Properties of Intramolecular Proton Transfer in Carbonic Anhydrase III

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ABSTRACT We investigated the efficiency of glutamic acid 64 and aspartic acid 64 as proton donors to the zinc-bound hydroxide in a series of site-specific mutants of human carbonic anhydrase III (HCA III). Rate constants for this intramolecular proton transfer, a step in the catalyzed dehydration of bicarbonate, were determined from the proton-transfer-dependent rates of release of $H_2{}^{18}O$ from the enzyme measured by mass spectrometry. The free energy plots representing these rate constants could be fit by the Marcus rate theory, resulting in an intrinsic barrier for the proton transfer of $\Delta G_o^{\ddagger} = 2.2 \pm 0.5$ kcal/mol, and a work function or thermodynamic contribution to the free energy of reaction $w^r = 10.8 \pm 0.1$ kcal/mol. These values are very similar in magnitude to the Marcus parameters describing intramolecular proton transfer from His^{64} and His^{67} to the zinc-bound hydroxide in mutants of HCA III. That result and the equivalent efficiency of Glu^{64} and Asp^{64} as proton donors in the catalysis by CA III demonstrate a lack of specificity in proton transfer from these sites, which is indirect evidence of a number of proton conduction pathways through different structures of intervening water chains. The dominance of the thermodynamic contribution or work function for all of these proton transfers is consistent with the view that formation and breaking of hydrogen bonds in such water chains is a limiting factor for proton translocation.

INTRODUCTION

There are at least seven functional isozymes of carbonic anhydrase in the α class that includes the human and animal carbonic anhydrases (Hewett-Emmett and Tashian, 1996). These enzymes provide an informative model for the study of proton transfer steps, because the overall catalysis contains rate-limiting proton transfers that can be both inter- and intramolecular, depending on conditions. These proton transfer steps have been subject to considerable study in determining the catalytic pathway of carbonic anhydrase (Christianson and Fierke, 1996; Silverman and Lindskog, 1988). The pathway for the dehydration of HCO_3^- catalyzed by carbonic anhydrase consists of two distinct and separate stages. The first is the binding of HCO_3^- to the form of the active site containing a zinc-bound water molecule, followed by conversion of HCO_3^- into CO_2 and its dissociation from the enzyme (Eq. 1). The departure of CO_2 leaves a zinc-bound hydroxide at the active site. The second stage comprises the series of proton transfer steps required to regenerate the zinc-bound water (Eq. 2). Here B is buffer in solution, and H^+ to the left of E indicates one or more shuttle residues of the enzyme itself that transfer protons between the zinc-bound water and buffer in solution. In carbonic anhydrase II (CA II), among the most efficient of the carbonic anhydrase isozymes, the predominant proton shuttle residue has been identified as His^{64} (Steiner et al., 1975; Tu et al., 1989).

$$HCO_3^- + EZnH_2O \rightleftarrows CO_2 + EZnOH^- + H_2O \quad (1)$$

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

The rate-limiting step in the overall maximum velocity of catalysis by CA II at steady state is nearly entirely dominated by the intramolecular proton transfer between the proton shuttle and the aqueous ligand of the zinc, provided there is an excess of buffer in solution (>25 mM) (Rowlett, 1984; Lindskog, 1984).

Carbonic anhydrases II, III, and V, three isozymes in the α class, share common features of catalysis and structure. They are all monomers of molecular mass near 30 kDa with one zinc per monomer and contain from 30% to 60% amino acid identity (Tashian, 1989). Moreover, their crystal structures are nearly superimposable, especially near the active site; for example, bovine CA III and human CA II have an rms difference in all backbone atoms of less than 1 Å (Eriksson and Liljas, 1993). In human CA II, the side chain of the proton shuttle His^{64} is located ~8 Å from the zinc, too distant for direct proton transfer. However, the crystal structures demonstrate an array of apparently hydrogen-bonded water molecules between His^{64} and the zinc-bound hydroxide (Eriksson et al., 1988; Scolnick and Christianson, 1996), which could serve as a proton wire for the translocation of protons, in a manner similar to that described for the water channel formed by gramicidin (Nagle and Morowitz, 1978; Pomès and Roux, 1996). Solvent hydrogen isotope effects on k_{cat} for the hydration of CO_2 by CA II are consistent with a role for this proton wire in catalysis (Venkatasubban and Silverman, 1980). Recently, the disruption of this water structure in the active-site cavity of isozyme II by the adjacent residue at position 65 has been

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shown to reduce proton transfer in catalysis (Jackman et al., 1996; Scolnick and Christianson, 1996).

Carbonic anhydrases III and V lack histidine at position 64 and are less active than CA II, in part because of their slower proton transfer between the active site and solution. However, in these two cases, human CA III (HCA III) (Jewell et al., 1991) and murine CA V (Heck et al., 1996), it has been possible to enhance maximum velocity by 7- to 50-fold by introduction of His^{64} through site-specific mutagenesis. Another property of CA III has allowed a greater breadth of study of the catalysis; the pK_a of the zinc-bound water molecule for HCA III is increased by the replacement of Phe^{198} by Leu or other residues (LoGrasso et al., 1991, 1993). Such substitutions alter the pK_a of the zinc-bound water in mutants of HCA III from near 5 to 9, and hence provide a sufficient range of pK_a values to construct a free energy plot (Silverman et al., 1993). Application of Marcus rate theory (Marcus, 1968; Kresge, 1975) to such a plot for mutants of HCA III containing His^{64} showed that the intrinsic kinetic barrier is small, near 1.5 kcal/mol, for intramolecular proton transfer between His^{64} and the zinc-bound water and analogous in magnitude to nonenzymic, bimolecular proton transfer between nitrogen and oxygen acids and bases (Kresge, 1975). The predominant energy required for the proton transfer came from the components of the free energy of reaction called work functions. These are in the range of 5–10 kcal/mol for variants of CA III and have been interpreted as the energy required to orient the proton donor and acceptor groups as well as the water in the active site for facile proton transfer (Silverman et al., 1993). Ren et al. (1995) continued this work by preparing mutants of HCA III containing the replacement $Arg^{67} \rightarrow His$; this site has a distance from the zinc approximately equivalent to that of position 64. The resulting Marcus parameters for intramolecular proton transfer from His^{67} to the zinc-bound hydroxide were very similar to those from His^{64}.

In this study we have extended this work to glutamic and aspartic acids at position 64 of HCA III. This allowed us the opportunity to investigate proton transfer from neutral proton donors of pK_a lower than that of the imidazolium cation of histidine. We found that the free energy plot for these rate constants could be adequately described by the Marcus rate theory. The resulting Marcus parameters for proton donation to the zinc-bound hydroxide by aspartic acid and glutamic acid at position 64 were very similar to those for His at 64 and His at 67 as proton donors. This indicates a significant capacity at the active site to accommodate a number of proton donors from at least two sites and suggests multiple proton transfer pathways of roughly equivalent efficiency through the water structure formed between these donors and the zinc-bound hydroxide.

MATERIALS AND METHODS

Enzymes

Site-specific mutants of human carbonic anhydrase III were prepared and purified as described earlier (Qian et al., 1997; Ren et al., 1995). Bacterial expression vectors based on the T7 expression vectors of Studier et al. (1990) were optimized for convenient site-directed mutagenesis and protein expression and were used as described by Tanhauser et al. (1992). Mutations were confirmed by DNA sequencing of the expression vector used to produce each mutant carbonic anhydrase. A two-step purification using gel filtration and ion exchange chromatography (Tu et al., 1986) resulted in enzymes with greater than 95% purity as determined by gel electrophoresis. Concentrations of wild-type HCA III and mutants were determined from the molar absorptivity of $6.2 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm (Engberg et al., 1985). For mutants of HCA III with the replacement $Phe^{198} \rightarrow$ Leu, potent inhibition with ethoxzolamide was observed ($K_I < 10^{-8}$ M), and we were able to determine enzyme concentrations by titration with this inhibitor, which were in excellent agreement with the absorptivity given above. The mutant Y64H/F65A murine CA V was prepared and purified as described by Heck et al. (1996). This was a truncated form identified as MCA Vc by Heck et al. (1994); in a sequence numbering scheme consistent with CA II and III, the expressed variant of MCA V began at residue 22, Ser.

Oxygen-18 exchange

The catalyzed rates 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 were measured by mass spectrometry. This isotope exchange method is carried out at chemical equilibrium and can be performed without buffers added to solution, because pH control is not a major problem. Two independent rates are determined in this method (Silverman, 1982; Koenig and Brown, 1981). The first is R_1, the rate of exchange of HCO_3^- and CO_2, as shown in Eq. 3:

$$HCOO^{18}O^- + EZnH_2O \rightleftarrows EZn^{18}OH^- + CO_2 + H_2O \quad (3)$$

$$H^+EZn^{18}OH^- \overset{k_B}{\rightleftarrows} EZn^{18}OH_2 \overset{H_2O}{\rightleftharpoons} EZnH_2O + H_2{}^{18}O \quad (4)$$

The substrate dependence of R_1 is given by an expression in the form of the Michaelis-Menten equation (Ren et al., 1995). The second rate is R_{H2O}, the proton-transfer-dependent rate of release from the active site of water bearing substrate oxygen, as shown in Eq. 4. 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_{H2O} are separate and distinct from those of the interconversion of CO_2 and HCO_3^- in Eq. 3. The rate constant k_B, describing intramolecular proton transfer from the donor group to zinc-bound hydroxide, was obtained by a nonlinear least-squares fit of Eq. 5 to the pH profiles for $R_{H2O}/[E]$. Equation 5 represents $R_{H2O}/[E]$ as dependent on the unprotonated form of the acceptor, zinc-bound hydroxide, and the protonated form of the donor, as in Eq. 4:

$$R_{H2O}/[E] = k_B/\{(1 + (K_a)_{donor}/[H^+])(1 + [H^+]/(K_a)_{ZnH2O})\} \quad (5)$$

$(K_a)_{ZnH2O}$ is the ionization constant of the zinc-bound water, and $(K_a)_{donor}$ 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 showed the application of Eq. 5 to HCA II (Silverman, 1982) and to mutants of HCA III containing the replacement $Lys^{64} \rightarrow$ His (Silverman et al., 1993) and $Arg^{67} \rightarrow$ His (Ren et al., 1995).

Measurements of the isotopic content of CO_2 were made using an Extrel EXM-200 mass spectrometer. Solutions contained 25 mM total substrate ($[CO_2] + [HCO_3^-]$) and 25 μM EDTA, but no buffers were added. Total ionic strength of solution was maintained at a minimum of 0.2 M with Na_2SO_4, and the temperature was 25°C.

RESULTS

Table 1 presents values of the rate constant k_B for intramolecular proton transfer from donor groups to the zinc-bound

TABLE 1 Rate constants, k_B, and corresponding pK_a values for proton transfer between the donors listed and the zinc-bound hydroxide in human carbonic anhydrase III*

Entry on Fig. 2	Enzyme	pK_a (donor)	pK_a (ZnH_2O)	k_B ($\times 10^{-3}$ s^{-1})
Basic group(s) of $pK_a \geq 8$ is proton donor				
a	Wild type[#]	$\gtrsim$9.0[§]	4.3[¶]	3[‖]
b	K64A	$\gtrsim$9.0[§]	4.3[¶]	2[‖]
c	R67N	$\gtrsim$9.0[§]	5.3[¶]	5[‖]
d	K64E/R67N	7.9 ± 0.2	5.2 ± 0.2	15 ± 1
Glu64 or Asp64 is proton donor				
1	K64E**	6.4 ± 0.3	5.3 ± 0.2	38 ± 8
2	K64D**	5.7 ± 0.2	~5.5	57 ± 3
3	K64E/F198V	6.3 ± 0.1	5.3 ± 0.1	27 ± 4
4	K64E/F198L**	5.8 ± 0.2	5.8 ± 0.2	48 ± 19
5	K64E/F198D	6.6 ± 0.1	8.7 ± 0.1	71 ± 26
6	K64D/F198D	6.9 ± 0.2	8.6 ± 1.1	51 ± 15
His64 is proton donor				
	K64H/R67N/F198L[##]	6.8	6.8	280 ± 50
	Wild-type HCA II	7.2 ± 0.2	6.8 ± 0.2	800 ± 40
	Y64H/F65A MCAV[§§]	6.5 ± 0.2	6.4 ± 0.2	400 ± 100

*Except the last two entries, which are human CA II and a murine CA V variant. These values were determined from the catalyzed rates of exchange of ^{18}O between CO_2 and water. Data were obtained by ^{18}O exchange at 25°C in the absence of buffers; total ionic strength of solution was maintained at 0.2 M by addition of the appropriate amount of Na_2SO_4. Rate constants k_B were determined by a least-squares fit of Eq. 5 to the data for R_{H20}, the rate of release of $H_2{}^{18}O$ from the enzyme.

[#]From Jewell et al. (1991).

[§]Proton donors in these cases are uncertain and possibly include Lys^{64}, Lys^{131}, Lys^{170}, and Tyr^{7}.

[¶]Because of enzyme denaturation at pH near 5, we were not able to observe the pK_a for the zinc-bound water in these variants. The values of pK_a listed were estimated from a linear free energy plot as described in Silverman et al. (1993).

[‖]The standard errors were less than ±20%.

**From Qian et al. (1997).

[##]From Silverman et al. (1993).

[§§]This variant of murine CA Vc was reported by Heck et al. (1996).

hydroxide in mutants of carbonic anhydrase, as well as estimates of the values of the pK_a of the donor and acceptor groups obtained from ^{18}O exchange experiments. These values of k_B were obtained by application of Eq. 5 to the ^{18}O exchange data, expressed as R_{H2O}/[E]. The data of Table 1 are presented according to the proton donor groups; data establishing Glu^{64} and Asp^{64} as proton donors in HCA III are given by Qian et al. (1997), and data establishing His^{64} as a proton donor for isozymes II, V, and III are given, respectively, by Tu et al. (1989), Heck et al. (1996), and Jewell et al. (1991). Catalytic constants for the following mutants are newly examined in this work and are presented in Table 1: K64E/R67N, K64E/F198V, K64E/F198D, and K64D/F198D.

In many cases for which the pK_a values of the donor group and the zinc-bound water were in the range of 6–8, the pH profiles for R_{H2O}/[E] were bell-shaped and, utilizing Eq. 5, yielded values of the pK_a of the donor and acceptor groups, as well as the rate constant for intramolecular proton transfer k_B (see, for example, Qian et al., 1997, and Silverman et al., 1993). We confirmed the value of the pK_a of the zinc-bound water molecule by measuring the pH dependence of k_{cat}/K_m obtained by ^{18}O exchange (Qian et al., 1997). The pK_a for k_{cat}/K_m is an accurate estimation of the ionization of the zinc-bound water (Simonsson and Lindskog, 1982), because it is the zinc-bound hydroxide that is the catalytic group in the hydration of CO_2 (Eq. 1).

For wild-type HCA III and several of its variants (entries a–c in Table 1), the pK_a of the zinc-bound water was near 5 and the pK_a of the donor groups was close to or above 9. In these cases the values of R_{H2O}/[E] were observed to be rather independent of pH (see, for example, figure 2 of Qian et al., 1997). From such data we gain an accurate estimate of k_B; but in the absence of other information, these experiments provide a poor estimate of the values of the pK_a of the proton donor and acceptor groups. In these cases, we have made estimates of the pK_a of the zinc-bound water by extrapolation of a linear free energy plot for k_{cat}/K_m for hydration observed for different mutants versus pK_a of the zinc-bound water; we estimated the pK_a of the zinc-bound water at 4.3 for wild-type HCA III (Silverman et al., 1993). Qian et al. (1997) have extended pH profiles to pH 5 and estimate a value closer to 5.3 ± 0.2 for the pK_a of the zinc-bound water in HCA III. The variants of this group (Table 1, entries a–c) also show evidence for a proton donor or donors of pK_a near or above 9, which is also observed by stopped flow (Jewell et al., 1991), and which establishes an intramolecular proton transfer rate constant 2–5 $\times 10^3$ s^{-1}. The identity of these basic donor groups has not been determined.

The variant K64E/R67N HCA III requires additional comment. We prepared this assuming that Glu^{64} would participate as a proton donor, as in the variants containing Glu^{64} described by Qian et al. (1997). Both of the rate

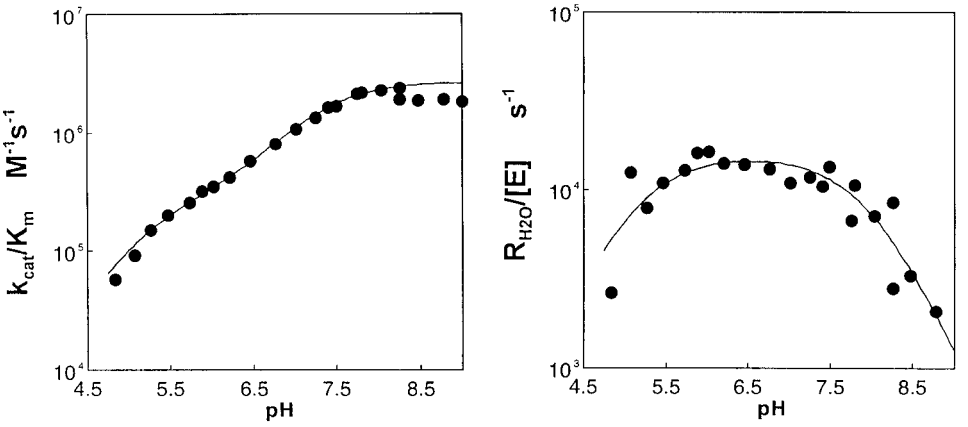

FIGURE 1 (*Left*) The constant k_{cat}/K_m for the hydration of CO_2 catalyzed by the mutant K64E/R67N HCA III. Data were obtained by the ^{18}O exchange method at 25°C in the absence of buffers. The total concentration of all species of CO_2 was 25 mM, and the total ionic strength of the solution was maintained at 0.2 M by the addition of the appropriate amounts of Na_2SO_4. The solid line is a least-squares fit representing the sum of two ionizations with $pK_a = 7.3 \pm 0.1$ and 5.3 ± 0.3, with maximum values of k_{cat}/K_m at $(2.4 \pm 0.1) \times 10^6$ M^{-1} s^{-1} and $(2.7 \pm 0.7) \times 10^5$ M^{-1} s^{-1}. (*Right*) Rate constants for the release of ^{18}O-labeled water, $R_{H2O}/[E]$, from the active sites of K64E/R67N HCA III. Data were measured under the conditions described above. The solid line is a fit representing the sum of two ionizations with $pK_a = 7.9 \pm 0.1$ and 5.2 ± 0.2. The rate constant for intramolecular proton transfer $k_B = (1.5 \pm 0.1) \times 10^4$ s^{-1} was obtained from this fit using Eq. 5.

constants k_{cat}/K_m and $R_{H2O}/[E]$, determined from ^{18}O exchange, show the influence of two ionizations (Fig. 1), one near pK_a 5.2 and another near 7.9. The pK_a near 5.2 is consistent with that for the zinc-bound water in wild type and R67N; however, the pK_a near 7.9 appears to be too high to be consistent with the pK_a assigned to Glu^{64} in the other variants of Table 1. Hence in Table 1 we have placed K64E/R67N among those variants of HCA III for which the identity of the donor group is uncertain.

The Marcus rate theory applied to proton transfer (Marcus, 1968; Kresge, 1975) describes the overall activation energy $\Delta G^{\ddagger}$ in terms of the standard free energy of reaction with the required active site conformation ΔG°_R and an intrinsic energy barrier $\Delta G^{\ddagger}_o$, which is the value of $\Delta G^{\ddagger}$ when $\Delta G^{\circ}_R = 0$:

$$\Delta G^{\ddagger} = w^r + \{1 + \Delta G^{\circ}_R/4\Delta G^{\ddagger}_0\}^2 \Delta G^{\ddagger}_0 \qquad (6)$$

The standard free energy of reaction ΔG°_R with the required conformation is then related to the measured overall free energy for the reaction by work terms: $\Delta G^{\circ} = w^r + \Delta G^{\circ}_R - w^p$. (For this calculation, $\Delta G^{\ddagger} = -RT \ln(hk_B/kT)$ and $\Delta G^{\circ} = RT \ln[(K_a)_{ZnH2O}/(K_a)_{donor}]$.) The work term w^r represents in the strictest sense the free energy that must be subtracted from ΔG° so that the observed free energy of activation can be made to fit the Marcus equation (Eq. 6). The energy w^r is for catalysis in the dehydration direction of Eq. 4, w^p is for the reverse direction. These work functions have been interpreted in the case of nonenzymic bimolecular proton transfer as the energy required to align acceptor, donor, and surrounding water for facile proton transfer (Kresge, 1975), and this interpretation has been extended into the enzymic case (Silverman et al., 1993).

The solid line in Fig. 2 is a least-squares fit of Eq. 6 to the values of k_B in Table 1, with Glu^{64} or Asp^{64} as donors (entries 1–6), and to the entries for which the donor is uncertain (entries a–d). In the fit to Eq. 6, entries were weighted by the inverse of the variance for each point. The intrinsic energy barrier for the intramolecular proton transfer resulting from this fit is $\Delta G^{\ddagger}_0 = 2.2 \pm 0.5$ kcal/mol, with work terms $w^r = 10.8 \pm 0.1$ and $w^p = 4.0 \pm 1.6$ kcal/mol. The quality of this fit depends on the variants a–d of Table 1 for which the proton donor or donors are uncertain; omitting these data results in a lower value of $\Delta G^{\ddagger}_0$ and larger w^p in the fit of the remaining points 1–6 to Eq. 6:

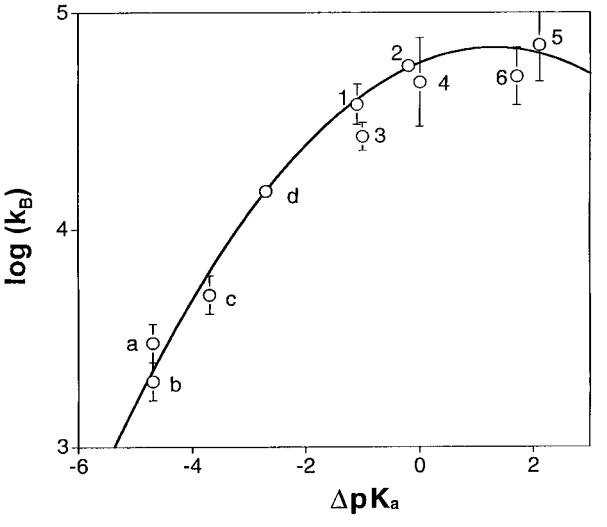

FIGURE 2 Dependence of the logarithm of k_B (s^{-1}) on ΔpK_a (the pK_a of the donor group subtracted from the pK_a of the zinc-bound water). k_B is the rate constant for donation of protons to the zinc-bound hydroxide accompanying the dehydration of HCO_3^- obtained from ^{18}O exchange experiments. The entries are wild type and mutants of HCA III listed in Table 1. The solid line is a least-squares fit of the Marcus equation (Eq. 6) to the entries of Table 1 (a–d, 1–6) giving $\Delta G^{\ddagger}_0 = 2.2 \pm 0.5$ kcal/mol, with work functions $w^r = 10.8 \pm 0.1$ kcal/mol and $w^p = 4.0 \pm 1.6$ kcal/mol.

$\Delta G_0^{\ddagger} = 1.1 \pm 0.4$ kcal/mol, with work terms $w^r = 10.8 \pm 0.1$ and $w^p = 7.8 \pm 1.3$ kcal/mol. In comparison, the Marcus parameters obtained for His^{64} (Silverman et al., 1993) and His^{67} (Ren et al., 1995) as donors in HCA III both had a better fit to Eq. 6 without entries a–c of Table 1.

The uncertainty in the true pK_a of the zinc-bound water in CA III has required in past studies an estimate of this pK_a at 4.3 in constructing the Marcus fits (Silverman et al., 1993; Ren et al., 1995). This was based on extrapolation of linear free energy plots for a number of mutants of HCA III with a range of values of the pK_a of the zinc-bound water. Qian et al. (1997) have extended activity measurements to pH < 6 and estimate the pK_a of the zinc-bound water in wild-type HCA III at 5.3 ± 0.2. We point out that changing the value of pK_a for wild-type HCA III from 4.3 to 5.3 in Fig. 2 yields these parameters: $\Delta G_o^{\ddagger} = 1.8 \pm 0.5$, $w^r = 10.9 \pm 0.1$, $w^p = 5.1 \pm 1.1$. Because the magnitude of these values is very similar to those for which the pK_a of 4.3 was used for HCA III (Table 2), we have retained the use of pK_a 4.3 for HCA III to be consistent with previous work.

DISCUSSION

Application of Marcus rate theory to intramolecular proton transfer in carbonic anhydrase first requires establishing a free energy plot with a range of pK_a values separating the donor and acceptor groups. In this study we have utilized aspartic and glutamic acids as donors; these residues provide little flexibility in altering their values of pK_a. Instead, we have altered the pK_a values of the zinc-bound water in HCA III, which is near 5 in the wild-type enzyme, by replacing Phe^{198} with Leu or Asp. Phe^{198} in CA III is located on the hydrophobic side of the active-site cavity with its Cδ ~6.4 Å from the zinc in bovine CA III (Eriksson and Liljas, 1993). Its replacement with Leu, the residue found at this position in CA II, increases the pK_a to 6.9, and its replacement with Asp increases its pK_a to 9.2 (LoGrasso et al., 1993).

A second requirement for application of Marcus theory is that the changes we make by site-specific mutagenesis in the active site do not alter the parameters of the Marcus theory itself, the intrinsic kinetic barrier and the thermodynamic contributions called the work functions. This is a much more difficult requirement to satisfy or even to determine. Our response has been to point out that the positions altered in these studies are on opposite sides of the active-site cavity and distant from each other. Moreover, the mutations considered in double-mutant cycles show mostly additive (i.e., indifferent) effects, indicating that the mutations at sites 64 and 198 do not influence each other in enhancement of catalysis. This is demonstrated in Table 3 for the rate constant for the changes in free energy of activation accompanying intramolecular proton transfer (measured by k_B). Among the replacements that introduce Glu or Asp at 64 (shown in Table 3), the only one that is not simply additive is K64D/F198L HCA III, which does not exhibit significantly enhanced proton transfer (Qian et al., 1997). Qian et al. (1997) concluded that Asp^{64} in this variant is not a shuttle residue for reasons not clear at this time. Some of these mutations at residues 64 and 198, resulting in K64E/F198L and K64E/F198D, have been found to interact in an antagonistic manner when k_{cat}/K_m is measured (Tu et al., 1994). The ratio k_{cat}/K_m is determined by steps in Eq. 1 that do not contain the proton transfer steps, but is a series of steps separate and distinct from the proton transfer steps. Therefore it is not counter to our aims that a series of mutations could interact in an antagonistic manner in k_{cat}/K_m but in an additive way for k_B.

A third requirement for accurate measurement of intramolecular proton transfer rates is the removal of buffers from solution. Buffers interfere by providing an alternative proton transfer pathway that may bypass His^{64} (Tu et al., 1989) or Glu or Asp^{64}. This was achieved in these studies by using an ^{18}O exchange method carried out at chemical equilibrium for the measurement of catalysis (Silverman, 1982). In this method pH control is not a problem and buffers are not used.

A fit of the Marcus theory to the mutants of Table 1 containing Glu or Asp at position 64 and other variants (entries 1–6 and a–d of Table 1) is shown by the solid line in Fig. 2. The corresponding parameters for the Marcus theory are given in Table 2. The most striking observation from Table 2 is the overall similarity in the Marcus parameters for the three series of mutants of HCA III in which data have been generated: 1) histidine residue at position 64, 2) histidine at 67, and 3) glutamic or aspartic acid residues at position 64. Although the Cα for positions 64 and 67 are equidistant from the zinc at close to 9.5 Å in CA III with side-chain positions that point into the active site cavity (Eriksson and Liljas, 1993), these two side chains experience different immediate environments due to adjacent residues. This and the equivalent rate constants for proton donation from Glu^{64} and Asp^{64} to the zinc-bound hydroxide found by Qian et al. (1997) demonstrate the overall accommodation of the active site to support proton translocation from these positions.

The magnitudes of the Marcus parameters in Table 2 give important clues to the capacity of the active site for proton donation. The predominant energy required for intramolecular proton transfer in these variants of HCA III occurs in the work functions w^r and w^p, contributions to the free

TABLE 2 Marcus theory parameters for intramolecular proton transfer in human carbonic anhydrase III and variants*

Proton donor	$\Delta G_o^{\ddagger}$ (kcal/mol)	w^r (kcal/mol)	w^p (kcal/mol)
His^{64}§	1.4 ± 0.3	10.0 ± 0.2	5.9 ± 1.1
His^{67}¶	1.3 ± 0.3	10.9 ± 0.1	5.9 ± 1.1
Glu, Asp^{64}	2.2 ± 0.5	10.8 ± 0.1	4.0 ± 1.6

*Data were obtained by least-squares fit of Eq. 6 to rate constants for intramolecular proton transfer k_B given in Fig. 2.
§Data from Silverman et al. (1993).
¶Data from Ren et al. (1995).

TABLE 3 Changes in free energies of activation obtained from maximum values of the rate constant for intramolecular proton transfer k_B accompanying the dehydration of HCO_3^- catalyzed by carbonic anhydrase III and mutants determined by ^{18}O exchange*

Mut_1 Mut_2	ΔG_1	ΔG_2	ΔG_{1+2} (kcal/mol)	$\Delta\Delta G_A$	$\Delta\Delta G_B$	Category
K64E + F198D	−1.5	−0.2	−1.7	−0.2	0.0	Additive
K64E + F198L	−1.5	−0.7	−1.7	−0.2	0.5	Additive (partially)#
K64D + F198D	−1.8	−0.2	−1.9	−0.1	0.1	Additive
K64D + F198L	−1.8	−0.7	−0.7	1.1	1.8	Antagonistic

*The notation and presentation format are those of Mildvan et al. (1992) for the effect of the less altering mutation (ΔG_2) on the more enhancing mutation (ΔG_1). ΔG_1 denotes the change in free energy of activation resulting from the first mutation listed; ΔG_2 is that for the second mutation; ΔG_{1+2} is that for the double mutation. $\Delta\Delta G_A = \Delta G_{1+2} - \Delta G_1$ and $\Delta\Delta G_B = \Delta G_{1+2} - \Delta G_1 - \Delta G_2$. Because we have observed increases in enzyme activity, the signs of $\Delta\Delta G_A$ and $\Delta\Delta G_B$ corresponding to synergistic and antagonistic interactions are reversed compared with the notation of Mildvan et al. (1992). The maximum value of k_B for wild-type HCA III is 3×10^3 s^{-1}.
#A second possible alternative.

energy of reaction. Previous experiments suggest that the rotation about the side-chain dihedral angles to attain the appropriate orientation of the proton donor is not an important contribution to w^r and w^p. The mobility of His^{64} in CA II is assumed from the multiple conformations available to this side chain in crystal structures (Nair and Christianson, 1991; Håkansson et al., 1992); moreover, adjacent residues of considerable bulk are needed to impede this mobility (Jackman et al., 1996; Scolnick and Christianson, 1996). Such bulky adjacent residues are lacking in human CA III (they are Gly^{63} and Thr^{65}, Cys^{66} and Val^{68}). In addition, there is enhancement by imidazole of catalysis in mutants of HCA III not containing a proton donor at position 64; this enhancement at large imidazole concentrations is roughly equivalent to the enhancement observed with mutants containing His^{64} (Silverman et al., 1993). Hence the work functions obtained from Marcus theory are assumed to have a small contribution from the energy needed to orient side chains.

An important component of the work functions w^r and w^p is believed to be the energy required to attain the proper orientation in the active-site cavity of the hydrogen-bonded water molecules that comprise the proton wire (Silverman et al., 1993; Kresge and Silverman, 1997). This is analogous to the conclusion of Pomès and Roux that formation and breaking of the hydrogen-bonded water chain is a limiting factor in proton translocation through the gramicidin channel (Pomès and Roux, 1996). Hence the variety of residues that can act as a proton donor from site 64 and His at site 67 is likely due to the accommodation of the active site to containing hydrogen-bonded water structures, each making a roughly equivalent contribution to the free energy of reaction w^r and w^p. Besides this, there is proton transfer in a number of variants of HCA III for which the proton donor is unknown, but the rate constant for proton transfer k_B lies on the free energy curve established for His^{64} or Glu^{64} (points a–d of Fig. 2). This similarity in work functions w^r for three classes of donors in Table 2 suggests that the work functions to establish water chains to other proton donors may also be similar to the values of Table 2. Such alternative proton donors might possibly include groups closer to the surface of the protein near the rim of the active-site cavity.

Although the parameters of the Marcus theory in Table 2 determined for intramolecular proton transfer in CA III are similar overall, there are intriguing and significant differences that may be clues to the mechanism of proton translocation through water chains. The work function w^r is significantly smaller by ~1 kcal/mol for proton transfer from His^{64}, as compared with His^{67} or Glu^{64} (Table 2). The larger values of w^r for His^{67} and Glu^{64} may indicate that proton translocation from these sites uses water chains different from those used by His^{64}. Although the standard errors are appreciable, the intrinsic barrier for the proton transfer $\Delta G_o^{\ddagger}$ could be larger for Glu^{64} and Asp^{64} as donors, compared with His^{64}. This emphasizes His^{64} as the most efficient proton shuttle among the sites studied, which is the translocation site that occurs naturally in CA II, among the most efficient of the carbonic anhydrases (Steiner et al., 1975; Tu et al., 1989). This conclusion was also supported by placing histidine residues at various positions in the active site of HCA II and noting that the wild-type with His^{64} has the largest catalytic turnover in CO_2 hydration (Liang et al., 1993).

It is useful to comment that there are nearby positions in the active-site cavity of HCA III from which proton transfer is not observed. The side chain of Asn^{62} is positioned along the active-site cavity of CA III and has its Cα located 12.7 Å from the zinc (Eriksson and Liljas, 1993). This Asn side chain extends out into solution with no residues between it and the zinc. The replacement $Asn^{62} \rightarrow His$ resulted in no appreciable enhancement of activity compared with wild-type HCA III; the mutant had k_{cat} for hydration and k_B both near 5×10^3 s^{-1} (Ren et al., unpublished observations). We thought that perhaps His^{62} in this mutant was impeded in mobility by the nearby bulky residues Lys^{64} and Arg^{67}. However, triple mutant N62H/K64A/R67A HCA III, in which these bulky residues are replaced with alanine, had values of k_{cat} and k_B identical to those of the single mutant N62H HCA III. We conclude that His^{62} is too distant from the zinc to facilitate proton transfer or has an inappropriate orientation with respect to an array of possible proton wires.

Another feature shown by these data pertains to the effect on proton transfer of rather distant (7–15 Å) side chains in the active site cavity. Here we note that three different isozymes (wild-type CA II and mutants of CA III and CA V, the last three entries of Table 1) containing His^{64} and all having ΔpK_a close to zero also have very similar values of the rate constant for intramolecular proton transfer k_B in the range $3–8 \times 10^5\ s^{-1}$ as measured by ^{18}O exchange. One appreciable difference in these enzymes is that the murine CA V we used lacked 20 residues from the amino terminus, including Tyr^7, which in CA II and CA III extends into the cavity and the side chain of which comes within 7 Å of presumed proton transfer pathways. Another notable difference is that HCA III has Arg at position 91, murine CA V has Lys^{91}, whereas HCA II has Ile^{91}. The Cα for position 91 is 12.7 Å from the zinc in CA III. Thus these results demonstrate that the rate constants k_B for intramolecular proton transfer are not exquisitely sensitive to the more distant surrounding residues.

CONCLUSIONS

1. The free energy plots for the intramolecular proton transfer from Glu^{64} and Asp^{64} to the zinc-bound hydroxide in mutants of HCA III can be fit to the Marcus rate theory. The resulting Marcus parameters show a small intrinsic barrier $\Delta G_o^{\ddagger}$ near 2.2 kcal/mol and a sizable work function near 11 kcal/mol in the dehydration direction, representing a thermodynamic contribution to the free energy of reaction.

2. These values of the Marcus parameters are very similar to those representing His^{64} and His^{67} as proton donors in mutants of HCA III, indicating a general capacity of the active site to accommodate proton translocation from these sites.

3. However, small differences of 1 kcal/mol in the work functions are consistent with less efficient proton donation from His^{67}, Glu^{64}, and Asp^{64} compared with His^{64}, and suggest that the intervening water conduction chain for His^{64} is more stabilized.

4. The similar values of the intramolecular rate constants from His^{64} to the zinc-bound hydroxide in CA II and variants of CA III and CA V show that the rate constant for this proton transfer is not greatly sensitive to more distant residues of the active site cavity.

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