Proton Transfer by Histidine 67 in Site-Directed Mutants of Human Carbonic Anhydrase III†

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ABSTRACT: The ability of a histidine residue at position 67 in human carbonic anhydrase III to transfer protons in the catalytic pathway for the hydration of CO_2 was investigated for a series of site-specific mutants. Wild-type carbonic anhydrase III has an arginine at this position with the Cα of residue 67 about 9.4 Å from the zinc. The active-site cavity contains no other residues capable of facile proton transfer. Rate constants for proton transfer from His 67 to the zinc-bound hydroxide were determined from the rate constants for the exchange of ^{18}O between CO_2 and water measured by mass spectrometry. A range of values for the pK_a of zinc-bound water was achieved by replacement of phenylalanine with leucine and aspartate at position 198 adjacent to the zinc. Application of Marcus rate theory showed that intramolecular proton transfer involving His 67 had an intrinsic energy barrier of 1.3 ± 0.3 kcal/mol and a thermodynamic work function for a preceding unfavorable equilibrium of 10.9 ± 0.1 kcal/mol. We previously showed that proton transfer from histidine 64 in carbonic anhydrase III could be described by Marcus rate theory [Silverman, D. N., Tu, C. K., Chen, X., Tanhauser, S. M., Kresge, A. J., & Laipis, P. J. (1993) *Biochemistry 32*, 10757−10762]. In comparison, proton transfer from His 67 must overcome a more unfavorable preceding equilibrium (a larger work function) that probably represents an energy requirement for proper alignment of donor and acceptor groups plus the intervening hydrogen-bonded water. Once this alignment is achieved, the intrinsic energy barrier appears the same for His 67 or His 64.

The catalysis of the dehydration of HCO_3^- by carbonic anhydrase occurs in two separate and distinct steps (Silverman & Lindskog, 1988). The first is the conversion of HCO_3^- into CO_2 at the zinc, leaving zinc-bound hydroxide at the active site.

$$HCOO^{18}O^- + EZnH_2O \rightleftarrows EZn^{18}OH^- + CO_2 + H_2O \quad (1)$$

In the second step, the zinc-bound water is regenerated by a series of proton transfer steps originating from buffer in solution. In the most efficient of the carbonic anhydrases, isozyme II, these steps involve both intramolecular and intermolecular transfer; the intramolecular transfer is rate determining in the presence of ample buffer (Steiner et al., 1975). The His 64 side chain in isozyme II is the predominant intramolecular shuttle transferring protons from buffer in solution to the zinc-bound hydroxide in the dehydration direction (Tu et al., 1989).

$$H^+\text{His 64}-EZn^{18}OH^- \overset{k_B}{\rightleftharpoons} \text{His 64}-EZn^{18}OH_2 \overset{H_2O}{\rightleftharpoons} \text{His 64}-EZnH_2O + H_2{}^{18}O \quad (2)$$

In the least efficient of the carbonic anhydrases, isozyme III, position 64 is occupied by a lysine which is too basic to be an efficient proton donor. This accounts in part for the slower activity of this isozyme.

The replacement of Lys 64 with His by site-specific mutagenesis in human carbonic anhydrase III (HCA III)[1] enhanced the maximum velocity of CO_2 hydration; the properties of this enhancement indicated that His 64 was also a proton shuttle, as it is in isozyme II (Jewell et al., 1991). Replacing phenylalanine at position 198, located near the metal center, with leucine or aspartate increased the pK_a of the zinc-bound water with the resulting range of values of pK_a varying from near 5 to 9 (LoGrasso et al., 1993). This allowed a better understanding of the function of His 64 in carbonic anhydrase by varying the pK_a of the zinc-bound water and observing the change in rate of intramolecular proton transfer (Silverman et al., 1993).

We now show how these features of proton transfer change when the proton donor is at a different position in the active-site cavity. Using site-specific mutagenesis, we have prepared a number of variants of HCA III with histidine at position 67, occupied by arginine in the wild-type enzyme. In the structure of wild-type bovine CA III the Cα positions of residues 64 and 67 are about equidistant from the zinc,

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[1] Abbreviations: HCA III, human carbonic anhydrase III; R67H HCA III, we use the single letter code in which this notation indicates the mutant of human carbonic anhydrase III in which Arg 67 has been replaced with His; 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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9.7 and 9.4 Å, respectively (Eriksson & Liljas, 1993). In both cases their side chains extend into the active-site cavity with Lys 64 forming a salt bridge to Glu 4 and Arg 67 without apparent interactions with other residues. The rate constants for proton transfer from His 67 to zinc-bound hydroxide were determined from the rates of ^{18}O exchange between CO_2 and water at chemical equilibrium determined by mass spectrometry. We have found that histidine at position 67 can transfer protons between the metal center and solution, but its maximal rate is about 4-fold less than for His 64. This difference is attributed by Marcus rate theory to the thermodynamic barrier required to attain alignment of donor and acceptor groups and intervening water for the transfer rather than to the intrinsic energy barrier for the proton transfer itself.

MATERIALS AND METHODS

Enzymes. Bacterial expression vectors optimized for efficient site-directed mutagenesis and protein synthesis as described by Tanhauser et al. (1992) were used to prepare human carbonic anhydrase III and mutants. These 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. 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 95% pure, determined by polyacrylamide gel electrophoresis. 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 ($K_I < 10^{-8}$ 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 absorption measurements.

Oxygen-18 Exchange. Mass spectrometry was used to measure the rate of catalyzed and uncatalyzed exchange of ^{18}O between CO_2 and water. This method is based on experiments of Mills and Urey (1940) and is described in its application to carbonic anhydrase by Silverman (1982). The advantage of the method is that the ^{18}O exchange is measured at chemical equilibrium and hence requires no external buffers to maintain pH. External buffers can participate in proton transfer in the catalytic pathway of carbonic anhydrase and are best avoided in measurements of intramolecular proton transfer.

The kinetic equations for the redistribution of ^{18}O from the CO_2–HCO_3^- system to water were solved to obtain two rates in the ^{18}O exchange catalyzed by carbonic anhydrase (Silverman et al., 1979; Koenig & Brown, 1981). The first is R_1 the rate of exchange of HCO_3^- and CO_2, as shown in eq 1. The substrate dependence of R_1 is given by

$$R_1/[E] = k_{cat}^{ex}[S]/(K_{eff}^{S} + [S]) \quad (3)$$

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^- (Simonsson et al., 1979). Values of $k_{cat}^{ex}/K_{eff}^{CO_2}$ for CO_2 hydration catalyzed by the enzymes in Table 1 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 within experimental uncertainty for hydration obtained by steady-state methods (Simonsson et al., 1979). The second rate is R_{H_2O} the rate of release from the active site of water bearing substrate oxygen, as shown in eq 2. In this mechanism, a proton converts zinc-bound hydroxide into zinc-bound water which then allows rapid exchange with solvent water. The steps measured by R_{H_2O} are separate and distinct from those of the interconversion of CO_2 and HCO_3^- in eq 1.

Table 1: 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 Figure 3	enzyme	p*K*a (donor)[b]	p*K*a (ZnH_2O)[b]	k_B[c] ($\times 10^{-3}$ s^{-1})
a	wild-type	9.0[f]	4.3[d]	3.0 ± 0.4
b	K64A	9.0[f]	4.3[d]	2.1 ± 0.4
c	R67N[g]	9.0[f]	5.3[e]	5 ± 1
	intramolecular (histidine 67 is proton donor)			
1	R67H	5.6	5.3	81 ± 14
2	K64A/R67H	6.2	6.0	59 ± 11
3	N62A/K64A/R67H	5.9	6.1	53 ± 13
4	K64A/R67H/F198L	8.4	6.4	36 ± 4
5	K64A/R67H/F198D	8.0	9.1	58 ± 14

[a] Rate constants, k_B, were obtained from ^{18}O exchange (eq 2) by a nonlinear least-squares fit of eq 4 to the data for R_{H_2O}. Experimental conditions were as described in the legend to Figure 2. [b] The standard errors in pK_a were ±0.2. [c] The rate constant for proton donation to the zinc-bound hydroxide, as in eq 2. [d] Because of enzyme denaturation under the conditions of these experiments, we were not able to observe sufficient titration data for a firm value of pK_a for zinc-bound water for these enzymes. The value pK_a 4.3 was estimated by Silverman et al. (1993). [e] The pK_a of 5.3 was not observed but estimated as described in Silverman et al. (1993). [f] Proton donors in this case are uncertain and probably include other proton donating residues near the active site. [g] Jewell et al. (1991).

Measurements of the isotopic content of CO_2 were made 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. Total ionic strength of solution was maintained at a minimum of 0.2 M with Na_2SO_4, and the temperature was 25 °C unless otherwise noted. For determination of solvent hydrogen isotope effects and NMR spectra, all pH measurements are presented as uncorrected pH meter readings. This procedure causes the approximate cancellation of the effects of D_2O on both pH meter and the ionization constant of titratable groups (Schowen & Schowen, 1982). Solvent hydrogen isotope effects were determined for several of the variants of Table 1 by measuring the pH profiles for $R_{H_2O}/[E]$ in 99.8% D_2O.

Steady-State Kinetics. Initial velocities of the hydration of CO_2 were determined by stopped-flow spectrophotometry (Applied Photophysics Model SF.17MV) measuring the rate of change of absorbance of a pH indicator (Khalifah, 1971). Saturated solutions of CO_2 were prepared by bubbling CO_2 into distilled H_2O or D_2O at 25 °C; dilutions were made using syringes with air-tight connections. 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); 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 maintained at a minimum of 0.1 M using Na_2SO_4. Kinetic constants for CO_2 hydration were estimated from initial velocities using ENZFITTER, a nonlinear least-squares analysis (Leatherbarrow, 1987). 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.

NMR Spectroscopy. Experiments were carried out using a Varian Unity 600 (600 MHz ^1H) NMR spectrometer at ambient temperature of about 21 °C. We utilized the MLEV-17 pulse sequence (Bax & Davis, 1985) with spin lock times near 80 ms to distinguish histidine $C^{\epsilon 1}H$ resonances from amide proton resonances as applied to proteins by Davis et al. (1994). The residual HDO signal was suppressed by presaturating the water resonance during a relaxation decay of 2.5 s. Enzyme concentration (R67H HCA III) was 1.0 mM with spectra recorded at 128 transients. Chemical shifts were measured from 2,2-dimethyl-2-silapentane-5-sulfonate as internal standard. For pH titrations of the histidine $C^{\epsilon 1}H$ resonance, uncorrected pH meter readings were recorded before and after NMR spectra were made, with spectra taken every 0.2−0.4 pH units. The pK_a was determined by nonlinear least-squares analysis of the chemical shift data (ENZFITTER; Leatherbarrow, 1987).

RESULTS

pK_a of the Zinc-Bound Water. The ratio k_{cat}/K_m for hydration of CO_2 catalyzed by carbonic anhydrase contains the rate constants of the pathway through the first irreversible step, which is the departure of HCO_3^- (Silverman and Lindskog, 1988); thus, this ratio contains the steps of eq 1 and is not expected to include the proton transfer steps of eq 2. The values of k_{cat}/K_m reported in Figure 1 were determined by ^{18}O exchange using solutions to which no buffers had been added. In each case the values of maximal k_{cat}/K_m and the apparent pK_a for catalysis were also measured by stopped-flow; these values agreed to within 30% for maximal k_{cat}/K_m and to within 0.3 units in pK_a. Since the catalysis of the hydration of CO_2 depends on the fraction of total active sites present as the zinc-bound hydroxide, the pH profile for k_{cat}/K_m gives an estimate of the pK_a for the zinc-bound water (Simonsson & Lindskog, 1982). In wild-type HCA III, this pK_a has not been observed because of enzyme denaturation at low pH. An estimate of pK_a 5 for this metal-bound water in CA III has been obtained by spectrometric methods using Co(II)-substituted bovine CA III (Ren et al., 1988). Silverman et al. (1993) have placed this pK_a at 4.3 based on extrapolation of observed linear free energy plots; to be consistent with the Brønsted plot of that work, we use the same pK_a in Table 1 and Figure 3. There is evidence observed at 10 °C for a pK_a near 5.0 for wild-type HCA III, as shown in Figure 1A. For R67H HCA III

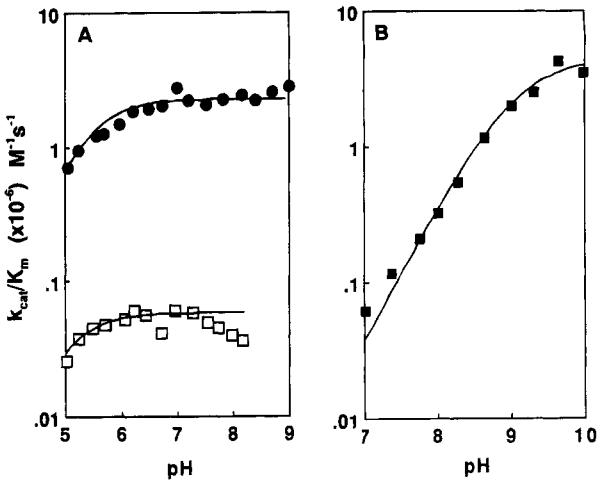

FIGURE 1: pH dependence of k_{cat}/K_m for the hydration of CO_2 catalyzed by (A, left) wild-type HCA III at 10 °C (□) and R67H HCA III at 25 °C (●); (B, right) K64A/R67H/F198D HCA III at 25 °C (■). Values were determined by ^{18}O exchange between CO_2 and water using solutions containing no external buffers. The total ionic strength of solution was maintained at 0.2 M by addition of Na_2SO_4. The solid lines are least-squares fits of the data with values of pK_a given in Table 1, with the exception of HCA III for which the solid line describes a single ionization of pK_a 5.0.

the pH profile of k_{cat}/K_m for hydration of CO_2 shows evidence of an ionization below pH 6 and provides an estimate of pK_a 5.3 ± 0.2 for the zinc-bound water (Figure 1A). For the mutant K64A/R67H/F198D HCA III, the value of the pK_a for the zinc-bound water was determined at 9.1 ± 0.1 (Figure 1B).

In this manner, we estimated values of pK_a for the zinc-bound water in a number of mutants of HCA III with His replacing Arg 67; these values are given in Table 1. The mutant K64A/R67H/F198L HCA III was able to catalyze the hydrolysis of 4-nitrophenyl acetate at an appreciable rate; in this case, the value of pK_a 6.5 ± 0.1 obtained agreed with that in Table 1.

pK_a of the Proton Donor Group. The rate constant R_{H_2O}/[E] for the release from the enzyme of $H_2{}^{18}O$ is limited in rate by the transfer of a proton to the zinc-bound hydroxide, as in eq 2, for both isozymes II and III (Silverman et al., 1993; Silverman, 1982). In these ^{18}O-exchange studies done in the absence of external buffer, this proton must come from another residue of the enzyme or possibly water in the active site.[2] For wild-type HCA III, R_{H_2O}/[E] at approximately 3 × 10^3 s^{-1} showed no pH dependence in the range of pH 6−9 (Figure 2A; Jewell et al., 1991; Tu et al., 1983). For R67H HCA III there was a strong pH dependence with as much as a 10-fold activation compared with wild-type (Figure 2A); K64A/R67H/F198D HCA III also showed a strong pH dependence (Figure 2B).

Equation 4 represents R_{H_2O}/[E] as dependent on the unprotonated form of the acceptor and protonated form of the donor, as in eq 2.

$$R_{H_2O}/[E] = k_B/\{(1 + K_B/[H^+])(1 + [H^+]/K_E)\} \quad (4)$$

[2] The substrate bicarbonate (pK_a 10.3) as proton donor to HCA III remains a possibility although a small rate would be anticipated as estimated from the Brønsted plot. Other residues of the enzyme with a high pK_a, such as Tyr 7 or Lys 132, are possibilities as intramolecular donors with small proton transfer rates.

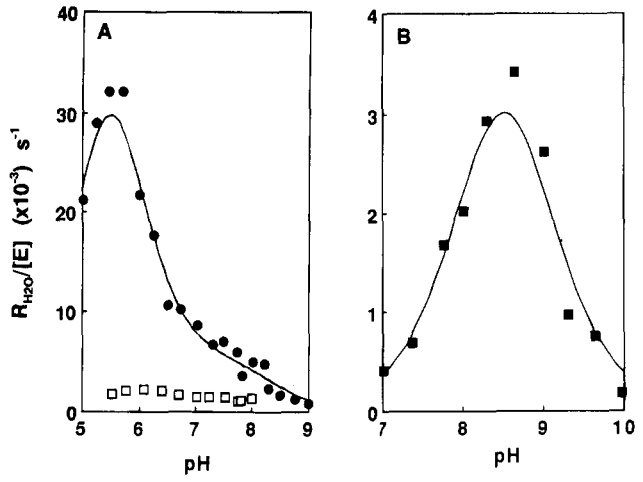

FIGURE 2: pH dependence at 25 °C of $R_{H_2O}/[E]$ the rate constant for release from the active site of $H_2{}^{18}O$ catalyzed by (A, left) wild-type HCA III (□) and R67H HCA III (●); (B, right) K64A/R67H/F198D HCA III (■). Values were determined by ^{18}O exchange between CO_2 and water using solutions containing no external buffers. The total ionic strength of solution was maintained at 0.2 M by addition of Na_2SO_4. The solid lines are least-squares fits of eq 4 to the data with values of pK_a for the zinc-bound water held at the values given in Table 1. Notice the different scales on the ordinate.

K_E is the ionization constant of the zinc-bound water and 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. Previous work shows the application of eq 4 to HCA II (Tu & Silverman, 1985) and to mutants of HCA III containing the replacement Lys 64 → His (Silverman et al., 1993). A least-squares fit of eq 4 to the data for $R_{H_2O}/[E]$ catalyzed by R67H HCA III (Figure 2A) resulted in a pK_a for the proton donor, His 67, of 5.6 ± 0.1; in this fit the pK_a of the zinc-bound water was held at the value found from k_{cat}/K_m for hydration and given in Table 1. The value of the pK_a of the side chain of His 67 was consistent with a comparison of the 600 MHz ^1H NMR spectrum of R67H HCA III with wild-type HCA III. This comparison confirmed the presence of a $C^{\epsilon 1}H$ resonance of histidine in R67H HCA III which titrated with a pK_a < 5.7 (7.86 ppm from internal 2,2-dimethyl-2-silapentane-5-sulfonate at pH 7 to 9) which was not present in the wild-type enzyme; protein denaturation at pH < 6.0 for the conditions of these NMR experiments precluded a precise measurement of this pK_a. For K64A/R67H/F198D HCA III the data of Figure 2B resulted in a pK_a for the proton donor, His 67, of 8.0 ± 0.2 with the pK_a for the zinc-bound water held at the value obtained from k_{cat}/K_m (Table 1).

The pH profiles for $R_{H_2O}/[E]$ were measured for additional mutants listed in Table 1. In each case, the pK_a of His 67 as well as k_B was determined by least-squares fit of eq 4 to the data for ^{18}O exchange measured in the absence of buffers; again, in these determinations the pK_a of the zinc-bound water was fixed at the value determined from the pH profile of k_{cat}/K_m for hydration of CO_2.

Although the situation is complicated by the necessity of external buffers in solution, the steady-state turnover number k_{cat} for hydration can also indicate the presence of proton acceptors in the catalysis (Silverman & Lindskog, 1988). For R67H HCA III, k_{cat} for hydration showed a plateau at 2 × 10^4 s^{-1} in the range of pH 6−8, to be compared with k_{cat} near 2 × 10^3 s^{-1} for wild-type HCA III (Jewell et al., 1991). Identification from k_{cat} of proton donor groups effective at pH > 8 has not been successful; there is an increment in k_{cat} of unknown source observed at pH > 8 for HCA III (Jewell et al., 1991) and for the mutants of Table 1 that masks effects due to the introduced proton donor groups in the active-site cavity.

One variant, N62D/K64A/R67H HCA III, had a pH profile of $R_{H_2O}/[E]$ too complex to be explained by the presence of a single proton donor group and was not included in Table 1. Another mutant, K64A/T65D/R67H/F198L HCA III was insufficiently stable for activity measurements under the conditions of Figure 2.

Rate Constants for Proton Transfer. The rate constants k_B for intramolecular proton transfer to the zinc-bound hydroxide were determined by least-squares fit of eq 4 to pH profiles of $R_{H_2O}/[E]$. The resulting rate constants are given in Table 1. For one mutant, K64A/R67H HCA III, we further confirmed the influence of His 67 using chemical modification by acrolein using the procedures of Pocker and Janjic (1988); this modification formylethylates exposed histidine and lysine residues. The resulting modified enzyme had ^{18}O-exchange constant k_B of 2 × 10^3 s^{-1} with a pH profile for $R_{H_2O}/[E]$ independent of pH in the range of 5.5−8.5, very similar to wild-type HCA III (Jewell et al., 1991).

DISCUSSION

A histidine at position 67 in HCA III can donate a proton to the zinc-bound hydroxide in the dehydration pathway, as was the case with histidine at position 64 in HCA III (Jewell et al., 1991). The following results support this conclusion. The proton transfer-dependent rate constant for the release of $H_2{}^{18}O$ from the active site, $R_{H_2O}/[E]$, for R67H HCA III was dependent on pH with a maximum at pH 5.5 that was 10-fold greater than that of wild-type HCA III (Figure 2A). Consistent with this result was the observation that the values of k_{cat} for hydration of CO_2 catalyzed by R67H HCA III were larger by approximately 10-fold than those of the wild-type HCA III. Moreover, chemical modification with acrolein of histidines in K64A/R67H HCA III abolished this activation with the chemically modified enzyme having kinetic constants similar to wild-type HCA III.

As described in Results, we have used eq 4 applied to pH profiles of ^{18}O-exchange to estimate the rate constants k_B for intramolecular proton transfer from His 67 to the zinc-bound hydroxide, with data presented in Table 1 and in the Brønsted plot of Figure 3. These values of k_B with His 67 as proton donor occur for enzymes with ΔpK_a close to zero and therefore are expected to be located at the maximum of the Brønsted plot (Bergman & Kresge, 1978; Cox & Jencks, 1978). We were not successful in preparing mutants that showed evidence for a single proton donor group outside the region $-2 < \Delta pK_a < 1$. The values of k_B in Table 1 form a free energy plot (Figure 3) for intramolecular proton transfer from His 67 to zinc-bound hydroxide that is qualitatively similar to proton transfer from His 64, which is shown as the dotted line on Figure 3 and is based on the data of Silverman et al. (1993). As with proton transfer from His 64, the data for His 67 can be described by Marcus rate theory.

The Marcus rate theory applied to proton transfer (Marcus, 1968; Kresge, 1975) describes the overall activation energy

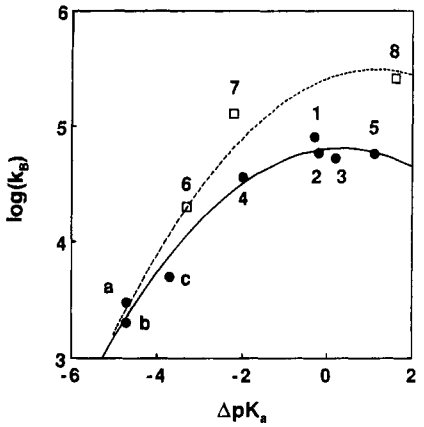

FIGURE 3: Dependence of the logarithm of k_B (s^{-1}) on ΔpK_a (pK_a-[zinc-bound water] − pK_a[donor group]) for mutants of HCA III. The entries are wild-type and mutants of HCA III numbered as in Table 1. Values of k_B and pK_a were obtained by application of eq 4 to the data for R_{H_2O}/[E]. The solid line is a least-squares fit of the Marcus equation (eq 5) to the entries of Table 1 giving $\Delta G_0^{\ddagger}$ = 1.3 ± 0.3 kcal/mol with work terms w^r = 10.9 ± 0.1 and w^p = 5.9 ± 1.1 kcal/mol. The dotted line is a comparable fit for His 64 as proton donor (including buffer imidazole) as determined from the data of Silverman et al. (1993). Points 6, 7, and 8 are from Silverman et al. (1993) and describe k_B for wild-type HCA III, R67N HCA III, and F198D HCA III, respectively, with 100−150 mM imidazole in solution as proton donor.

$\Delta G^{\ddagger}$ in terms of the standard free energy of reaction with the required active site conformation $\Delta G_R°$ and an intrinsic energy barrier $\Delta G_o^{\ddagger}$, which is the value of $\Delta G^{\ddagger}$ when $\Delta G_R°$ = 0.

$$\Delta G^{\ddagger} = w^r + \{1 + \Delta G_R°/4\Delta G_o^{\ddagger}\}^2 \Delta G_o^{\ddagger} \qquad (5)$$

The standard free energy of reaction $\Delta G_R°$ with the required conformation is then related to the measured overall free energy for the reaction by work terms: $\Delta G° = w^r + \Delta G_R° - w^p$. The work term w^r is the energy required to align acceptor, donor, and surrounding water for facile proton transfer. The energy w^p is this work term for the corresponding reorganization for the reverse process.

The solid line in Figure 3 is a least-squares fit of eq 5 to all of the values of k_B in Table 1 with entries weighted by the inverse of the variance of each point. The intrinsic energy barrier for the intramolecular proton transfer resulting from this fit is $\Delta G_0^{\ddagger}$ = 1.3 ± 0.3 kcal/mol with work terms w^r = 10.9 ± 0.1 and w^p = 5.9 ± 1.1 kcal/mol. This fit includes points a, b, and c of Table 1 which do not contain histidine at position 67, for which the proton donors have not been determined,[2] and for which the pK_a of the zinc-bound water is uncertain. When these points are omitted, a fit of the data yields $\Delta G_0^{\ddagger}$ = 1.2 ± 0.7 kcal/mol with work terms w^r = 10.9 ± 0.1 and w^p = 6.0 ± 2.9 kcal/mol.

These results are very similar to those obtained with mutants of HCA III containing the replacement Lys 64 → His (Silverman et al., 1993). For the case of intramolecular proton transfer from His 64 to zinc-bound hydroxide in mutants of HCA III the constants from the fit to the Marcus equation are $\Delta G_0^{\ddagger}$ = 1.4 ± 0.3 kcal/mol with work terms w^r = 10.0 ± 0.2 and w^p = 5.9 ± 1.1 kcal/mol. For both cases of histidine at 64 or 67, the relatively low magnitude of $\Delta G_0^{\ddagger}$ is comparable to that found for the bimolecular proton transfers between nitrogen and oxygen acids and bases in solution (near 2 kcal/mol; Kresge, 1975). The substantial energy needed to achieve proton transfer in these mutants is indicated by the work term $w^r \approx$ 10 kcal/mol for the dehydration direction. This corresponds to the energy required to align the donor and acceptor groups and the water between them into an appropriate orientation for the proton transfer. It is unlikely that these work terms contain energy to desolvate the donor and acceptor groups since too great a distance separates them for direct proton transfer to occur. More likely, the work terms contain energy required to orient the side chain of histidine and establish the hydrogen-bonded water network between the donor and acceptor through which the protons transfer.

The solvent hydrogen isotope effects on k_B measured for two mutants containing His 67 were also compared with the data for mutants containing His 64. These isotope effects [$(k_B)_{H_2O}/(k_B)_{D_2O}$] were 4.1 ± 0.4 for R67H HCA III and 3.7 ± 0.4 for K64A/R67H/F198L HCA III and fall on the curve describing the fit of Marcus rate theory to the isotope effects for mutants containing His 64 [Figure 4 of Silverman et al. (1993)]. These results are consistent with the observed qualitative similarity of proton transfer from histidine at positions 64 and 67.

Although the data of Figure 3 show that proton donation from histidine at residue 64 and 67 are qualitatively similar, it is clear that the maximal level of the rate constant k_B is less for proton donation from His 67 by about 4-fold (Figure 3). This is emphasized in experiments, also shown in Figure 3 (points 6−8), in which catalysis by mutants lacking a histidine proton donor were enhanced by 100−150 mM imidazole in solution [wild-type, R67N, and F198D HCA III, data from Table 1 of Silverman et al. (1993)]. These data fall on the Brønsted curve for His 64 as donor rather on the curve for His 67 emphasizing that His 67 is not as efficient in proton transfer as imidazolium ion in solution; in contrast, His 64 is as efficient as imidazolium ion in solution.

The intrinsic energy barriers $\Delta G_0^{\ddagger}$ for proton transfer from His 67 and His 64 are indistinguishable with values near 1.3 kcal/mol. The difference in these proton transfers is more apparent in the work functions, for which w^r is significantly greater for proton transfer from His 67 (10.9 ± 0.1 kcal/mol) compared with His 64 (10.0 ± 0.2 kcal/mol). The greater value of w^r for proton donation by His 67 indicates a less favorable pre-equilibrium; its position in the active site is less favorable for alignment of donor and acceptor and intervening water. However, once an appropriate alignment is achieved, the equivalent values of $\Delta G_0^{\ddagger}$ indicate that the intrinsic energy barrier for proton transfer is the same from His 67 as from His 64.

Application of the Marcus rate theory in this manner assumes that other parameters of the system do not change as a result of mutations near the active site. The mutations discussed in this work are located in the active site cavity with side chains extended into the cavity. One measure of their influence at the active site itself is the change caused in the steady-state rate k_{cat}/K_m containing the rate constants of eq 1, the interconversion of CO_2 and HCO_3^-. The data of Table 2 demonstrate that the mutations we have made at residues 67 and 198 are additive in their effects on k_{cat}/K_m; that is, these are noninteracting residues. Table 2 is based on double-mutant cycles (Carter et al., 1984) and is presented in the format and nomenclature of Mildvan et al. (1992), with the exception that we are observing enzyme activations

Table 2: Changes in Free Energies of Activation Obtained from Maximal Values of k_{cat}/K_m for the Hydration of CO_2 and Maximal Values of k_B, the Rate Constant for Proton Transfer between His 67 and Zinc-Bound Hydroxide, in Mutants of Carbonic Anhydrase III Each Containing the Replacement Lys 64 → Ala (i.e., K64A)[a]

mutation 1	mutation 2	ΔG_1[b] (kcal/mol)	ΔG_2[c] (kcal/mol)	ΔG_{1+2}[d] (kcal/mol)	$\Delta\Delta G_A$[e] (kcal/mol)	$\Delta\Delta G_B$[f] (kcal/mol)	category
			Data Obtained from k_{cat}/K_m				
R67H	F198D	−0.7	−1.1	−1.7	−1.0	0.1	additive
R67H	F198L	−0.7	−2.1	−2.7	−2.0	0.1	additive
			Data Obtained from k_B				
R67H	F198D	−2.0	−1.0	−2.0	0.0	1.0	none
R67H	F198L	−2.0	−1.0	−1.7	0.3	1.3	none

[a] This table uses the format and notation of Mildvan et al. (1992). [b] Free energy change for the first replacement; for example, the first entry is $\Delta G_1 = -0.7$ kcal/mol $= -RT \ln[(k_{cat}/K_m)_{Ala64/His67}/(k_{cat}/K_m)_{Ala64}]$. [c] Free energy change for the second replacement; for example, the first entry is $\Delta G_2 = -1.1$ kcal/mol $= -RT \ln[(k_{cat}/K_m)_{Ala64/Asp198}/(k_{cat}/K_m)_{Ala64}]$. [d] Free energy change for the double replacement; for example, the first entry is $\Delta G_{1+2} = -1.7$ kcal/mol $= -RT \ln[(k_{cat}/K_m)_{Ala64/His67/Asp198}/(k_{cat}/K_m)_{Ala64}]$. [e] $\Delta\Delta G_A = \Delta G_{1+2} - \Delta G_1$. [f] $\Delta\Delta G_B = \Delta G_{1+2} - \Delta G_1 - \Delta G_2$.

in each case. These activations are linked to the enhancement of the pK_a of the zinc-bound water which is linearly correlated with the logarithm of k_{cat}/K_m in mutants of HCA III containing replacements at 198 (LoGrasso et al., 1993).

Table 2 demonstrates that, unlike k_{cat}/K_m, the contributions to the rate constants for proton transfer k_B were not additive. These fell in a category labeled "none" (Mildvan et al., 1992) in which mutation 2 has no effect in the double mutant beyond that found with mutation 1 alone, even though mutation 2 alone enhanced activity. This is another consequence of the location of our mutants near the maximum in the Marcus plot of Figure 3, a region in which there is a small dependence of k_B on ΔpK_a. That is, the second mutation alters the pK_a of the zinc-bound water but results in no additional change in k_B. Together, these double-mutant cycles on k_{cat}/K_m and k_B demonstrate a well-behaved system with no indication of cooperative or opposing structural effects on the catalysis or proton transfer.

The replacement of Asn 67 → His in HCA II, among the most efficient of the carbonic anhydrases, has been studied by steady-state measurements in a mutant H64A−N67H HCA II which exhibits proton transfer capability 5−20% that of wild-type HCA II, determined from k_{cat} and depending on the buffer used (Liang et al., 1993). In this case of a mutant of HCA II, His 67 is also a proton transfer group but again less efficient than the His 64 of wild-type HCA II. Widely different effects of similar or mirror mutations in HCA II and III have already been noted (LoGrasso et al., 1993; Ren et al., 1991) so different results from similar mutations in isozymes II and III is not unexpected. However, in this case proton donation from His 67 occurs in mutants of both isozyme II and III. It is also notable that histidine 67 appears in wild-type HCA I (with a pK_a of 6.0; Campbell et al., 1974) in which it, along with His 200, may have a proton transfer role, albeit less efficient than His 64 in HCA II (Behravan et al., 1990).

Conclusion. The intramolecular proton transfer from His 67 to zinc-bound hydroxide in carbonic anhydrase III and site-specific mutants was described by the Marcus rate theory. The intrinsic energy barrier for this proton transfer was quite small at about 1.3 kcal/mol; a work term possibly including the energy required to align the pertinent groups on the protein and to reorganize water molecules near the active site was larger near 11 kcal/mol. These data are qualitatively similar to those from a comparable study of intramolecular proton transfer utilizing His 64 in HCA III. When considered in detail, transfer via His 67 was less efficient than via His 64 due to a higher energy required for solvent and active site reorganization; the intrinsic energy barriers for the proton transfers from these two sites were equivalent.

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