Influence of Amino Acid Replacement at Position 198 on Catalytic Properties of Zinc-Bound Water in Human Carbonic Anhydrase III†

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ABSTRACT: Carbonic anhydrase III, found predominantly in skeletal muscle, is the least efficient of the mammalian carbonic anhydrases in catalyzing the hydration of CO_2. Phenylalanine-198 is located on the hydrophobic side of the active-site cavity with its phenyl ring in the proximity of the catalytically active zinc-bound water. We replaced phenylalanine-198 in human carbonic anhydrase III with seven other amino acids (Ala, Asn, Asp, His, Leu, Tyr, Val) using site-directed mutagenesis. The catalytic properties of these enzymes were determined by stopped-flow spectrophotometry, and the exchange of ^{18}O between CO_2 and water was measured by mass spectrometry. All of the mutants had maximal values of k_{cat}/K_m for the hydration of CO_2 enhanced, and five of the mutants had the pK_a of the zinc-bound water increased compared with the wild-type enzyme. The largest effects were observed with the replacement Phe-198 → Asp which increased the maximal k_{cat}/K_m 140-fold and increased the pK_a of the zinc-bound water from near 5 to 9.2. A Brønsted correlation was observed between $\log(k_{cat}/K_m)$ for hydration of CO_2 and the pK_a of the zinc-bound water (correlation coefficient $r = 0.92$); in addition, this pK_a was inversely correlated with hydrophobicity of the residue at 198 (correlation coefficient $r = -0.83$). A direct correlation between the logarithm of the maximal k_{cat}/K_m for hydration and the logarithm of the pH-independent value of K_i for inhibition by cyanate ($r = 0.95$) indicated that the effect of the mutations at residue 198 occurred in large part by enhancement of the rate of dissociation of the enzyme–bicarbonate complex.

The most and least catalytically efficient isozymes of carbonic anhydrase are isozymes II and III, respectively. Although they have very similar backbone conformations (Eriksson & Liljas, 1993; Eriksson, 1988), isozyme III is less active in the hydration of CO_2 by 300-fold at physiological pH (Silverman & Lindskog, 1988). In addition, isozyme III is less susceptible to inhibition by sulfonamides (Sanyal et al., 1982; Engberg et al., 1985; Kararli & Silverman, 1985) and has a lower pK_a of zinc-bound water (Engberg & Lindskog, 1984; Ren et al., 1988a), which plays a critical role in the catalysis. The replacement by directed mutagenesis of three residues near the active site of human carbonic anhydrase III (HCA III)[1] with the amino acids at the corresponding positions in HCA II (Lys-64 → His, Arg-67→ Asn, and Phe-198 → Leu) is sufficient to give the resulting triple mutant properties in catalysis and inhibition very similar to HCA II (LoGrasso et al., 1991). A key residue in determining the catalytic properties of HCA III is position 198 which is phenylalanine in HCA III and leucine in HCA II. Compared with wild-type HCA III, the mutant F198L HCA III has a 25-fold enhancement in catalytic hydration of CO_2 as well as an enhancement in hydrolysis of 4-nitrophenyl acetate, and has an increase of at least 1 unit in the pK_a of the zinc-bound water (LoGrasso et al., 1991). The current study was designed to take advantage of the sensitivity of catalysis and inhibition to the residue at position 198 in HCA III and to use this sensitivity to elucidate features of the catalytic mechanism. We made seven replacements (Ala, Asn, Asp, His, Leu, Tyr, and Val) at position 198 in HCA III and measured their effect on catalysis and inhibition. We have found that all of these replacements caused an increase in maximal values of k_{cat}/K_m for hydration of CO_2, and some caused substantial increases in the pK_a of catalysis. The activity of hydration measured by maximal k_{cat}/K_m correlated with the inhibition of catalysis by cyanate and with the apparent pK_a of the zinc-bound water in a Brønsted plot.

The refined structure of bovine CA III at 2.0-Å resolution has been reported (Eriksson & Liljas, 1993; Eriksson, 1988). Position 198 in this CA III is located on the hydrophobic side of the active-site cavity with its Cζ 8.1 Å from the zinc (Eriksson & Liljas, 1993). The phenyl ring of Phe-198 is not buried, and a water molecule appears to be hydrogen-bonded to its π-ring; this water is in turn hydrogen-bonded to other water molecules in the cavity forming a network that leads to the vicinity of the zinc (Eriksson & Liljas, 1993). This residue plays no direct role in the catalytic pathway, but it is apparent that because of Phe-198 the volume of the active-site cavity is much reduced compared with HCA II.

Catalysis of the hydration of CO_2 by both CA II and CA III occurs in two stages. The first is the conversion of CO_2 into HCO_3^-, leaving water as a ligand on the zinc (eq 1). This stage is currently viewed as involving the direct nucleophilic attack of zinc-bound hydroxide on CO_2 (Silverman & Lindskog, 1988).

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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, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

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$$CO_2 + EZnOH^- \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EZnOH^-(CO_2) \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EZnHCO_3^- \underset{k_{-3}}{\overset{k_3[H_2O]}{\rightleftarrows}} EZnH_2O + HCO_3^- \quad (1)$$

The second stage of catalysis is the transfer of a proton from the zinc-bound water to solution to regenerate the active form of the enzyme as shown in eq 2, where B is a proton

$$EZnH_2O + B \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} EZnOH^- + BH^+ \quad (2)$$

acceptor (Silverman & Lindskog, 1988; Tu et al., 1990). This is an essential step in the catalysis and requires that the proton transfer occurs at least as rapidly as the maximal turnover number. Different pathways for the proton transfer in CA III can predominate, depending on conditions. (1) Proton transfer between zinc-bound water and water in the active site has been suggested for the pH-independent value $k_{cat} = 2 \times 10^3$ s^{-1} for hydration in the pH range of 6–8 (Silverman & Lindskog, 1988). (2) Intramolecular proton transfer from zinc-bound water to another side chain, such as Lys-64, is suggested to have the same role at pH >8 as His-64 in HCA II (Kararli & Silverman, 1985; Rowlett et al., 1991; Jewell et al., 1991). The proton is then transferred from this side chain to buffer in solution (Silverman & Lindskog, 1988). (3) Finally, proton transfer occurs directly from zinc-bound water in HCA III to small buffers such as imidazole and phosphate that have access to the active site (Tu et al., 1990; Paranawithana et al., 1990).

METHODS

Enzymes. Mutants of human carbonic anhydrase III 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 absorptivity of 6.2×10^4 M^{-1} cm^{-1} at 280 nm (Engberg et al., 1985). The mutants of HCA III with Ala and Leu at position 198 showed potent inhibition with ethoxzolamide ($K_i = 2 \times 10^{-8}$ M). In this situation, we were able to confirm the concentration of enzyme to within 15% of that determined from the absorptivity by titration with ethoxzolamide using a Henderson plot (Segel 1975).

Steady-State Measurements. 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). The buffer–indicator pairs (with the wavelengths observed) were 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 the total ionic strength of the solution maintained at a minimum of 0.1 M using Na_2SO_4. For hydration of CO_2, kinetic constants were estimated from initial velocities using a weighted, linear least-squares method with v^4 weights, where v is the 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 a minimum of 0.1 M with Na_2SO_4. Solutions contained 33 mM of one of the buffers used in the measurements of CO_2 hydration.

Oxygen-18 Exchange. 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 was measured by mass spectrometry (Silverman, 1982). The ^{18}O method is also useful because two independent rates can be obtained, R_1 and R_{H_2O}. R_1 is the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium. The dependence of R_1 on total substrate concentration was used to determine values of k_{cat}/K_m for comparison with those determined by stopped-flow.[2] R_{H_2O} is the rate of release of ^{18}O-labeled water from the active site. R_{H_2O} involves proton transfer to the zinc-bound hydroxide, forming a zinc-bound water which is readily exchangeable with solvent water (Tu et al., 1983).

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 25 mM total substrate ($[CO_2] + [HCO_3^-]$) and 25 μM EDTA unless otherwise noted. No buffers were used. Total ionic strength of the solution was maintained at 0.2 M with Na_2SO_4. Silverman and Tu (1986) reported a biphasic depletion of ^{18}O from CO_2 when catalyzed by HCA III; when observed in this study, the slower of the two phases was reported. For R_{H_2O}, the standard errors were generally in the range of 15–25%. For R_1, the standard errors were less than 15%.

RESULTS

Interconversion of CO_2 and HCO_3^-. Two of the mutants, F198Y and F198V HCA III, were similar to wild-type HCA III in that the k_{cat}/K_m for hydration of CO_2 did not vary in the range of pH 6–9 (shown for wild-type HCA III in Figure 1). This is consistent with many studies indicating that the pK_a for catalysis by HCA III is less than 5.5 (Tu et al., 1983; Engberg et al., 1985; Kararli & Silverman, 1985; Ren et al., 1988a). The pH dependence of k_{cat}/K_m for the mutants F198H, F198N, F198A, F198L, and F198D HCA III could be described by an ionization with a maximum at high pH (Figure 1 for F198H and F198D, other data not shown); the values of this pK_a are given in Table I. The largest change compared with wild-type HCA III was for F198D HCA III which had a pK_a for catalysis approximated at 9.2 ± 0.2 (Figure 1). The maximal value of k_{cat}/K_m for F198D HCA III was greater than that for HCA III by over 100-fold and was comparable in magnitude to k_{cat}/K_m for the most efficient of the carbonic anhydrases, wild-type HCA II (Table I). For

[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 bicarbonate or CO_2. Values of k_{cat}^{ex}/K_{eff}^S for the enzymes were determined by nonlinear least-squares regression fit of the above expression for R_1 to the data or by measurement of R_1 at values of [S] much smaller than K_{eff}^S. In theory and in practice, k_{cat}^{ex}/K_{eff}^S is equal to k_{cat}/K_m obtained by steady-state methods (Simonsson et al., 1979).

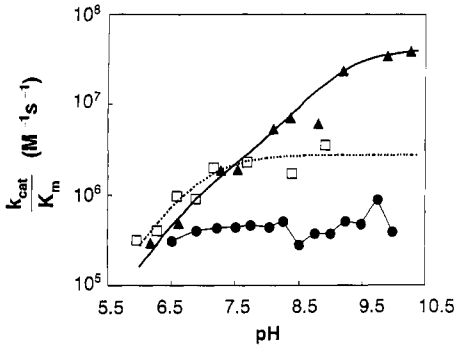

FIGURE 1: pH profile of k_{cat}/K_m for the hydration of CO_2 catalyzed by (●) HCA III, (□) F198H HCA III, and (▲) F198D HCA III. The buffers used in these stopped-flow experiments were the following: from pH 6.0 to 7.5, Mops; from pH 7.6 to 8.8, Taps; from pH 8.9 to 10.3, Ches. All buffer concentrations were 50 mM, the temperature was 25 °C, and the total ionic strength of solution was maintained at a minimum of 0.1 M by the addition of Na_2SO_4. The solid line for F198D HCA III corresponds to the influence of two ionizations: $pK_a = 9.2 \pm 0.2$, $(k_{cat}/K_m)_{max} = (3.9 \pm 0.2) \times 10^7$ M^{-1} s^{-1}; and $pK_a = 7.1 \pm 0.2$, $(k_{cat}/K_m)_{max} = (1.7 \pm 1.5) \times 10^6$ M^{-1} s^{-1}. The dotted line for F198H HCA III corresponds to the data in Table I.

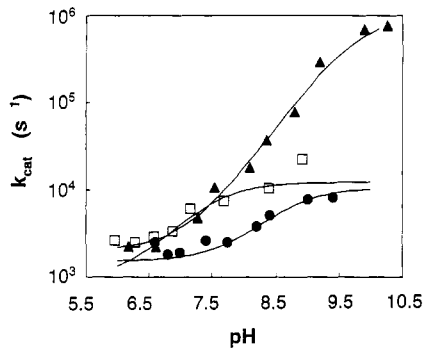

FIGURE 2: pH profile of k_{cat} for the hydration of CO_2 catalyzed by (●) HCA III, (□) F198H HCA III, and (▲) F198D HCA III. Experimental conditions were as described for Figure 1. The solid line for F198D HCA III was obtained by a nonlinear least-squares fit of the data, resulting in $pK_a = 9.6 \pm 0.1$, $(k_{cat})_{max} = (9 \pm 1) \times 10^5$ s^{-1}, and $(k_{cat})_{min} = 1500$ s^{-1}.

Table I: Maximal Values of k_{cat}/K_m, Values of the Apparent pK_a, and Selected Values of the Turnover Number, k_{cat}, for the Hydration of CO_2 Catalyzed by Wild-Type and Mutant Carbonic Anhydrases[a]

enzyme	k_{cat}/K_m ($\times 10^{-6}$ M^{-1} s^{-1})	k_{cat}[b] ($\times 10^{-4}$ s^{-1})	pK_a
HCA III[c]	0.3	0.2	5.0[d]
F198Y HCA III	0.5	0.2	5.0[d]
F198V HCA III	1.6	1.0	5.0[d]
F198H HCA III	2.7	0.3	7.0
F198N HCA III	6.2	0.2	7.1
F198A HCA III	6.5	2.5	6.5
F198L HCA III[e]	7.4	2.2	6.9
F198D HCA III	43	0.2	9.2
HCA II[f]	150	*f*	7.0

[a] Values of the apparent pK_a for catalysis were determined from k_{cat}/K_m for the hydration of CO_2 with standard errors in the pK_a of 0.2. Values of k_{cat} had standard errors less than 20% and k_{cat}/K_m less than 12%. [b] These values are for the pH-independent plateau at low pH and omit an increase in k_{cat} at pH >8. [c] From Jewell et al. (1991). [d] This rough estimate of pK_a is based on the spectroscopic and inhibition data of Ren et al. (1988a) for Co(II)-substituted bovine CA III. These enzymes denature at pH <5.5, and the values of pK_a have not been measured directly. [e] From LoGrasso et al. (1991). [f] From Khalifah (1971). There is no low-pH plateau for k_{cat} catalyzed by HCA II.

all the enzymes of Table I, values of k_{cat}/K_m for hydration of CO_2 were determined both by stopped-flow and by ^{18}O exchange,[2] the latter of which was carried out in the absence of buffer. These values determined at steady-state and chemical equilibrium were generally in agreement, within 30% of each other. An improved fit to the smaller values of k_{cat}/K_m catalyzed by F198D HCA III was obtained when we assumed a second ionization affected the catalysis (Figure 1). This was apparent both in the stopped-flow and in the ^{18}O-exchange data, but only for F198D HCA III, and probably indicates the influence of a second ionization at low pH, perhaps that of Asp-198.

Only two of the mutants of HCA III listed in Table I had significant catalysis of the hydrolysis of 4-nitrophenyl acetate. F198A and F198L HCA III had maximal values of k_{cat}/K_m for hydrolysis at 60 and 1000 M^{-1} s^{-1}, respectively [data for F198L from LoGrasso et al. (1991)]. The data could be fit to a single ionization; the values of the pK_a for this catalysis (6.0 ± 0.2 and 6.4 ± 0.2 for F198A and F198L, respectively) were somewhat lower than 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 maximal value of k_{cat}/K_m for this hydrolysis catalyzed by wild-type HCA III is 11 M^{-1} s^{-1} (Tu et al., 1986).

Maximal Velocity. Values of k_{cat} for hydration of CO_2 were generally pH-dependent with a plateau at low pH. Values of k_{cat} for this low-pH plateau varied over a 10-fold range for mutants of HCA III (Table I). In the higher pH range, values of k_{cat} increased with increasing pH. Such high pH values of k_{cat} are shown for wild-type HCA III, F198H HCA III, and F198D HCA III in Figure 2. The values of k_{cat} for F198D HCA III could be described by a titration curve with a maximum at high pH and a pK_a of 9.6 ± 0.1 (Figure 2). The maximal value of k_{cat} catalyzed by this mutant was 8×10^5 s^{-1}, nearly equal to that observed for HCA II, which is 1.4×10^6 s^{-1} (Khalifah, 1971). This pH dependence was very similar to that reported for k_{cat} catalyzed by bovine CA III with a pK_a of 8.8 (using 50 mM Na_2SO_4; Rowlett et al., 1991), feline CA III with a pK_a of 9.0 (Kararli & Silverman, 1985), and HCA III with a pK_a of 8.5 (Jewell et al., 1991).

Isotope Effects. The solvent hydrogen isotope effect (the ratio of values measured in H_2O to that in 98% D_2O) on the proton transfer dependent water off-rate $R_{H_2O}/[E]$ was 3.3 ± 0.3 for F198D HCA III, measured at an uncorrected pH meter reading of 7.0 with other conditions as described under Methods except that the total substrate concentration was 100 mM. Corresponding values for HCA III and F198L HCA III were 2.4 ± 0.4 and 2.7 ± 0.7 as reported by LoGrasso et al. (1991). In each case, the solvent hydrogen isotope effect on $R_1/[E]$ was unity.

Inhibition. The inhibition by ethoxzolamide and cyanate was determined using ^{18}O exchange at chemical equilibrium. The values of K_i in Table II were obtained by least-squares fit of the catalytic velocity to the expression for competitive inhibition as a function of inhibitor concentration under the conditions that the total substrate concentration ($[CO_2] + [HCO_3^-] = 25$ mM) was much less than the apparent binding constant for the total substrate, K_{eff}^S.[2] At the pH of these measurements, 7.3–7.5, K_{eff}^S is greater than 100 mM for wild-type HCA III and the mutants of Table I as determined by substrate dependence studies (data not shown). We have converted the apparent values of K_i for inhibition by cyanate to pH-independent binding constants for the zinc-bound water form of the enzyme by dividing each value of the apparent K_i by $(1 + K_a/[H^+])$ using the values of pK_a given in Table I. The usefulness of this procedure for the inhibition of F198L HCA III is demonstrated by the fit of these inhibition data to a titration curve corresponding to the binding of cyanate to the zinc-bound water form of the enzyme, assuming

Table II: Inhibition Constants, K_i, of the Interconversion of CO_2 and HCO_3^- at Chemical Equilibrium Catalyzed by Wild-Type and Mutant Carbonic Anhydrases at pH 7.3–7.5[a]

enzyme	K_i ethoxzolamide (μM)	K_i cyanate (mM)
HCA III	8.1	0.030 (0.0001)
F198Y HCA III	8.9	0.030 (0.0001)
F198V HCA III	2.4	0.41 (0.0016)
F198H HCA III	0.28	0.058 (0.014)
F198N HCA III	0.26	1.5 (0.50)
F198A HCA III	0.18	0.71 (0.082)[b]
F198L HCA III[c]	0.11	0.05 (0.01)
F198D HCA III	140	11 (11)
HCA II[c]	0.008	30 (10)

[a] Values of the inhibition constants were determined from the decrease in R_1 caused by addition of inhibitor, other conditions as described for ^{18}O exchange under Methods. Maximal pH-independent inhibition constants for cyanate (in parentheses) were calculated using the values of pK_a given in Table I as described under Results. [b] This value was obtained for the double mutant R67N–F198A HCA III. [c] From LoGrasso et al. (1991).

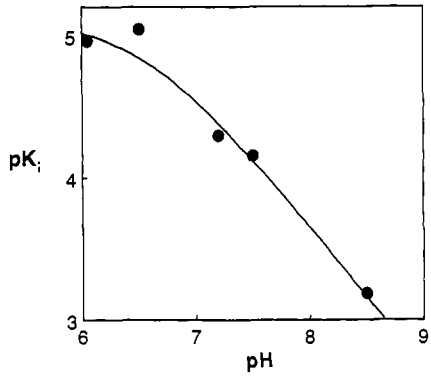

FIGURE 3: pH dependence of pK_i, the negative logarithm of the inhibition constant (M) of sodium cyanate, determined from measurements of ^{18}O exchange catalyzed by F198L HCA III at 25 °C. The line is a least-squares fit of these data to a titration curve with a pK_a of 6.6 ± 0.2 and a maximal value of $K_i = (1.3 \pm 0.4) \times 10^{-5}$ M.

negligible binding to the zinc-bound hydroxide form (Figure 3). The estimates of the pH-independent K_i for cyanate are given in parentheses in Table II. The values of K_i determined from R_{H_2O} in the same manner agreed to within 25% with the values determined from R_1.

The experiments reported here used Na_2SO_4 to maintain the ionic strength at 0.1 or 0.2 M. Rowlett et al. (1991) have shown sulfate to be a partial, hyperbolic inhibitor of bovine CA III with an apparent dissociation constant for sulfate near 1.0 mM. Maximal inhibition of bovine CA III with sulfate resulted in a 30% decrease in the k_{cat} for hydration and an increase of 0.8 unit in the apparent pK_a for k_{cat}. We obtained very similar results with HCA III and the mutant F198L HCA III (for F198L HCA III, the presence of 66 mM Na_2SO_4 caused a 30% decrease in the maximal R_{H_2O} with the apparent pK_a increased 0.6 unit). We observed no effect of sulfate on the k_{cat}/K_m for hydration catalyzed by HCA III or F198L HCA III in the pH range 6.5–9.0.

DISCUSSION

Ionization of the Activity-Controlling Group. The current study was designed to elucidate features of the catalytic mechanism of HCA III using the sensitivity of catalysis and inhibition to the residue at position 198. In the mechanism described by eq 1 and 2, the pH dependence of activity k_{cat}/K_m for hydration of CO_2 is determined predominantly by the pK_a of the zinc-bound water. There is considerable support

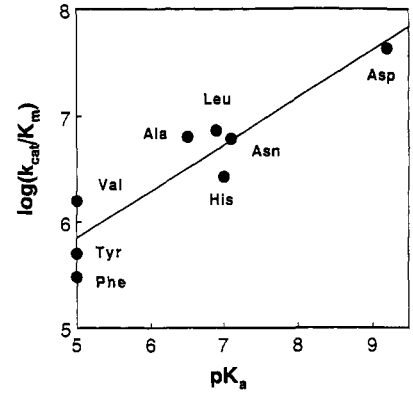

FIGURE 4: Brønsted plot showing the correlation between the pK_a for the zinc-bound water and the logarithm of the maximal value of k_{cat}/K_m (M^{-1} s^{-1}) for the hydration of CO_2 catalyzed by variants of human carbonic anhydrase III. Individual points are labeled with the identity of the residue at position 198. The line is a least-squares fit to all the data (slope = 0.44 ± 0.07; correlation coefficient 0.92).

from isozymes II and III that such titration behavior of k_{cat}/K_m accurately represents the pK_a of the activity-controlling zinc-bound water (Lindskog, 1983; Simonsson & Lindskog, 1982; Engberg & Lindskog, 1984). Possible perturbations are caused by the ionization of nearby groups, most notably His-64 in CA II (Simonsson & Lindskog, 1982; Bertini et al., 1985). In mutants containing replacements at position 198, the pK_a of the zinc-bound water varied from approximately 5.0 for the wild-type HCA III to 9.2 for F198D HCA III (Table I) and provided an opportunity to assess the influence of position 198 on the properties of the zinc-bound water in catalysis. Among the mutants studied here, evidence for a second ionization was marginally present only for F198D HCA III, which was slightly better fit by introduction of a second ionization with a pK_a of 7.1 (Figure 1). Although evidence is weak, this could possibly be the ionization of Asp-198 in a hydrophobic environment.

The assignment of pK_a 9.2 for F198D HCA III to zinc-bound water is reasonable considering that a pK_a of 9.2 is close to that observed for ionization of water in simple aqueous ions of zinc (Sillen et al., 1971) and for macrocyclic complexes of zinc that are useful as models of carbonic anhydrase (Brown, 1990). On the other hand, the observed pK_a of 9.2 is far from that of the carboxylate side chain of Asp, anticipated to have a pK_a between 4 and 5 in most proteins.

Also consistent with the assignment of the pK_a of 9.2 to zinc-bound water is the similarity in magnitude of $\Delta\Delta G$ ($\Delta G_{F198D} - \Delta G_{wild\text{-}type}$) based on the observed values of pK_a of Table I ($\Delta\Delta G$ = 5.8 kcal/mol) and on the pH-independent values of the equilibrium dissociation constants for the binding of cyanate $K_i(OCN^-)$ of Table II ($\Delta\Delta G$ = –6.8 kcal/mol). These large values observed for $\Delta\Delta G$ suggest that the charged side chain of Asp-198 in F198D HCA III destabilizes the zinc-bound hydroxide and the zinc-bound cyanate to approximately the same extent. The values of $\Delta\Delta G$ also suggest that the charge on the Asp side chain is not neutralized in a salt bridge, as might be formed with the side chains of Lys-64 or Arg-67. Moreover, the large values of k_{cat}/K_m for F198D HCA III (Figure 1) suggest that the Asp-198 side chain is not binding to the zinc in a way to inhibit hydration of CO_2.

The pH profile for k_{cat}/K_m catalyzed by F198H HCA III (Figure 1) is consistent with a single ionization of pK_a 7.0 for the zinc-bound water, an assignment that is consistent with the trends in Figures 4 and 5 discussed below. The ionization of His-198 expected at pK_a 6–8 may be superimposed and not detectable from the data of Figure 1. In K64H HCA III, there is no evidence for an effect of His-64 in the data for

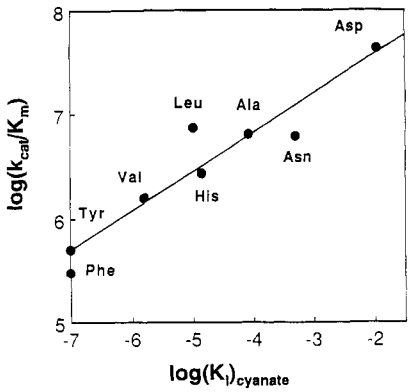

FIGURE 5: Correlation of the maximal value of k_{cat}/K_m (M^{-1} s^{-1}) for the hydration of CO_2 catalyzed by variants of human carbonic anhydrase III with K_i (M) for the inhibition by cyanate. Individual points are labeled with the identity of the residue at position 198. The line is a least squares fit to all the data (slope = 0.38 ± 0.05; correlation coefficient 0.95).

k_{cat}/K_m (Jewell et al., 1991), although position 64 is further from the zinc than position 198. The ionization of His-64 in HCA II affects the pH profile of k_{cat}/K_m for catalysis, but is masked by the presence of sulfate ions (Simonsson & Lindskog, 1982). The pH profile for k_{cat} is expected to reflect predominantly the pK_a of the group accepting a proton from the zinc-bound water in the hydration direction (Silverman & Lindskog, 1988). The pH profile for k_{cat} (Figure 2) shows a very small increase at pH near 7 which might be due to ionization of His-198.

In addition to these effects of charged groups on the catalytic activity, the apparent pK_a of catalysis measured from k_{cat}/K_m for hydration appears to be influenced by the hydrophobicity of its environment. The values of pK_a in Table I show a correlation (correlation coefficient $r = -0.83$) with the solvation free energies ΔG_R of Eisenberg and McLachlan (1986). This correlation with solvation free energies emphasizes the strong effect of a nearby residue on the pK_a of the zinc-bound water in HCA III, but does not include specific electrostatic interactions.

Interconversion of CO_2 and HCO_3^-. The ratio k_{cat}/K_m for hydration of CO_2 contains rate constants for the steps in the interconversion of CO_2 and HCO_3^- shown in eq 1. This constant, k_{cat}/K_m, does not include the proton transfer steps of eq 2. There was a dependence of maximal values of k_{cat}/K_m for CO_2 hydration on the pK_a of the zinc-bound water, as shown in Figure 4. Here the values of pK_a for HCA III and the mutants F198V and F198Y HCA III cannot be measured by the methods used here because of denaturation of the enzyme at low pH; instead, they are roughly estimated at 5.0 based on the spectroscopic and inhibition properties of bovine CA III (Ren et al., 1988a). This Brønsted plot of Figure 4 shows a linear correlation ($r = 0.92$) between $\log(k_{cat}/K_m)$ and pK_a, and has a slope of 0.44 ± 0.07. Such correlations are well known for nucleophilic attack on a carbonyl (Hudson & Klopman, 1974) and for hydration of CO_2 by metal-bound hydroxides in inorganic complexes (Martin, 1976). However, the Brønsted plot of Figure 4 may not entirely reflect the rate of step 2 of eq 1, the nucleophilic attack on CO_2. Instead, it may reflect rate-contributing dissociation of product HCO_3^- from the active site. A substantial contribution to k_{cat}/K_m from the dissociation of HCO_3^- is consistent with the tighter binding of HCO_3^- to CA III (Ren et al., 1988b) compared with CA II (Simonsson et al., 1979) determined by ^{13}C NMR and the known tight binding of anions to CA III (Sanyal et al., 1982). The data are less consistent with a rate-limiting interconversion of CO_2 and HCO_3^- (step 2 of eq 1); the presence of a polar group at position 198 or the carboxylate group of Asp-198 would not favor the formation of the negatively charged HCO_3^- and would be expected to decrease the rate of catalysis, contrary to the observations of Table I.

Further evidence of the steps contributing to the maximal k_{cat}/K_m for hydration of CO_2 is in the linear correlation ($r = 0.95$) between $\log(k_{cat}/K_m)$ and $\log(K_i)$ for the pH-independent cyanate inhibition (Figure 5), suggesting that the same features at position 198 affect the binding of HCO_3^- and cyanate. The slope of the correlation in Figure 5 is 0.38 ± 0.05, indicating that the K_i for cyanate is more sensitive to the changes at residue 198 than is the k_{cat}/K_m for hydration. Extension of the discussion to K_m is difficult for these enzymes because K_m is a steady-state constant and not a binding constant (Silverman & Lindskog, 1988). Moreover, the correlation ($r = 0.87$) between $\log(K_m)$ and $\log(K_i)$ is weaker than for $\log(k_{cat}/K_m)$ and $\log(K_i)$ ($r = 0.95$).

In the case that dissociation of HCO_3^- from the active site is rate-limiting, k_{cat}/K_m for hydration is given by $k_3/[K_1K_2(1 + [H^+]/K_E)]$ where $K_1 = k_{-1}/k_1$, $K_2 = k_{-2}/k_2$, and K_E is the ionization constant of the zinc-bound water. [This was derived from the steady-state expressions of Simonsson et al. (1979).] Aspartate at position 198 might be expected to suppress formation of bound HCO_3^- and increase K_2; however, this effect considered alone would contribute to a decrease of k_{cat}/K_m and is contrary to observations.

For carboxypeptidase and other enzymes, correlations between $\log(k_{cat}/K_m)^{-1}$ and $\log(K_i)$ for different inhibitors have demonstrated that the inhibitors mimic aspects of the transition state for the chemical transformation (Phillips et al., 1992). The data in Figure 5 are different in that the inhibition by a single inhibitor, cyanate, is represented for a number of mutants of HCA III. There is a linear correlation with negative slope between the values of $\log(k_{cat}/K_m)^{-1}$ for hydration of CO_2 and $\log(K_i)$, indicating that cyanate is not mimicking the transition state for the interconversion of CO_2 and HCO_3^-. We conclude from Figure 5 that the binding of cyanate mimics the binding of HCO_3^- in the enzyme–product complex and that destabilization of this complex enhances the catalysis by lessening a thermodynamic well in the pathway.

The binding site of cyanate on HCA III is not determined. Eriksson (1988) has suggested that the orientation which thiocyanate adopts in BCA III would be similar to that for HCO_3^-. In HCA II, thiocyanate binds to the zinc and is in van der Waals contact with Leu-198 (Eriksson et al., 1988). There is evidence that cyanate does not bind directly to the zinc in HCA II; the crystal structure of the complex shows that cyanate binds nearby, forming a hydrogen bond with the backbone NH of Thr-199 with the other end of the cyanate at a distance of 3.1 Å from the zinc (Lindahl et al., 1993).

Although the variants of HCA III with the large and hydrophobic residues Phe-198 and Tyr-198 had the smallest activity, we found no strong correlation of the k_{cat}/K_m for hydration with volume or hydrophobicity: the correlation r was –0.74 for the linear association between $\log(k_{cat}/K_m)$ and the amino acid volume, V_R, of Richards (1977); the correlation r was –0.78 between $\log(k_{cat}/K_m)$ and the amino acid solvation energy, ΔG_R, of Eisenberg and McLachlan (1986). These results can be compared to recent findings of Fierke et al. (1991) in which no correlation was found between the k_{cat}/K_m for hydration of CO_2 and the hydrophobicity of the residues replacing valine at position 143 in HCA II. Nair et al. (1991) did find a correlation between the k_{cat}/K_m for the hydration of CO_2 and the hydrophobicity of the side chain at position 121 in HCA II, a valine in both HCA II and HCA III.

[3] X. Chen, C. K. Tu, S. Taoka, P. J. Laipis, and D. N. Silverman, unpublished observations.

Proton Transfer. The steady-state constant k_{cat} for hydration contains rate constants for steps from the enzyme–substrate complex through the regeneration of the zinc-bound hydroxide. For CA III, k_{cat} is limited by the proton transfer of eq 2 (Silverman & Lindskog, 1988; Rowlett et al., 1991). Solvent hydrogen isotope effects observed for F198D and F198L HCA III (see Results) are consistent with the same conclusion for these mutants. We observed increases of k_{cat} at the higher pH regions in catalysis by variants of HCA III. Jewell et al. (1991) and Rowlett et al. (1991) made this observation for wild-type HCA III for which k_{cat} reached a value near 1×10^4 s^{-1} at high pH and have suggested that the data are consistent with a basic group with a pK_a near 9 acting as a proton shuttle to enhance proton transfer to solution, as in eq 2. The enhancement of the k_{cat} for hydration catalyzed by F198D HCA III approached 1×10^6 s^{-1} at pH >9.0 (Figure 2). This is also consistent in pH profile with proton transfer from zinc-bound water to such an acceptor which appears to be as efficient in proton transfer as His-64 in HCA II. The pH dependence of k_{cat} for F198D HCA III follows a titration curve having a single ionization (pK_a = 9.6 ± 0.2). This value is near the microscopic pK_a of 9.0 for the proton shuttle group in bovine CA III as determined by computer simulations (Rowlett et al., 1991). Jewell et al. (1991) have shown a strong pH dependence of k_{cat} for the mutant H64K HCA II which is qualitatively very similar to these results for F198D HCA III, supporting the suggestion that Lys-64 contributes to proton transfer in these mutants. However, recent steady-state and ^{18}O-exchange experiments with K64A HCA III show maximum velocity and R_{H_2O}/[E] similar to those of HCA III, indicating that Lys-64 in HCA III may not be the only contributor to the intramolecular proton transfer.[3] Other proton acceptor groups contributing to k_{cat} in HCA III could include Cys-66 or Tyr-7, which are near the active site and have not yet been studied as possible proton acceptors in catalysis by CA III.

The portion of k_{cat} for hydration catalyzed by HCA III and mutants that is independent of pH in the range of pH 6–8 has values of k_{cat} that vary from 2 to 25 ms^{-1} (Table I). This portion of k_{cat} for HCA III is suggested to involve rate-limiting proton transfer from zinc-bound water to water in the active site (Silverman & Lindskog, 1988). A turnover number limited by proton transfer in the range 10^3–10^4 s^{-1} for the enzymes of Table I is consistent with diffusion-controlled dissociation of the proton from zinc-bound water to water in the active-site cavity, assuming diffusion-controlled protonation by H_3O^+ in the reverse direction at 10^8–10^{10} M^{-1} s^{-1}. The exception is F198D HCA III; with a pK_a of 9.2, the zinc-bound water is too basic to contribute a proton to water at a rate above about 10 s^{-1}. Here some other group near the active site, perhaps Asp-198, acts as proton acceptor.

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