Rate–Equilibria Relationships in Intramolecular Proton Transfer in Human Carbonic Anhydrase III†

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ABSTRACT: Maximal turnover rates for the dehydration of HCO_3^- catalyzed by the zinc metalloenzyme carbonic anhydrase III are limited by a proton transfer to zinc-bound hydroxide in the active site. We have used site-directed mutagenesis to place a proton donor, histidine, at position 64 and used ^{18}O exchange between CO_2 and water measured by mass spectrometry to determine the rates of intramolecular proton transfer to the zinc-bound hydroxide. In a series of site-specific mutants, the values of pK_a of the zinc-bound water ranged from approximately 5 to 9. The rate constants for proton transfer obeyed a Brønsted correlation and showed sharp curvature characteristic of facile proton transfers. Application of Marcus rate theory shows that this proton transfer has the small intrinsic energy barrier (near 1.5 kcal/mol) characteristic of rapid proton transfer between nitrogen and oxygen acids and bases, but has an observed overall energy barrier (near 10 kcal/mol), indicating the involvement of accompanying, energy requiring processes such as solvent reorganization or conformational change.

The Brønsted relation is a linear free energy relationship correlating rate constants for proton transfer k_B with the difference in acid or base strength of the proton acceptor and donor, as shown in the equation (Brønsted and Pederson, 1924; Kresge, 1973, 1975):

$$\log(k_B) = \beta[pK_a(\text{acceptor}) - pK_a(\text{donor})] + \text{constant} \quad (1)$$

The slope β of a Brønsted plot can be used to characterize reaction mechanisms and provide an estimate of the degree of charge transfer between reactants and products in the transition state. An observed variation in slope β over a range of pK_a values can be further interpreted through Marcus rate theory to give an intrinsic energy barrier for the proton transfer and two work terms, the energy required to align the reactants into the reaction complex for both forward and reverse directions (Marcus, 1968; Kresge, 1975).

Applications of these concepts to proton transfer in enzymic catalysis have been difficult because of chemical and structural constraints imposed at the active site. Rowlett and Silverman (1982) and Pocker et al. (1986) were able to obtain a Brønsted plot for intermolecular proton transfer between buffers in solution and a proton shuttle residue (His 64) near the surface of carbonic anhydrase II. Some of the constraints in Brønsted analysis of enzymic reactions can be removed by use of site-directed mutagenesis. Toney and Kirsch (1989) replaced a lysine residue in the active site of aspartate aminotransferase and showed that enhancement of catalysis by external amines in solution follows a Brønsted relation. Tu et al. (1989) replaced the proton shuttle residue histidine 64 with alanine in human carbonic anhydrase II (HCA II);[1] the low activity of the resulting mutant was then enhanced by imidazole in solution.

The carbonic anhydrases (EC 4.2.1.1) are zinc metalloenzymes that catalyze the hydration of CO_2 to form HCO_3^- and a proton. Carbonic anhydrase III is a predominant cytosolic protein in skeletal muscle (Gros & Dodgson, 1988). It is the least efficient of the seven known isozymes of mammalian CA, with a catalytic turnover of 2×10^3 s^{-1} in the hydration of CO_2 at pH 7.4 (Jewell et al., 1991; Ren et al., 1988; Tu et al., 1983), 300-fold less than observed for the more efficient CA II. The catalytic pathways for CA II and CA III are qualitatively similar and occur in two stages. The first is the interconversion of CO_2 and HCO_3^-; the second is the transfer of a proton between the enzyme and solution (Silverman & Lindskog, 1988). In dehydration, the substrate HCO_3^- binds to the form of the active site with water bound to zinc; the subsequent release of CO_2 leaves a hydroxide ion on the metal:

$$HCOO^{18}O^- + EZnH_2O \rightleftarrows EZn^{18}OH^- + CO_2 + H_2O \quad (2)$$

To regenerate the active form of the enzyme for the next dehydration step, the hydroxide accepts a proton (eq 3) by three possible pathways: from buffer in solution, from water, or from another residue of the enzyme which is then reprotonated from solution. In eq 3, BH can be water, buffer

$$EZn^{18}OH^- + BH \underset{k_{-B}}{\overset{k_B}{\rightleftarrows}} EZn^{18}OH_2 + B^- \overset{H_2O}{\rightleftarrows} EZnH_2O + H_2{}^{18}O + B^- \quad (3)$$

in solution, or a side chain of the enzyme such as histidine 64. The release of a proton from the zinc-bound water in the hydration direction, and the protonation of the zinc-bound hydroxide in the dehydration direction, is a rate-limiting step

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[1] Abbreviations: HCA III, human carbonic anhydrase III; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Taps, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid.

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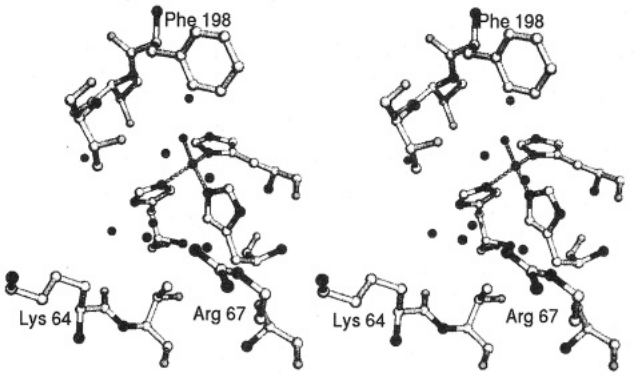


FIGURE 1: Residues near the zinc in bovine carbonic anhydrase III from the crystal structure of Eriksson and Liljas (1993). The oxygens of water molecules are shown as black spheres in the active-site cavity, and a bond is shown connecting the zinc to its water ligand. The three other ligands of the zinc are His 94, His 96 , and His 119.

in the overall catalysis by CA II and III (Silverman & Lindskog, 1988).

Jewell et al. (1991) showed that a histidine replacing the lysine at position 64 in wild-type HCA III can act as a proton acceptor and donor in the catalyzed hydration of CO_2 and dehydration of HCO_3^-, taking on a proton-transfer role in the pathway similar to that of His 64 in HCA II. Position 64 is in the active-site cavity, with the Cα located about 9.7 Å from the zinc in both bovine CA III (see Figure 1; Eriksson & Liljas, 1993) and HCA II (Eriksson et al., 1988). The lysine at this position in the active site cavity of wild-type CA III is shown in Figure 1 (Eriksson & Liljas; 1993). The present experiments use ^{18}O exchange to show that a Brønsted relation describes the intramolecular proton transfer between histidine at position 64 as proton donor and zinc-bound hydroxide as proton acceptor in several site-specific mutants of HCA III. Application of Marcus rate theory shows that this proton transfer has the small intrinsic energy barrier (near 1.5 kcal/mol) characteristic of rapid proton transfer between nitrogen and oxygen acids and bases, but has an observed overall energy barrier (near 10 kcal/mol) indicating the involvement of accompanying, energy requiring processes such as solvent reorganization or conformational change.

METHODS

Enzymes. Human carbonic anhydrase III and mutants were prepared using bacterial expression vectors optimized for efficient site-directed mutagenesis and protein synthesis as described by Tanhauser et al. (1992). The vectors were derived from the T7 expression vectors of Studier et al. (1990) and contained a bacteriophage f1 origin of replication for production of single-stranded DNA. Both single-site and cassette mutants were prepared with these vectors. Expression ranged from 1 to 20 mg/L, depending on the mutant. All mutations were confirmed by DNA sequencing of the expression vector used to produce the mutant protein. Modified and unmodified carbonic anhydrases III were purified by gel filtration followed by ion exchange chromatography as described by Tu et al. (1986). The resulting enzymes were greater than 98% pure, determined by polyacrylamide gel electrophoresis. The concentrations of wild-type HCA III and mutants were determined from the molar absorbtivity of 6.2×10^4 $M^{-1}cm^{-1}$ at 280 nm (Engberg et al., 1985). For mutants of HCA III with the replacement Phe 198 → Leu, potent inhibition with acetazolamide was observed ($K_I < 10^{-7}$ M). In this situation we were able to confirm the concentration of enzyme by titration with the inhibitor, using a Henderson plot (Segel 1975), to within 10% of that determined from the absorbtivity.

Oxygen-18 Exchange. We measured the rate of exchange of ^{18}O between CO_2 and water and of ^{18}O between ^{12}C- and ^{13}C-containing species of $CO_2$with mass spectrometry (Silverman, 1982). This ^{18}O-exchange method is carried out at chemical equilibrium and can therefore be performed without buffers, an advantage in studying intermolecular proton transfer. The ^{18}O method is also useful because two independent rates can be obtained, R_1 and R_{H_2O} (Silverman, 1982). R_1 is the rate of interconversion of CO_2 and HCO_3^-at chemical equilibrium (eq 2). This step during dehydration provides a transitory labeling of the active site with ^{18}O. R_{H_2O} is the rate of release of ^{18}O-labeled water from the active site (eq 3). R_{H_2O} involves a proton transfer to the zinc-bound hydroxide, forming a zinc-bound water, which is readily exchangeable with solvent water. Water containing oxygen-18 is greatly diluted by $H_2{}^{16}O$, resulting in net depletion of ^{18}O from species of CO_2 (eq 3).

Measurements of the isotopic content of CO_2 were made at 25°C using an Extrel EXM-200 mass spectrometer or a Dycor M-100 gas analyzer with a membrane inlet probe (Silverman, 1982) . Solutions contained 5–100 mM total substrate ($[CO_2] + [HCO_3^-]$) and 25 μM EDTA unless otherwise noted. Total ionic strength of solution was maintained at a minimum of 0.2 M with Na_2SO_4. For R_{H_2O} the standard errors were generally in the range of 10–20%. (Errors as large as 30% occurred in worst cases for values of R_{H_2O} at pH > 8, a range in which concentrations of CO_2 are small). For R_1 the standard errors were less than 15%.

Steady-State kinetics. The rate of hydration of CO_2 was determined by stopped-flow spectrophotometry (Applied Photophysics Model SF.17MV) measuring the rate of change of absorbance of a pH indicator (Khalifah, 1971; Rowlett & Silverman, 1982). The buffer–indicator pairs (with the wavelengths observed) included the following: Mes (pK_a 6.1) and chlorophenol red (pK_a 6.3, 574 nm); Mops (pK_a 7.2) and *p*-nitrophenol (pK_a 7.1, 400 nm); Taps (pK_a 8.4) and *m*-cresol purple (pK_a 8.3, 578 nm); and Ches (pK_a 9.3) and thymol blue (pK_a 8.9, 590 nm). Experiments were carried out at 25 °C with 50 mM buffer, and total ionic strength of solution was maintained at a minimum of 0.1 M using Na_2SO_4. Kinetic constants for CO_2 hydration were estimated from initial velocities using a weighted, linear least-squares method with v^4 weights, where v is initial velocity (Cleland, 1967). Initial velocities of the hydrolysis of 4-nitrophenyl acetate were measured (Beckman DU7 spectrophotometer) by the method of Verpoorte et al. (1967) in which the increase in absorbance was followed at 348 nm, the isosbestic point of nitrophenol and the conjugate nitrophenolate ion. Measurements were made at 25°C, and ionic strength was maintained at 0.1 M with Na_2SO_4. Solutions contained 33 mM of one of the buffers used in the CO_2 measurements.

RESULTS

pK_a of Zinc-Bound Water. In carbonic anhydrase, the pH dependence of k_{cat}/K_m for hydration of CO_2 is an accurate measure of the pK_a of the catalytic group, the zinc-bound water (Simonsson & Lindskog, 1982; Lindskog, 1983). Moreover, k_{cat}/K_m contains rate constants for the steps in eq 2, the interconversion of CO_2 and HCO_3^-, and does not depend on buffer concentration (Silverman & Lindskog, 1988). Using a nonlinear least squares fit to a single ionization with a maximum at high pH, we determined the apparent pK_a values of the zinc-bound water from the pH dependence of k_{cat}/K_m

Table I: Rate Constants, k_B, for Proton Transfer between Proton Donors and the Proton Acceptor, Zinc-Bound Hydroxide, in Carbonic Anhydrase III and Mutants Determined by the Rates of Release of $H_2{}^{18}O$ from the Enzymes[a]

entry on Figures 3 and 4	enzyme	pK_a (donor)[b]	pK_a (ZnH_2O)[b]	k_B[c] ($\times 10^{-3}$ s^{-1})
a	wild-type[g]	9.0[f]	4.3[d]	3
b	K64A	9.0[f]	4.3[d]	2
	Intramolecular (Histidine 64 Is Proton Donor)			
1	K64H[g]	7.5	4.3[d]	20
2	K64H-R67N[g]	7.5	5.3[e]	100
3	K64H-F198L	8.6	6.7	30
4	K64H-R67N-F198L[h]	6.8	6.8	280
5	K64H-F198D	6.5	8.4	200
6	K64H-R67N-F198D	6.4	8.7	380
	Intermolecular (Imidazolium Ion Is Proton Donor)			
7	wild-type[i]	7.6	4.3[d]	20
8	R67N[g]	7.5	5.3[e]	130
9	F198D	6.8	8.4	260

[a] Rate constants, k_B, were obtained from ^{18}O exchange (eq 3) by a nonlinear least-squares fit of eq 5 to the data for R_{H_2O}. Experimental conditions were as described in the legend to Figure 2 for H_2O as solvent. [b] The standard errors in pK_a were ±0.2. [c] The standard errors in k_B were less than ±20%. [d] Because of enzyme denaturation, we were not able to observe the pK_a for zinc-bound water for these enzymes. The value pK_a 4.3 was estimated from a linear free energy relationship as described in the text. [e] The pK_a of 5.3 was not observed but estimated as described in the text. [f] Proton donors in this case are uncertain and possibly include Lys 64 and HCO_3^-. [g] Jewell et al. (1991). [h] LoGrasso et al. (1991). [i] Tu et al. (1990).

for hydration of CO_2 using stopped-flow (Table I). These values agreed within 15% with values of k_{cat}/K_m determined using ^{18}O exchange;[2] thus, this pK_a was measured by at least two different methods. For two variants of HCA III, K64H-F198L and K64H-R67N-F198L, the pK_a of the zinc-bound water was further confirmed by measurement of the rate of hydrolysis of 4-nitrophenyl acetate. The values of the apparent pK_a for this catalysis (6.6 ± 0.1 for both K64H-F198L and the triple mutant) were in suitable agreement with the values determined from CO_2 hydration given in Table I. These catalyses of ester hydrolysis were inhibited by addition of 10^{-6} M ethoxzolamide. The other mutants showed negligible ester hydrolysis.

^{18}O Exchange in the Absence of Buffers. The pH dependence of R_{H_2O}, the rate of release of ^{18}O-labeled water from the active site, was measured in the absence of buffer for the variants of HCA III given in Table I (numbered 1–6 and a and b); some of these data have appeared in previous reports as indicated in Table I. For the mutant K64H-R67N-F198L HCA III, the pH dependence of $R_{H_2O}/[E]$ is shown in Figure 2. These experiments were carried out in the absence of external buffers, although the solutions did contain substrate HCO_3^- at concentrations of 5–100 mM.

^{18}O Exchange in the Presence of Imidazole Buffers. It is a general feature of HCA III that its catalysis can be enhanced by buffers of small size such as imidazole and phosphate. This has been observed at steady state; it has also been observed

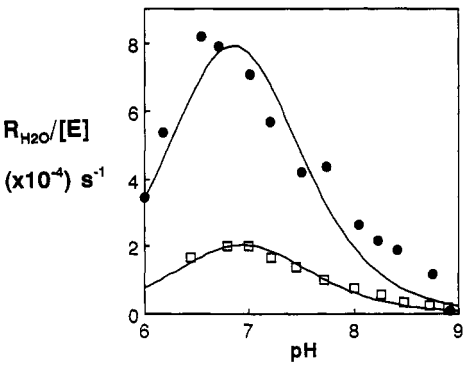


FIGURE 2: Variation with pH (uncorrected pH meter reading) of $R_{H_2O}/[E]$ catalyzed by the triple mutant K64H-R67N-F198L HCA III: (●) in H_2O; and (□) in 99% D_2O. R_{H_2O} is the rate of release from enzyme of ^{18}O-labeled water, and [E] is total concentration of enzyme. Data were obtained at 25°C with solutions containing 25 mM of all species of CO_2 and 25 μM EDTA, and with total ionic strength of solution maintained at 0.2 M with Na_2SO_4. No buffers were added to solution. Solid lines for experiments in H_2O were obtained from a nonlinear least-squares fit of eq 5 to the data (with pK_a of zinc-bound water fixed at that determined by measurements of k_{cat}/K_m) and resulted in the entries of Table I.

by catalyzed ^{18}O exchange at chemical equilibrium (eq 3) in which R_{H_2O} for HCA III and mutants is enhanced by imidazole and phosphate in a saturable manner consistent with proton transfer to the zinc-bound hydroxide (Tu et al., 1990, 1983; Paranawithana et al., 1990).

These observations can be described by eq 4, which assumes proton transfer from buffers, designated BH, to the active site and provides a satisfactory fit to the data (Tu et al., 1990).

$$R_{H_2O} = k_B[E][BH]/(K_{eff}^{B} + [BH]) \quad (4)$$

Here k_B is the maximal rate constant for that component of water release enhanced by the proton-transfer agent and K_{eff}^{B} is an apparent binding constant of the proton-transfer agent to the enzyme. [E] and [BH] are the concentrations of total enzyme and total buffer. Values for k_B and K_{eff}^{B} for the enhancement of R_{H_2O} by imidazole (Tu et al., 1990) and phosphate (Paranawithana et al., 1990) have been reported.[3]

The pH profile of R_{H_2O} catalyzed by HCA III and two mutants (numbered 7–9 in Table I) was measured using solutions containing concentrations (100–150 mM) of imidazole buffer near saturation of the enhancement of R_{H_2O}. This is similar to the pH profiles of R_{H_2O} catalyzed by HCA III and R67N HCA III in the presence of large concentrations (150 mM) of imidazole [see Figure 2 of Tu et al. (1990) and Figure 5 of Jewell et al. (1991)], and to R_{H_2O} catalyzed by HCA III in the presence of 200 mM phosphate [Figure 2 of Paranawithana et al. (1990)].

DISCUSSION

Site-specific mutagenesis at positions near the zinc in the active-site cavity of HCA III provided a series of variants with values of the apparent pK_a for the zinc-bound water ranging from approximately 5 for wild-type HCA III to 8.7 for a mutant of HCA III obtained with the replacement

[2] The substrate dependence of R_1 is given by $R_1/[E] = k_{cat}^{ex}[S]/(K_{eff}^{S} + [S])$, in which k_{cat}^{ex} is a rate constant for maximal interconversion of CO_2 and HCO_3^-, K_{eff}^{S} is an apparent substrate binding constant, and [S] is the concentration of substrate, CO_2, or HCO_3^-. Values of $k_{cat}^{ex}/K_{eff}^{CO_2}$ for CO_2 hydration catalyzed by the enzymes in Table I were determined by nonlinear least-squares fit of the above expression for R_1 to the data or by measurement of R_1 at values of $[CO_2]$ much smaller than $K_{eff}^{CO_2}$. In theory and in practice, $k_{cat}^{ex}/K_{eff}^{CO_2}$ is equal to k_{cat}/K_m for hydration obtained by steady-state methods (Simonsson et al., 1979).

[3] The rate constant for proton transfer $k_{H_2O}^{B}$ in Tu et al. (1990) and Paranawithana et al. (1990) is written as k_B in this work. Equation 4 has no term representing ^{18}O exchange in the absence of buffer caused by intermolecular proton transfer. Such ^{18}O exchange is believed to be negligible since proton transfer from water to zinc hydroxide is not a prominent pathway. For mutants designated a and b and 7–9 in Table I, R_{H_2O} was indistinguishable from zero at low total substrate concentration (<1 mM) and no buffers.

Phe 198 → Asp (LoGrasso et al., 1993). We have taken advantage of this diversity in pK_a to investigate the properties of proton transfer in the dehydration of HCO_3^- catalyzed by carbonic anhydrase III.

Intramolecular Proton Transfer. The imidazole side chain of His 64 in carbonic anhydrases II and III can act as a proton-transfer residue (Tu et al., 1989; Jewell et al., 1991; Jonsson et al., 1976), donating a proton to the zinc-bound hydroxide in the dehydration direction. In the ^{18}O-exchange method, performed at chemical equilibrium, this is measured by R_{H_2O}, the rate of release from the active site of water bearing substrate oxygen as shown in eq 3. These experiments have the advantage over steady-state experiments that no buffers are used and there is less concern about separating the effects of inter- and intramolecular proton transfer.

The pH profile for R_{H_2O} is adequately fit by eq 5 which describes the release of ^{18}O-labeled water from the active site as limited in rate by transfer of a proton from a donor group, the imidazolium group of His 64, to the (labeled) zinc hydroxide (Tu & Silverman, 1985; Silverman, 1982). The rate-limiting nature of this proton transfer has been confirmed by solvent hydrogen isotope effects (Tu & Silverman, 1982; Tu et al., 1983), buffer enhancement of R_{H_2O} (Jewell et al., 1991; Tu et al., 1990; Paranawithana et al., 1990), and simulations of catalysis (Rowlett et al., 1991; Lindskog, 1984; Rowlett 1984).

$$R_{H_2O}/[E] = k_B/\{(1 + K_B/[H^+])(1 + [H^+]/K_E)\} \quad (5)$$

K_E is the ionization constant of the zinc-bound water, K_B is that of the proton shuttle group, [E] is total enzyme concentration, and k_B is the rate constant for the proton transfer. Figure 2 shows the application of eq 5 to R_{H_2O} catalyzed by K64H-R67N-F198L HCA III. Previous work shows the application of eq 5 to HCA II [Figure 3 of Tu and Silverman (1985)] and to mutants of HCA III containing His 64 [K64H HCA III and K64H-R67N HCA III, Figure 4 of Jewell et al. (1991)]. The constants obtained by least-squares fit of eq 5 for these and additional variants of HCA III containing His 64 are given in Table I. In the fitting of eq 5, the value of K_E was fixed to that obtained from observation of the pH dependence of k_{cat}/K_m for hydration of CO_2.

The values of the pK_a for the zinc-bound water in wild-type CA III and some other variants indicated in Table I have not been measured directly because of enzyme denaturation at pH < 5.5. A pK_a near 5.0 has been roughly estimated for the metal-bound water of Co(II)-substituted bovine CA III using spectrophotometric and inhibition data (Ren et al., 1988). LoGrasso et al. (1993) observed a correlation (correlation coefficient 0.92) between k_{cat}/K_m for hydration and pK_a of catalysis for a series of site-specific mutants at position 198 of HCA III; the values of pK_a 4.3 for wild-type, K64H, and K64A, and pK_a 5.3 for K64H-R67N HCA III, given in Table I were obtained by extrapolation from these data. In cases such as K64H and K64H-R67N HCA III for which the values of pK_a for the donor and acceptor groups are well separated (Table I), the value of k_B obtained from a fit to eq 5 is not significantly affected by substantial uncertainties in the ionization constants of the acceptor group, the zinc-bound hydroxide. However, in these cases (pK_a of zinc-bound water less than 6 as indicated in Table I) the uncertainties in ΔpK_a are a source of scatter in the Brønsted plot.

Proton Transfer from Imidazole Buffer. It is a general feature of catalysis by HCA III that proton transfer to the active site can be enhanced by buffers of small size such as imidazole and phosphate. This has been observed for hydration of CO_2 at steady state and for ^{18}O-exchange experiments at equilibrium in which R_{H_2O} for HCA III and mutants is enhanced by imidazole and phosphate in a saturable manner consistent with proton transfer to the zinc-bound hydroxide as described by eq 3 (Tu et al., 1990, 1983; Paranawithana et al., 1990). At saturating imidazole concentrations the pH profile of R_{H_2O} was adequately described by eq 5, with data given for HCA III and two variants in Table I (numbers 7–9).

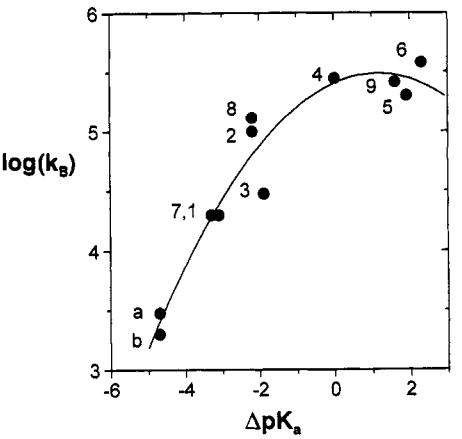


FIGURE 3: Brønsted plot of the logarithm of k_B (s^{-1}) versus ΔpK_a (pK_a[zinc-bound water] – pK_a[donor group]). The entries are wild-type and mutants of carbonic anhydrase III numbered as in Table I. Values of k_B and pK_a's were obtained by a nonlinear least-squares fit of eq 5 to data for $R_{H_2O}/[E]$. The solid line is a least-squares fit of the Marcus equation (eq 6) to all of the data, resulting in $\Delta G^{\ddagger}_0$ = 1.4 ± 0.3 kcal/mol, w^r = 10.0 ± 0.2 kcal/mol, and w^p = 5.9 ± 1.1 kcal/mol.

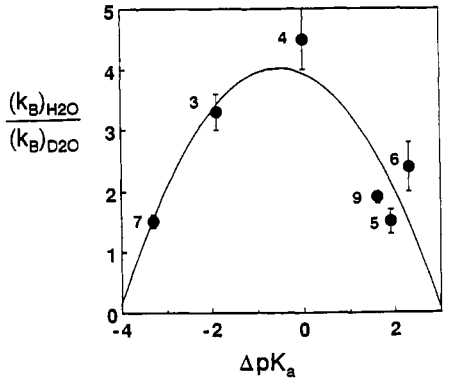
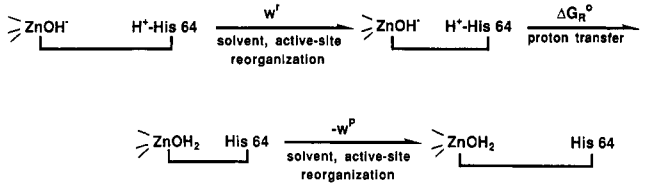


FIGURE 4: Solvent hydrogen isotope effect of the rate constant for proton transfer as a function of ΔpK_a (pK_a[zinc-bound water] – pK_a[donor group]). The entries are wild type and mutants of carbonic anhydrase III numbered as in Table I. Conditions as described in the legend to Figure 2. The data are means and standard errors of 3–5 measurements. The solid line is a least-squares fit to the quadratic form of eq 7.

Brønsted Correlation. The rate constant k_B for intramolecular transfer of a proton from histidine at position 64 to the zinc-bound hydroxide in mutants of HCA III correlated with ΔpK_a in a Brønsted plot (Figure 3, Table I). Interpretation of the Brønsted plot is obscured somewhat because of an uncertainty in the pK_a of the zinc-bound water for the forms of HCA III with slower proton transfer, as described above. Also, we have not made statistical corrections to account for multiple proton donation sites on the imidazolium ring; these may be nonequivalent sites because of preferred orientations of the histidine side chain. Nevertheless, the values of pK_a are not grossly in error, and the overall curvature of the Brønsted plot is confirmed by the maximum in solvent hydrogen isotope effect for k_B (Figure 4). This maximum is expected for proton transfer when the values of pK_a for the

Scheme I



donor and acceptor group are nearly equal (Bergman & Kresge, 1978; Cox and Jencks, 1978). It is also expected that for this case the maximum of Figure 4 occurs near the triple mutant K64H-R67N-F198L HCA III, the mutant in which the pK_a of zinc water is near 7, matching approximately the pK_a of the imidazolium ring of His 64 (Table I).

Marcus Rate Theory. A better understanding of the meaning of the Brønsted plot is obtained through application of Marcus theory for the proton transfer of Scheme I. Here w^r is a work term giving the energy required for solvent reorganization and side-chain conformational change required prior to the proton transfer. Structural data (Eriksson et al., 1988) and isotope effects (Venkatasubban & Silverman, 1980) for HCA II suggest proton transfer between His 64 and the zinc-bound hydroxide proceeds through hydrogen-bonded water molecules in a proton relay mechanism. The side chain of His 64 in HCA II cannot extend close enough to the zinc-bound hydroxide for direct proton transfer (Eriksson et al., 1988). These features are a likely possibility for CA III as well, especially since the backbone conformations of these two isozymes are very similar (Eriksson & Liljas, 1993). The work term w^r is then the energy required to align acceptor, donor, and intervening hydrogen-bonded water for facile proton transfer. The energy w^p is this work term for the corresponding reorganization for the reverse process (Scheme I). The standard free energy of reaction with the required active-site conformation is $\Delta G_R°$, with the measured overall free energy for the reaction given by $\Delta G° = w^r + \Delta G_R° - w^p$.

The observed overall activation barrier for the proton transfer $\Delta G^‡$ is given in Marcus theory by eq 6, which relates this to an intrinsic energy barrier $\Delta G^‡_0$ which is the value of $\Delta G^‡$ when $\Delta G_R° = 0$ (Marcus, 1968). The slope β of a

$$\Delta G^‡ = w^r + \{1 + \Delta G_R°/4\Delta G^‡_0\}^2 \Delta G^‡_0 \quad (6)$$

Brønsted plot is given by Marcus theory as $d\Delta G^‡/d\Delta G_R°$. Brønsted plots for proton transfer between nitrogen and oxygen acids and bases show sharp curvature, changing slope from zero to one over the span of ΔpK_a −2 to 2 (Kresge, 1975). Analysis by Marcus theory shows that such proton transfers have a low intrinsic energy barrier $\Delta G^‡_0$, near 2 kcal/mol, with a work term w^r also near 2 kcal/mol, representing energy required to form the reaction complex (Kresge, 1973, 1975).

The Brønsted plot of Figure 3 represents proton transfer between nitrogen and oxygen acids and bases, and it displays the sharp curvature characteristic of such processes. However, it is unusual because the rate of proton transfer is relatively slow, less than 10^6 s^{-1}, and the magnitude of the overall energy barrier for the proton transfer is large, near 10 kcal/mol. A least-squares fit of the Marcus equation, eq 6, to all of the data of Figure 3 gives an intrinsic energy barrier for the proton transfer of $\Delta G^‡_0 = 1.4 \pm 0.3$ kcal/mol, with work terms $w^r = 10.0 \pm 0.2$ kcal/mol and $w^p = 5.9 \pm 1.1$ kcal/mol. (For this calculation, $\Delta G^‡ = -RT \ln(hk_B/kT)$ and $\Delta G° = RT \ln[(K_a)_{ZnH_2O}/(K_a)_{donor}]$.) That is, the data are consistent with a rather low intrinsic energy barrier for the transfer accompanied by substantial work functions. Points a and b of Table I and Figure 3 (wild-type and K64A HCA III) were included in this fit although it is clear that an imidazolium group is not the proton donor in these cases. For these enzymes the donor group (or groups) is uncertain, but the pH dependence of R_{H_2O} in both cases shows an apparent pK_a near 9 and could possibly involve HCO_3^-, which under our solution conditions has a pK_a of 9.8 for the equilibrium between HCO_3^- and CO_3^{2-} (Butler, 1982). Comparison of wild-type with K64A HCA III (Table 1) and C66A HCA III shows that Lys 64 and Cys 66 are not predominant proton donor groups.[4] The values of $\Delta G^‡_0$ and the work terms are not significantly altered by omitting the intermolecular processes 7–9 of Table I ($\Delta G^‡_0 = 1.6 \pm 0.5$ kcal/mol; $w^r = 10.0 \pm 0.2$ kcal/mol; and $w^p = 5.6 \pm 1.6$ kcal/mol), nor are they significantly altered (although uncertainties are greater) by omitting the points 7–9 for examples of intermolecular proton transfer and points a and b which do not involve imidazole as proton donors ($\Delta G^‡_0 = 1.9 \pm 1.8$ kcal/mol; $w^r = 10.0 \pm 0.4$ kcal/mol; and $w^p = 5.0 \pm 5.2$ kcal/mol).

The solvent hydrogen isotope effects (SHIE) on k_B are also consistent with a low value of the intrinsic energy barrier $\Delta G^‡_0$, as shown by the following considerations. The data in Figure 4 were fit to the quadratic form of eq 7, an expression

$$\ln[(k_B)_{H_2O}/(k_B)_{D_2O}] = \ln[(k_B)_{H_2O}/(k_B)_{D_2O}]_{max}[1 - (\Delta G_R°/4\Delta G^‡_0)^2] \quad (7)$$

for the dependence of the SHIE on $\Delta G_R°$ (Kresge et al., 1977; see this reference for the assumptions inherent in eq 7). The fit of this expression to the SHIE data of Figure 4 gave an intrinsic energy barrier for the proton transfer of $\Delta G^‡_0 = 1.3 \pm 0.3$ kcal/mol with $[(k_B)_{H_2O}/(k_B)_{D_2O}]_{max} = 4.7 \pm 0.7$ and work terms $w^r - w^p = 0.6 \pm 0.5$ kcal/mol. This latter value is different from the value of $w^r - w^p$ near 4 kcal/mol determined from the data of Figure 3. The reasons for this difference are uncertain but may reflect experimental errors in the SHIE or assumptions used to derive eq 7 which are not fully applicable to this enzymic catalysis.

The transition from a slope near unity to a slope near zero in the Brønsted plot of Figure 3 is shifted to the negative side of $\Delta pK_a = 0$. The point of slope 0.5 ($d[\ln k_B]/d[\Delta pK_a] = 0.5$) occurs at $\Delta pK_a = -3.0$. This is also reflected in a slight shift of the maximum in the isotope effects of Figure 4 away from $\Delta pK_a = 0$; the maximum in Figure 4 occurs at $\Delta pK_a = -0.5$. These observations are related to the unequal values of the work terms for proton transfer in the dehydration (w^r) and hydration (w^p) directions in the Marcus equation. This inequality can be rationalized in a qualitative manner by noting that in the dehydration direction (Scheme I) the proton transfer is from a charged imidazolium group to a zinc-bound hydroxide, whereas in the reverse direction the proton transfer is from zinc-bound water to an uncharged imidazole group. Electrostatic effects in the active-site cavity could make significant contributions to these different work terms.

It is these work terms that make intramolecular proton transfer in carbonic anhydrase III much slower than the maximal values near 10^{12} s^{-1} observed, for example, in the proton transfers in electronically excited states (Barbara et al., 1989). A major clue may be that in many examples of intramolecular proton transfer at 10^{12} s^{-1}, dynamic solvent involvement is not observed. The source of the sizable work terms in CA III is not clear. Since the intramolecular proton transfer between His 64 and the zinc-bound hydroxide is

[4] Unpublished results from this laboratory.

believed to proceed through hydrogen-bonded water molecules in the active site, the work term need not contain energy to desolvate the proton donor and acceptor. However, it is possible that energy is required for solvent reorganization to provide a pathway for proton transfer through hydrogen-bonded water molecules that are restricted in motion by the narrow active-site cavity. Because imidazole in solution can substitute for His 64, giving nearly identical kinetic parameters for the catalysis (see points 7–9 of Table I), it is unlikely that orientation of the imidazolium ion or histidine side chain is involved. This is consistent with the results for the mutant T200S HCA II, for which Krebs et al. (1991) showed that a change in conformation (in the crystal structure) of the side chain of His 64 compared with wild type had no effect on the proton-transfer dependent turnover for catalysis. The SHIE associated with the work terms is expected to be very near unity; thus the SHIE observed for k_B catalyzed by K64H-F198L and K64H-R67N-F198L HCA III are due to the primary proton transfer.

Three values of k_B for intermolecular proton transfer utilizing imidazolium ion as proton donor also fell on the same correlation for the Brønsted plot (Figure 3, Table I, points 7–9). They were obtained from R_{H_2O} measured in the presence of total concentrations of imidazole (100–150 mM) that approached saturation in the binding of this proton donor to enzyme and represent proton transfer within the bound enzyme–buffer complex. This ability of imidazole to substitute for histidine 64 explains a previous result for the mutant K64H HCA III in which the proton shuttle residue His 64 is placed in isozyme III. R_{H_2O} catalyzed by wild-type HCA III is activated by imidazole in solution to the level of R_{H_2O} for K64H HCA III (Tu et al., 1990); a similar effect was observed for H64A HCA II in the presence of imidazolium ions (Tu et al., 1989).

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