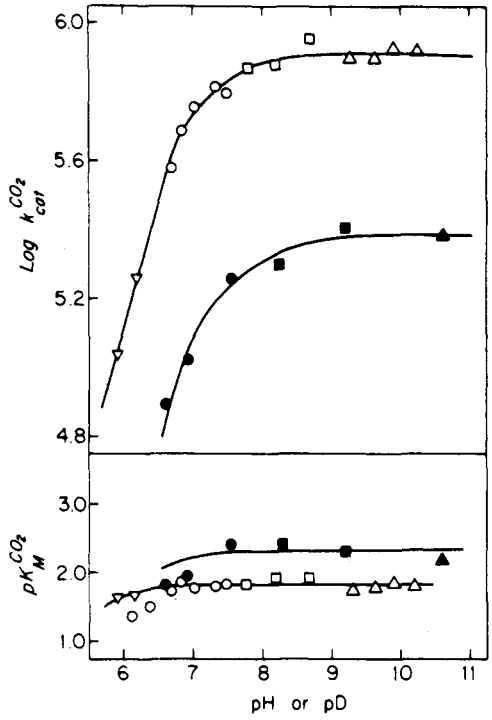

Figure 2. The pH dependence of k_{cat} and K_m for the hydration of CO_2 catalyzed by bovine red cell carbonic anhydrase II in H_2O (open symbols) and D_2O (filled symbols). The temperature was 25 °C, and the ionic strength was maintained at 0.10 M with Na_2SO_4. The following buffers were present at concentrations between 0.02 and 0.05 M: (▽) 3-picoline, (○) phosphate, (□) 1,2-dimethylimidazole, and (△) *N,N*-dimethylglycine. Reproduced from ref 17.

now generally accepted. A simplified model of the catalytic mechanism is shown in eq 3 and 4. In this

$$EZnOH^- + CO_2 \rightleftharpoons EZn(OH^-)CO_2 \rightleftharpoons EZnHCO_3^- \rightleftharpoons EZnH_2O + HCO_3^- \quad (3)$$

$$EZnH_2O \rightleftharpoons EZnOH^- + H^+ \quad (4)$$

mechanism, which involves a direct nucleophilic attack of zinc-bound OH^- on CO_2, the interconversion between CO_2 and HCO_3^- is temporally separated from the release of a proton to the reaction medium. This is referred to as a Ping-Pong scheme. The support for this

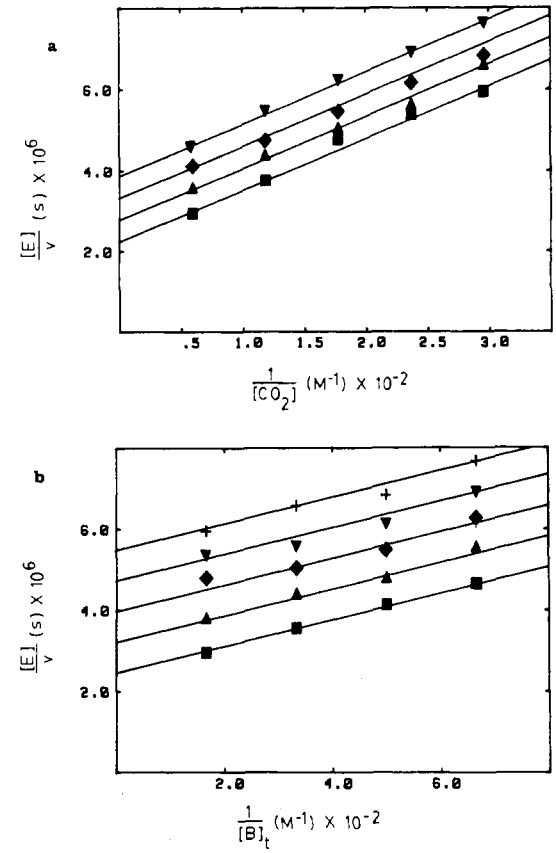

Figure 3. Two double-reciprocal plots showing the parallel patterns characteristic of Ping-Pong mechanisms for both CO_2 (top) and buffer (bottom) as substrates. The initial velocity of catalyzed hydration of CO_2 was measured by stopped flow using a changing pH indicator method; $[B]_t$ is the total concentration of buffer. Human red cell carbonic anhydrase II was present at 69 nM, and ionic strength was maintained at 0.2 M with Na_2SO_4. Temperature was 25 °C and pH 8.5. (top) [1,2-dimethylimidazole] = 6.0 mM (■), 3.0 mM (▲), 2.0 mM (◆), 1.5 mM (▼). (bottom) Replot of the same data with B = 1,2-dimethylimidazole, $[CO_2]$ = 17 mM (■), 8.5 mM (▲), 5.6 mM (◆), 4.2 mM (▼), 3.4 mM (+). Reproduced from ref 13.

mechanistic hypothesis is extensive and is the topic of this review.

Intermolecular Proton Transfer

The formulation of the release of H^+ from the active site of isozyme II to solution as in eq 4 presents a dilemma because the maximal rate constant for the transfer of a proton from a catalytic group of $pK_a = 7$ to bulk water is about 10^3 s^{-1} (assuming a diffusion-limited rate constant of 10^{10} M^{-1} s^{-1} for the recombination of H^+ with the basic form of the catalytic group), a thousandfold slower than the maximal turnover number for hydration of CO_2 (see Table I). Moreover, hydroxide ion, which is a good proton acceptor, is present at too small a concentration in solution at physiological pH to provide an explanation of the large catalytic turnover. A general solution to the problem was provided by Alberty[7] and Eigen and Hammes,[8] who suggested that buffers in solution are involved in such a catalysis since they are much better proton acceptors than H_2O and much more concentrated in most kinetic studies at physiological pH than hydroxide ion. This suggestion was applied specifically to carbonic anhy-

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(8) Eigen, M.; Hammes, G. G. *Adv. Enzymol.* **1963**, *25*, 1.

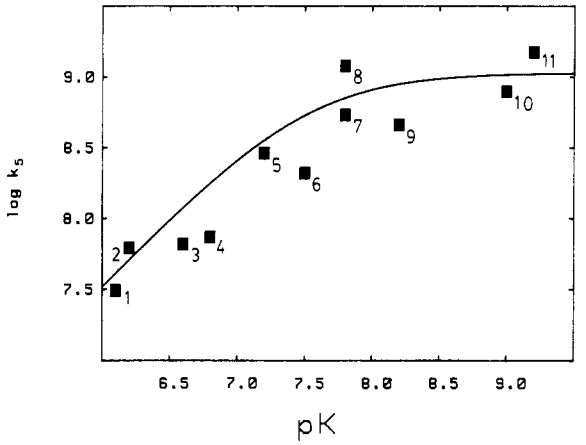

Figure 4. The variation of the logarithm of k_5 of eq 5 showing a dependence on the pK_a of the external buffers as proton acceptors very similar to the plots of proton transfer between small molecules described by Eigen.[25] The external buffers are (1) Mes, (2) 3,5-lutidine, (3) 3,4-lutidine, (4) 2,4-lutidine, (5) 1-methylimidazole, (6) Hepes, (7) triethanolamine, (8) 4-methylimidazole, (9) 1,2-dimethylimidazole, (10) Ted, and (11) Ches. The curve drawn through the points was calculated for $k_5 = 1.1 \times 10^9$ M^{-1} s^{-1} and a pK_a for the donor group on the enzyme of 7.6. Reproduced from ref 13.

drase in 1973[5,9,10] and was subsequently verified by experiments that showed a decrease in the catalyzed, initial velocity of hydration of CO_2[11] (Figure 3) and a decrease in the catalyzed exchange of ^{18}O between CO_2 and H_2O[12] as the buffer concentration was decreased below 10 mM.

Thus, buffers in solution are able to participate as proton-transfer agents in the hydration of CO_2 catalyzed by carbonic anhydrase II, and eq 4 for this isozyme contributes much less to the catalysis than eq 5:

$$EZnH_2O + \text{buffer} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EZnOH^- + \text{bufferH}^+ \quad (5)$$

The initial velocity patterns obtained with varying buffer concentrations (Figure 3) are consistent with the Ping-Pong mechanism of eq 3 and 5 and verify the initial hypothesis that the intermolecular proton transfer occurs in a step separate from the interconversion of CO_2 and HCO_3^-.[11-13] In addition, ^{13}C NMR measurements of rates of the interconversion between CO_2 and HCO_3^- at chemical equilibrium showed no buffer effect[14] in accordance with the Ping-Pong model. A Brønsted plot of values of k_5 obtained from the buffer concentration dependence of the rate of CO_2 hydration catalyzed by isozyme II is shown in Figure 4 and indicates that the interaction between enzyme and buffer has little structural specificity. When the pK_a of the buffer is greater than the pK_a of the catalytic group, k_5 appears independent of the pK_a value of the buffer with a value near 10^9 M^{-1} s^{-1}, indicating a diffusion-controlled process. When the pK_a values of the donor and acceptor are approximately equal, there is a transition leading to a region of the plot of slope equal to unity when the pK_a of the buffer is much less than that of the enzyme as donor. Analysis of the data in Figure 4 indicates a pK_a of the donor group on the enzyme of 7.6 ± 0.6;[13] this is consistent with similar data from Pocker et al.,[15] who also provide values for k_{-5} for proton transfer in the dehydration direction with the enzyme as proton acceptor.

Intramolecular Proton Transfer

We present now the hypothesis that the proton donor of pK_a near 7 in the experiments described above is not the zinc-bound water at all but another residue closer to the surface of the enzyme. This is an hypothesis that was initially made by Steiner et al.[16] to explain solvent hydrogen isotope effects in the catalysis and has since provided a framework in which to understand many aspects of the catalysis that we cover below, including the implications of a rate-limiting intramolecular proton transfer. Using sufficiently high buffer concentrations to ensure that intermolecular proton transfer is not rate-limiting, Steiner et al.[16] compared the Michaelis–Menten parameters for the hydration of CO_2 catalyzed by carbonic anhydrase II in ^1H_2O and ^2H_2O. Using human carbonic anhydrase II, they observed an isotope effect of 3.8 in k_{cat} for hydration and an isotope effect of unity in the ratio k_{cat}/K_m; using bovine carbonic anhydrase II, Pocker and Bjorkquist[17] made similar observations shown in Figure 2. In a sequential mechanism such as shown in eq 3 and 5 the ratio k_{cat}/K_m contains rate constants for steps from the initial encounter of substrate with enzyme through the first irreversible step. In these experiments, the first irreversible step is the departure from the enzyme of the product HCO_3^-.

Thus, the ratio k_{cat}/K_m contains rate constants for the processes given in eq 3 only, not in eq 4 and 5. The implication is that the steps in eq 3 do not involve a change in bonding to hydrogen in a rate-contributing step. This was later supported by similar results using entirely different kinetic methods. Techniques that measure the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium determined that this rate was the same when measured in ^1H_2O or ^2H_2O. The techniques measured exchange broadening of the ^{13}C resonances in CO_2 and HCO_3^-[14] and the exchange of ^{18}O between CO_2 and H_2O.[18] These results are the basis for the suggestion that the interconversion of CO_2 and HCO_3^- occurs by direct nucleophilic attack of zinc-bound hydroxide on CO_2 without rate-contributing proton transfer and not by a general base mechanism in which zinc-bound hydroxide abstracts a proton from an adjacent water while the incipient hydroxide reacts with CO_2.

A solvent hydrogen isotope effect of 3.8 on k_{cat} is large enough to indicate a primary intramolecular proton transfer in the catalysis, that is, the transfer of a proton in the transition state of a rate-contributing step. A rate-contributing intermolecular proton transfer is eliminated by the large concentrations of buffer used

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in the experiments (between 20 and 50 mM; see Figure 2). The parameter k_{cat} contains rate constants for steps from the initial enzyme–substrate complex through the remaining steps of the catalysis including the proton transfers. Since the steps in eq 3 have an overall isotope effect of unity, there is an intramolecular proton transfer involved in the steps of eq 5. This was the hypothesis of Steiner et al.,[16] who then suggested that the proton transfer so indicated was the protolysis of zinc-bound water by transfer of a proton to a nearby residue of the enzyme with a similar pK_a value near 7. Consideration of the known structure of carbonic anhydrase II from X-ray diffraction results[19] suggested His-64 as the foremost candidate for this nearby residue. It has a pK_a of 7.1 as determined by NMR[20] and is situated 6 Å from the zinc. From its position on

$$\text{His-64}\quad EZnH_2O \rightleftharpoons H^+\text{His-64}\quad EZnOH^- \qquad (6)$$

His-64, the proton is transferred to buffer in solution completing the catalytic cycle. Thus, His-64 appears to act as a proton shuttle in the mechanism of isozyme II, providing a facile pathway for release of H^+ to solution. Yet, the transfer of H^+ between the metal site and His-64 seems to be the "most difficult" step in the catalysis.

A number of other experiments support the hypothesis of a rate-limiting intramolecular proton transfer in the maximal velocity for hydration of CO_2 catalyzed by carbonic anhydrase II. Product inhibition by HCO_3^- of catalyzed hydration shows the presence of a kinetically significant isomerization step in the catalytic cycle.[21] Furthermore, it has been shown by ^{18}O exchange experiments[18] that ^{18}O abstracted from substrate HCO_3^- transiently labels the active site of carbonic anhydrase II. The pH profile and solvent isotope effect on the rate of release of ^{18}O from the active site to bulk water are consistent with a rate-limiting, intramolecular proton transfer between two groups of the enzyme both with values of pK_a near 7,[22] presumably His-64 and zinc-bound water. The rate constant for this proton transfer is 7×10^5 s^{-1}, similar to k_{cat} for catalyzed hydration. And finally, a thorough investigation of the solvent hydrogen isotope effect on k_{cat} for hydration showed that this steady-state constant has an exponential dependence on the atom fraction of deuterium in solvent water.[23] This observation requires a change in bonding to two or more hydrogens in the transition state or states that determine k_{cat}. If the hypothesis of an intramolecular proton transfer between His-64 and zinc-bound water is assumed, then this result strongly suggests proton transfer through intervening water bridges. This suggestion was later made more plausible by detailed interpretation of the refined crystal structure of carbonic anhydrase II.[24]

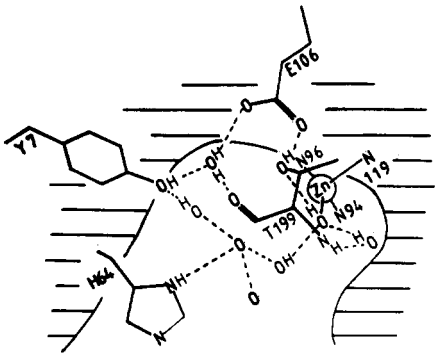

Figure 5. A schematic diagram of the active site of human carbonic anhydrase II as determined from the crystal structure. The zinc atom is shown with its ligands to the three histidine residues at positions 94, 96, and 119. The other residues shown are tyrosine-7, histidine-64, glutamate-106, and threonine-199. Also shown by the O's are the positions of the oxygens of water in the hydrogen-bond network near the zinc as determined from the electron densities of some of the active-site water molecules. Reprinted with permission from ref 24. Copyright 1986 Birkhäuser.

This structure revealed a number of ordered water molecules in the cavity between the zinc ion and the side chain of His-64, which forms a gate separating the exterior solution from the active site. The zinc ion is connected to His-64 by water bridges (Figure 5). Although a number of these ordered water molecules, as well as the amide group of Asn-62, are closer to side chain atoms of His-64 than van der Waals distances, the distances are longer than normal hydrogen bonds. This may indicate that the crystal structure shows an average position of the side chain of His-64 which is flipping between two or more positions and that only one of these is connected to the zinc-bound water through regular hydrogen bonds. The narrow line width of the ^1H NMR resonance of the C_2 proton of His-64 suggests that the side chain is rather mobile.[20] Such flipping of His-64 could explain why proton transfer between zinc-bound water and His-64 proceeds with a rate constant of 10^6 s^{-1} rather than 10^{13} s^{-1} or so expected for a rigid, ice-like array of water-bridged hydrogen bonds.[8,25]

Inhibition of the Proton Shuttle

The inhibition of the interconversion of CO_2 and HCO_3^- (eq 3) by sulfonamides and anions is known to occur by the binding of these inhibitors to the metal of carbonic anhydrase; in the case of sulfonamides the sulfonamido nitrogen is bound directly to the metal.[26] Specific inhibitors of the proton shuttle would be of significance in verifying the proposed mechanism and in demonstrating the properties of catalysis by carbonic anhydrase II when the enzyme must function without the hypothesized pathway for transfer of protons and from the active site.

In fact, such inhibitors of the proton shuttle are available in the Cu^{2+} and Hg^{2+} ions. Observation of inhibition by cupric and mercuric ions was made by measuring ^{18}O exchange. With this method, the rates of two steps in the catalytic pathway can be estimated.[18] The first is the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium, in principle the same

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(26) Evelhoch, J. L.; Bocian, D. F.; Sudmeier, J. L. *Biochemistry* **1981**, *20*, 4951.

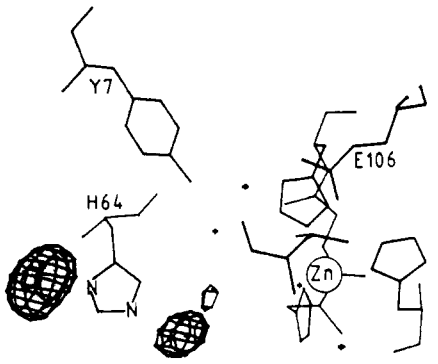

Figure 6. A diagram determined from X-ray diffraction data showing the binding of Hg^{2+} to the imidazole side chain of histidine-64 in human carbonic anhydrase II. The interpretation is that Hg^{2+} binds to either ND2 or NE2 of histidine-64. Kinetic studies showed that the binding of Hg^{2+} to isozyme II inhibits the proton shuttle pathway in the catalysis. Reprinted with permission from ref 24. Copyright 1986 Birkhäuser.

rate that can be calculated from the exchange broadening of the ^{13}C NMR resonances in CO_2 and HCO_3^- at chemical equilibrium.[14,18] The second, designated $R(H_2O)$, is the rate of release from the enzyme of H_2O bearing the oxygen abstracted from substrate bicarbonate. As discussed in the previous section, this process most likely involves a proton transfer between His-64 and the zinc site. Such a proton transfer converts $Zn^{18}OH^-$ into $ZnH_2{}^{18}O$ and facilitates the release of $H_2{}^{18}O$ to the solvent. Tu et al.[27] discovered that Cu^{2+} and Hg^{2+} ions at concentrations below 10^{-6} M were inhibitors of $R(H_2O)$ without having an effect on the rate of exchange between CO_2 and HCO_3^-. They suggested that this inhibition could be caused by the coordination of these metal ions to His-64, the proposed proton shuttle group. The implication is that with metal bound at the imidazole ring of His-64 this side chain would not function to transfer protons from the active site to solution. It was known that this binding did not influence zinc at the active site since the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium was not affected. Such a suggestion is consistent with the pH profile for the inhibition by Cu^{2+} [27] and with the noncompetitive binding interaction of Cu^{2+} and 5-(dimethylamino)naphthalene-1-sulfonamide (DNSA) on carbonic anhydrase II.[28] DNSA, as a sulfonamide, is expected to bind to the zinc of carbonic anhydrase, and the noncompetitive binding interaction is consistent with the binding of cupric ion at another site of the enzyme.

These suggestions were confirmed by Eriksson, Jones, and Liljas,[24] who were able to obtain crystals of the complex of carbonic anhydrase II with Hg^{2+} in solutions containing poly(ethylene glycol) and collected X-ray diffraction data to a resolution of 3.1 Å. The analysis showed a mixture of two complexes; half of the protein showed mercuric ion bound at one of the nitrogens (ND1) of the imidazole ring of His-64 and half with mercuric ion bound at the other nitrogen of His-64 (NE2) with no indication of chelation (Figure 6). Hence, this combination of structural with kinetic data provides a confirmation of the role of the side chain of His-64 as a proton shuttle in the pathway for the hydration of CO_2 catalyzed by carbonic anhydrase II.

Anion Inhibition

There is overwhelming spectroscopic and crystallographic evidence that anions such as NCO^-, N_3^-, and I^- that inhibit the CO_2 hydration activity of carbonic anhydrase bind to the metal ion either by displacing a coordinated water or by expansion of the coordination sphere.[4,29] The affinity of anions for carbonic anhydrase in its high pH form is so weak that the pH dependence of anion binding to the active site can formally be described as the competition between the anion and hydroxide ion for a coordination site on the metal. Many reviews cover the topic of inhibition of carbonic anhydrase by anions.[30-32] Our purpose here is to comment on the implications of rate-limiting intramolecular proton transfer on the inhibition.

One very pertinent observation is that the mode of anion inhibition of CO_2 hydration catalyzed by carbonic anhydrase II changes as pH is increased from 7 to 9.[33,34] At pH <7 the mode of inhibition is noncompetitive or very close to it for anions such as I^-,N_3^-, and NCS^-, meaning that in steady-state catalysis these anions affect only k_{cat} and not K_m. At pH 9, however, the inhibition changes to a mode which is very close to uncompetitive, meaning that in steady state these anions affect k_{cat} and K_m to the same extent, leaving the ratio k_{cat}/K_m unchanged. The transition between these two regions is continuous.[33,34] The uncompetitive mode of inhibition is usually attributed in general biochemistry texts to the binding of inhibitor only in a ternary complex between enzyme, substrate, and inhibitor. However, the possibility of a ternary complex is not consistent with studies in which anions (I^-, NCS^-, and NCO^-) inhibited the 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.[22] A similar result has been observed with human carbonic anhydrase I measuring Cl^- inhibition of the catalytic interconversion at chemical equilibrium by ^{13}C resonance broadening.[35] These competitions mean that inhibitor and substrate do not bind to the active site simultaneously and suggest that another mechanism of inhibition perhaps related to the Ping-Pong hypothesis is involved.

The observed uncompetitive inhibition at pH 9 is consistent with the hypothesis of the rate-limiting intramolecular proton transfer of eq 6. Thus, at saturating CO_2 concentrations the form of the enzyme that precedes this rate-limiting step, a form with zinc-bound water, should accumulate. This is the form with a high affinity for anions. At low CO_2 concentrations, when $[CO_2] \ll K_m$, the $EZnOH^-$ form will dominate at pH > pK_a and there will be little or no inhibition. Consequently, an uncompetitive inhibition pattern is predicted. When pH < pK_a, the $EZnH_2O$ form will dom-

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inate during the steady state at all CO_2 concentrations and there will be little or no dependence of the apparent K_i on substrate concentration. Hence, classical noncompetitive inhibition is predicted in the limit of low pH.

The mechanistic model presented here takes into account only the major features of catalysis. A more elaborate model has been worked out, and this model has been tested by computer simulations in which rate constants for catalysis are assigned according to data in the literature to reproduce a wide body of kinetic and equilibrium properties of isozyme II.[36,37] These simulations confirm in a quantitative fashion the anion inhibition patterns discussed qualitatively above.

Before leaving this topic it is appropriate to comment that an entire array of inhibition patterns have been reported for the hydration of CO_2 catalyzed by carbonic anhydrase II. Besides the example above of uncompetitive inhibition of CO_2 hydration at pH 9, inhibitors have been found giving rise to noncompetitive or competitive patterns under these conditions.[38-40] All these inhibitors probably interact with the same site at or near the metal ion and compete with substrate at equilibrium, but computer simulations indicate that the diverse inhibition patterns at steady state are a consequence of the rate-limiting protolysis of metal-bound water and reflect diverse kinetic pathways of inhibitor binding[40] to this site.

Hydration of CO_2 Catalyzed by Carbonic Anhydrase III

The catalysis of the hydration of CO_2 by carbonic anhydrase III from bovine and feline skeletal muscle has kinetic parameters, for both steady-state and equilibrium conditions, which are independent of pH in the region of pH from 5.5 to 8.0.[41,42] This behavior is quite different from that described above for isozyme II which in CO_2 hydration has k_{cat} and k_{cat}/K_m roughly described by a titration curve with pK_a near 7. We have proposed that the CO_2 hydration activity of isozyme III is controlled by the ionization of zinc-bound water as in isozyme II and that the pK_a of $EZnH_2O$ is below 5.5 in isozyme III.[41] This low pK_a for isozyme III is perhaps due to stabilization of the zinc-bound hydroxide by positive charges in the active site; that is, bovine isozyme III has several basic amino acid residues, Lys-64, Arg-67, and Arg-91 which in isozyme II are His-64, Asn-67, and Val-91.[43] A low pK_a for the zinc-bound water in isozyme III is also supported by the observation of a visible absorption spectrum for Co(II)-substituted carbonic anhydrase III in the range of pH 5–9 which is very similar to that observed for the high-pH form of the analogous Co(II)-substituted isozyme II (that observed for pH > pK_a of the active site group).[44] It is also supported by the observation of uncompetitive inhibition by anions of the hydration of CO_2 catalyzed by carbonic anhydrase III in the region of pH 6–9.[42] This result also suggests that, like isozyme II, isozyme III has a rate-limiting proton transfer that causes the accumulation at steady state of the enzyme species with protonated active site, $EZnH_2O$. This suggestion is supported by solvent hydrogen isotope effects which show a rough parallel between isozymes II and III, both of which have an isotope effect near unity on k_{cat}/K_m for hydration of CO_2 and an isotope effect very significantly different from unity for k_{cat}.[42] There is no buffer effect on the catalysis by carbonic anhydrase III and no evidence for an intramolecular proton transfer. Moreover, there is no residue in the active site crevice for isozyme III that could serve as a likely proton shuttle. Isozyme III has a lysine in position 64 where there is a histidine in isozyme II. It is a possibility that isozyme III during catalytic hydration of CO_2 transfers a proton directly to water, a step consistent with its turnover number in the hydration of CO_2 of 3×10^3 s^{-1}.

Other data consistent with the hypothesis of a rate-limiting proton transfer in catalysis of CO_2 hydration by carbonic anhydrase III come from anion inhibition at steady state and at chemical equilibrium. As mentioned above, anions inhibit isozyme III uncompetitively. The apparent K_i value for the inhibition of k_{cat} by azide at pH 7.5 is 13 μM.[45] The experiment measuring ^{18}O exchange (at chemical equilibrium) using solutions of the same pH and ionic strength showed an apparent inhibition constant of 330 μM for inhibition by azide.[45] The experiments at chemical equilibrium have a ratio of zinc-bound water to zinc-bound hydroxide which is very low because the corresponding pK_a of the active site is less than 5.5. At saturating CO_2 concentrations in the steady state, $EZnH_2O$, which binds anions tightly, accumulates as the species before a rate-limiting step, resulting in smaller inhibition constants as discussed in the section on anion inhibition of isozyme II. Such experiments have been repeated, with qualitatively similar results, using the inhibitors cyanate and chlorzolamide.[46]

The experiments we describe next are the first direct evidence for the accumulation of the enzyme species with metal-bound water at steady state. They take advantage of the relatively slow turnover in hydration of CO_2 catalyzed by isozyme III to observe the coordination state of cobalt at the active site during the reaction. The visible absorption spectrum for Co(II)-substituted carbonic anhydrase III is similar in form to that observed for isozyme II,[44] with absorption peaks at 510, 551, 619, and 642 nm. As described above, this is the high-pH form of the active site with metal-bound hydroxide. The absorption spectrum of the low-pH form of isozyme II is less intense and lacks the maxima at 619 and 642 nm.

Tu and Silverman[47] mixed bovine Co(II)–isozyme III at pH 7.5 with a saturated CO_2 solution in a stopped-flow apparatus and followed the reaction at several wavelengths. After the dead time, a decreased absor-

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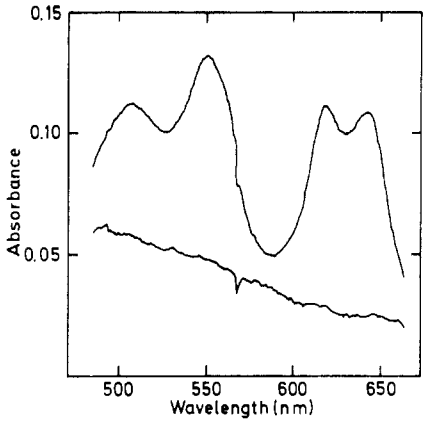

Figure 7. Rapid-scan stopped-flow spectra of thiol-modified bovine Co(II)–carbonic anhydrase III. Spectra are composed of two overlapping 96-nm regions with 1-ms scanning time at 11 °C. Concentrations were after mixing: 0.41 mM enzyme; 75 mM Mops–NaOH buffer, pH 7.2; 100 mM Na_2SO_4. Top spectrum, no CO_2 present. Bottom spectrum, with 16 mM CO_2. Both spectra were recorded at the stop of flow.

bance was observed consistent with an accumulation of the enzyme form $ECoH_2O$. As the hydration reaction approached equilibrium, the spectrum returned toward that of the $ECoOH^-$ form. However, in this experiment the maxima at 619 and 642 nm were still present during the steady state, indicating that only part of the enzyme had been converted to the $ECoH_2O$ form. This was probably due to incomplete substrate saturation, since the initial CO_2 concentration was only about $1.8K_m$. To achieve a more complete saturation with CO_2, Ren et al.[48] used bovine Co(II)–isozyme III that had been modified by reaction with methyl methanethiosulfonate. This reagent modifies thiol groups, and the resulting modified enzyme has a higher catalytic activity and a lower K_m for CO_2. Figure 7 shows absorption spectra of modified Co(II)–isozyme III recorded in a rapid-scan stopped-flow apparatus in the absence and presence of 16 mM CO_2, respectively. The spectrum obtained at the stop of flow in the presence of CO_2 is quite featureless and lacks the maxima at 619 and 642 nm, suggesting that practically no $ECoOH^-$ form is present under these conditions. The $ECoH_2O$ and $ECoHCO_3^-$ forms are anticipated to have similar absorption spectra with weak absorbances. As a result, these experiments alone cannot differentiate between a rate limitation by protolysis of metal-bound water or by dissociation of the enzyme–bicarbonate complex. However, in combination with the significant solvent hydrogen isotope effect of 2.5 on k_{cat} for the hydration catalyzed by carbonic anhydrase III[42] and the observed uncompetitive anion inhibition patterns, these results support rate limitation by protolysis of metal-bound water.

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Concluding Remarks

In this Account we have emphasized one aspect of the catalytic mechanism of carbonic anhydrase, the protolysis of water. This very simple chemical process, which is an elementary step in a multitude of enzymic reactions, might appear trivial but seems to limit the maximal rate of CO_2 hydration catalyzed by carbonic anhydrase isozymes II and III and probably contributes to rate limitation in the case of isozyme I.[49] In future studies the systematic variation of active-site residues by directed mutagenesis will, hopefully, lead to a better understanding of relations between structural and functional differences in these isozymes and the fine tuning of the catalytic activity. However, while the chemical aspects of carbonic anhydrase catalysis are beginning to be understood in detail, a number of biological questions remain essentially unanswered. Thus, the physiological roles of isozymes I and III are virtually unknown. Not until these roles have been defined will it be possible to understand why the "less than perfect" isozymes I and III have survived at least 300 million years of evolution in the presence of the "perfectly evolved" isozyme II.

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