Interaction and Influence of Phenylalanine-198 and Threonine-199 on Catalysis by Human Carbonic Anhydrase III†

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ABSTRACT: Site-directed mutants of human carbonic anhydrase III were used to examine the role of Thr-199 and its interaction with Phe-198 in the catalyzed hydration of CO_2. Threonine-199 is a hydrogen bond acceptor for the zinc-bound water, and Phe-198 forms part of the hydrophobic side of the active-site cavity of carbonic anhydrase III. Catalytic activity for a total of five single and double mutants at residues 198 and 199 was determined by stopped-flow spectrophotometry and ^{18}O exchange between CO_2 and water measured by mass spectrometry. The replacement Thr-199 → Ala resulted in a 4-fold decrease in the k_{cat}/K_m for hydration of CO_2. We tested the hypothesis that the 25-fold increase in the k_{cat}/K_m for hydration of CO_2 accompanying the replacement Phe-198 → Leu in isozyme III is caused by changes in the interaction of Thr-199 with the zinc-bound water or the transition state for catalysis. Comparison of hydration of CO_2 by the single and double mutants of isozyme III containing the replacements Thr-199 → Ala and Phe-198 → Leu was consistent with an interaction between these two sites.

Carbonic anhydrase III is found predominantly in skeletal muscle (Gros & Dodgson, 1988) and is the least catalytically efficient of the seven known isozymes of mammalian carbonic anhydrase (Tashian, 1989). Human carbonic anhydrase III (HCA III)[1] has a maximal turnover which is 300-fold smaller than that for HCA II at physiological pH (Jewell et al., 1991; Engberg et al., 1985; Tu et al., 1983). Catalysis by isozyme III occurs in two separate stages and shares many common features with catalysis by isozyme II (Silverman & Lindskog, 1988). The first stage is the conversion of CO_2 into HCO_3^- (eq 1) and is believed to occur by the direct nucleophilic attack of the zinc-bound hydroxide on CO_2 (Silverman & Lindskog, 1988). The second stage is the proton-transfer steps which

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

regenerate the zinc hydroxide form of the active site (eq 2).

$$EZnH_2O + B \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} EZnOH^- + BH^+ \quad (2)$$

Here B is the proton acceptor and can be a small buffer molecule (Tu et al., 1990; Paranawithana et al., 1990), water in the active-site cavity (Kararli & Silverman, 1985), or a side chain of the enzyme such as His-64 in the mutant K64H[2]

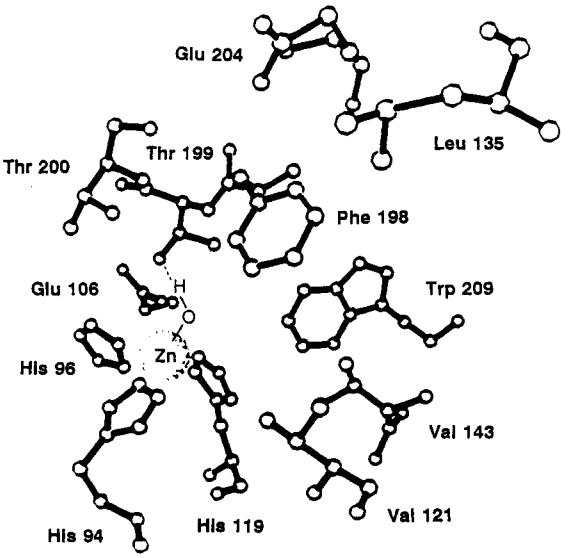

FIGURE 1: Residues near the zinc in bovine carbonic anhydrase III according to the crystal structure of Eriksson and Liljas (1993).

HCA III which then transfers the proton to buffer in solution (Jewell et al., 1991). The crystal structure of bovine CA III shows a backbone conformation very similar to that of HCA II, with a root mean squared difference in backbone atoms of 0.92 Å (Eriksson & Liljas, 1993; Eriksson et al., 1988; Eriksson, 1988), and the amino acid identity between these two enzymes is 56%. In both of these structures, the zinc-bound water is a hydrogen bond donor to the Oγ1 of the side chain hydroxyl group of Thr-199 (Figure 1). This residue is invariant in all forms of carbonic anhydrase sequenced to date (Tashian, 1989), except for the plant carbonic anhydrases which have an entirely different primary structure [see, for example, Fawcett et al. 1990)]. In turn, the hydroxyl of Thr-199 is a hydrogen bond donor to the carboxylate of Glu-106

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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.

[2] The single-letter amino acid abbreviations are used in which K64H HCA III refers to the mutant of human carbonic anhydrase III in which Lys-64 has been replaced with His.

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(Eriksson & Liljas, 1993). Merz (1990) has discussed the importance of the interaction between the hydrogen bond acceptor, the hydroxyl side chain of Thr-199, and the donor, the zinc-bound water or hydroxide in isozyme II; its elimination through mutagenesis is expected to decrease catalysis.

Phenylalanine-198 is unique to isozyme III and appears in all forms sequenced to date [human, bovine, equine, mouse, rat; see Eriksson (1988) and Eriksson & Liljas (1993) for references]. Phenylalanine-198 is not a buried side chain but forms part of the hydrophobic side of the active-site cavity, with the Cζ of Phe-198 8.1 Å from the zinc (Figure 1) and its phenyl ring hydrogen-bonded to water in the active site (Eriksson, 1988; Eriksson & Liljas, 1993). This residue is not believed to play a direct role in the catalytic pathway but does influence catalysis (LoGrasso et al., 1991; Silverman & Lindskog, 1988). The replacement of Phe-198 with Leu in HCA III enhanced the k_{cat}/K_m for hydration by 25-fold and increased the pK_a of zinc-bound water by at least 1 pK_a unit compared with wild-type (LoGrasso et al., 1991). The corresponding residue at position 198 in HCA II is Leu; thorough studies of the catalytic and structural consequences of amino acid substitution at this position have been reported (Krebs et al., 1993; Nair & Christianson, 1993).

We have examined the function of Thr-199 in catalysis by HCA III using site-directed mutagenesis. The mutant T199A HCA III had k_{cat} and k_{cat}/K_m for the hydration of CO_2 decreased 4-fold compared with wild-type HCA III; the apparent pK_a for catalysis increased from near 5 to 6.3. The mutant T199S had no change in k_{cat} and apparent pK_a with a 3-fold increase in k_{cat}/K_m. For isozyme III, this identifies the role of the hydroxyl group at position 199 as stabilizing the zinc-bound hydroxide and the transition state for catalysis. Our results also support the hypothesis that the increase in reactivity and pK_a of zinc-bound water caused by the replacement Phe-198 → Leu in HCA III is related to the interaction of Phe-198 with Thr-199.

METHODS

Mutagenesis and Expression. 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 depended on the mutant and ranged from 0.1 to 20 mg/L on the basis of kinetic measurements. All mutations were confirmed by DNA sequencing of the expression vector used to produce the mutant protein.

Enzyme Purification. Variants of carbonic anhydrase III were purified by gel filtration (Ultrogel AcA 44, LKB) followed by anion-exchange chromatography (DEAE-Sephacel, Pharmacia) with minor modifications of the procedure of Tu et al. (1986). The resulting mutant enzymes were estimated to be at least 96% pure, determined by 0.1% SDS–12% polyacrylamide gel electrophoresis with staining by Coomassie Brilliant Blue. The concentrations of HCA III and these mutants were determined from the molar absorptivity of 6.2×10^4 M^{-1} cm^{-1} at 280 nm determined for bovine CA III (Engberg et al., 1985). The mutants of HCA III with the replacement Phe-198 → Leu showed potent inhibition with ethoxzolamide ($K_i \approx 2 \times 10^{-8}$ M). In this situation, we were able to confirm the concentration of enzyme to within 10% of that determined from the absorptivity by titration with ethoxzolamide using a Henderson plot (Segel, 1975). Each of these variants was stable for many days when stored at 4 °C in solutions of 50 mM Tris, pH 8.4.

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 Mes[1] (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), Hepes (pK_a 7.5) and phenol red (pK_a 7.5, 557 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 (unless otherwise noted), and the total ionic strength of the solution was maintained at a minimum of 0.1 M using Na_2SO_4. Initial velocities of the hydrolysis of 4-nitrophenyl acetate were measured (Beckman DU 650 or DU 7 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 the ionic strength was maintained at a minimum of 0.2 M with Na_2SO_4. Solutions contained a 50 mM aliquot of one of the buffers used in the measurements of CO_2 hydration. For both hydration of CO_2 and hydrolysis of 4-nitrophenyl acetate, kinetic constants were estimated from initial velocities using a least-squares method (Enzfitter, Elsevier-BIOSOFT).

Oxygen-18 Exchange. The 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 using an Extrel EXM-200 mass spectrometer or a Dycor M-100 gas analyzer with a membrane inlet probe (Silverman, 1982). Solutions were at 25 °C and contained 25 μM EDTA to complex potentially inhibitory metal ions. No buffers were used. The total ionic strength of the solution was maintained at 0.2 M with Na_2SO_4.

The ^{18}O method is useful because it measures the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium, R_1, as shown in eq 3. The substrate dependence of R_1 is given

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

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 all species of CO_2 (Simonsson et al., 1979). Values of k_{cat}^{ex}/K_{eff} for the enzymes were determined by nonlinear least-squares fit of the above expression for R_1 to the data for varying substrate concentration as in Figure 3 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; Silverman, 1982).

RESULTS

Because of the low activity and lower expression levels for the two mutants containing Ala-199, a more complete set of data for k_{cat}/K_m was obtained by ^{18}O exchange (Figure 2) which requires about 10-fold less enzyme than stopped-flow; these values were in agreement within 25% with values of k_{cat}/K_m obtained by stopped-flow. The remaining values in Figure 2 were determined by stopped-flow as were the values for the mutants containing Ser-199.

Kinetic Constants. Maximal values of k_{cat}/K_m for the hydration of CO_2 catalyzed by HCA III and mutants are

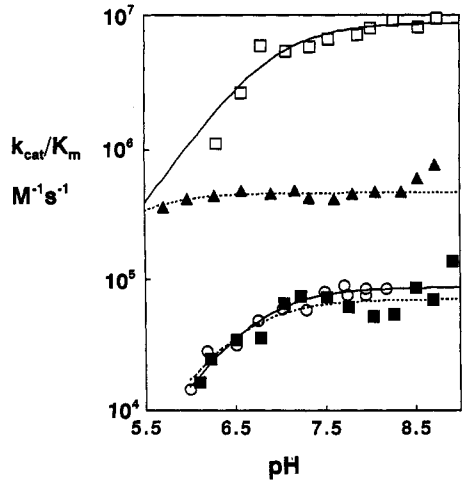

FIGURE 2: Dependence on pH of k_{cat}/K_m for the hydration of CO_2 catalyzed by (▲) HCA III, (■) T199A HCA III, (□) F198L HCA III, and (○) F198L-T199A HCA III. Measurements were made by stopped-flow spectrophotometry (HCA III and F198L HCA III) and ^{18}O exchange (T199A and F198L-T199A HCA III) at 25 °C using Na_2SO_4 to maintain the total ionic strength of the solution (see text). The stopped-flow experiments used 50 mM buffer as described in the text; the ^{18}O-exchange experiments used no buffers.

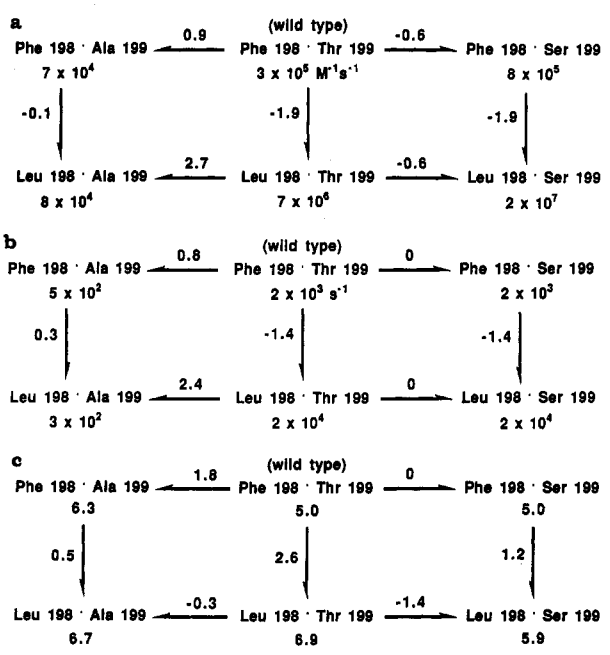

FIGURE 3: (a) Comparisons of the maximal values of k_{cat}/K_m (M^{-1} s^{-1}) for the hydration of CO_2 at 25 °C catalyzed by variants of HCA III obtained by site-directed mutagenesis at positions 198 and 199 in the active-site cavity. Values of k_{cat}/K_m appear beneath each designated mutant. The values adjacent to the arrows are the changes in free energy barriers (kcal/mol) for the catalysis corresponding to the designated mutations. Free energy changes were determined using $\Delta\Delta G = -RT\ln[(k_{cat}/K_m)_{mut2}/(k_{cat}/K_m)_{mut1}]$. (b) Comparisons of k_{cat} (s^{-1}, given beneath each designated mutant) for the hydration of CO_2 catalyzed by variants of HCA III obtained by site-directed mutagenesis. Values of k_{cat} are those observed in a pH-independent region at pH <8. (c) Comparisons of the apparent pK_a (given beneath each designated mutant) obtained from k_{cat}/K_m for hydration of CO_2 (except for F198L-T199S HCA III which is based on the hydrolysis of 4-nitrophenyl acetate). Values for HCA III and T199S HCA III are estimates (see text).

shown in Figures 2 and 3a. Values of k_{cat} for the hydration of CO_2 catalyzed by wild-type HCA III were independent of pH between pH 6.0 and 8.0 and increased above pH 8 (Jewell et al., 1991); qualitatively similar behavior was observed for the mutants F198L, T199S, and F198L-T199S. The values of k_{cat} reported for these enzymes and appearing in Figure 3b are for the plateau region (pH <8). Values of k_{cat} for T199A and F198L-T199A HCA III showed no pH dependence in the range of pH 7.8–8.6 (using Hepes and Taps buffer) with the average of four (T199A) and three (F198L-T199A) values given in Figure 3b.

Removal of the hydroxyl group at position 199 in HCA III by the replacement of Thr with Ala resulted in a 4-fold decrease in maximal values of k_{cat}/K_m and k_{cat} for the hydration of CO_2 (Figures 2 and 3a,b). The double mutant F198L-T199A HCA III showed a greater decrease in k_{cat}/K_m and k_{cat}, 60–80-fold, compared with the single mutant F198L HCA III, as given in Figures 2 and 3a,b. Values of the steady-state constants for wild-type and F198L HCA III were also reported by LoGrasso et al. (1991).

The conservative replacement of Thr-199 with Ser in HCA III caused a nearly 3-fold increase in maximal values of k_{cat}/K_m (Figure 3a) and caused no change in the k_{cat} (Figure 3b). Identical results were observed when Thr-199 was replaced with Ser in the variant F198L to make the double mutant F198L-T199S HCA III (Figure 3a,b).

The ratio of maximal values of k_{cat}/K_m measured in H_2O to that measured in D_2O (99% deuterium) was $(k_{cat}/K_m)_{H_2O}/(k_{cat}/K_m)_{D_2O} = 0.85 \pm 0.09$, determined by ^{18}O exchange using solutions of T199A HCA III containing no buffer. The ratio $(k_{cat})_{H_2O}/(k_{cat})_{D_2O}$ measured by stopped-flow was dependent on the buffer used; this ratio was 4.3 ± 0.6 at pH 7.6 using 25 mM Hepes. The ratio of maximal values of the proton-transfer-dependent water off-rate was $R_{H_2O}/R_{D_2O} = 1.6 \pm 0.4$ for T199A HCA III measured in the absence of buffer.

pK_a for Catalysis. Values of the pK_a for catalysis were determined from the pH profile of k_{cat}/K_m for hydration of CO_2. This is demonstrated for three mutants of HCA III in Figure 2. The pK_a values of wild-type HCA III and T199S HCA III could not be determined because of denaturation of the enzyme at low pH; this pK_a for CA III has been estimated at 5.0 on the basis of spectroscopic and inhibition data for cobalt(II)-substituted bovine CA III (Ren et al., 1988a). The replacement Thr-199 → Ser in HCA III did not place the apparent pK_a into the pH range of these measurements. For F198L-T199S HCA III, the pK_a of catalysis was not clear from stopped-flow and ^{18}O-exchange measurements, and the pK_a of 5.9 (Figure 3c) was obtained from hydrolysis of 4-nitrophenyl acetate.[3] The mutants T199A HCA III and F198L-T199A HCA III had the apparent pK_a for k_{cat}/K_m increased to 6.3 and 6.7 (Figures 2 and 3c).

Figure 4 demonstrates the total substrate concentration ($[CO_2] + [HCO_3^-]$) dependence of $R_1/[E]$ catalyzed by T199A compared with wild-type at pH 7.2. The variation of $R_1/[E]$ with total substrate concentration is fit by nonlinear least-squares methods to an effective binding constant of all substrate, K_{eff}^S of 38 ± 3 mM for T199A HCA III. This indicates a tighter binding of substrate than for wild-type HCA III and for T199S HCA III (data not shown) for which the value of K_{eff}^S is too great to estimate from the current data with total substrate as high as 100 mM.

DISCUSSION

The ratio k_{cat}/K_m for hydration of CO_2 contains rate constants from the binding of CO_2 to the first irreversible

[3] Of all the variants of HCA III in Figure 3, significant catalysis of the hydrolysis of 4-nitrophenyl acetate was observed for F198L HCA III and F198L-T199S HCA III. This catalysis for F198L HCA III is described by LoGrasso et al. (1991).

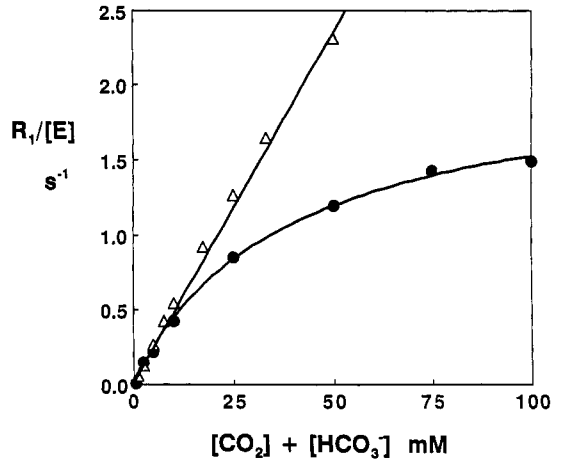

FIGURE 4: Dependence on total substrate concentration ($[CO_2] + [HCO_3^-]$) of $R_1/[E]$ catalyzed by (△) HCA III and (●) T199A HCA III. R_1 is the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium measured by ^{18}O exchange. Solutions at pH 7.2 and 25 °C contained Na_2SO_4 to maintain the total ionic strength at a minimum of 0.2 M. No buffers were used.

step, the departure of HCO_3^-, and describes the steps in the conversion of CO_2 to HCO_3^- given in eq 1. The replacement Thr-199 → Ala in HCA III caused a decrease by 4-fold in maximal values of k_{cat}/K_m for hydration of CO_2 and an increase in the apparent pK_a of the catalysis from a value near 5 to 6.3 (Figures 2 and 3). These data are qualitatively similar to those obtained with the replacement Thr-199 → Ala in HCA II for which there is an increase in the pK_a from 6.8 for HCA II to near 8 for T199A HCA II, accompanied by a 100-fold decrease in the k_{cat}/K_m for hydration (Liang et al., 1993; Lindskog et al., 1991). These results confirm a role for the hydroxyl side chain at position 199 in catalyzing the hydration of CO_2. However, it is interesting to note that despite a 100-fold decrease in activity, the mutant T199A HCA II has a higher maximal value of k_{cat}/K_m in the hydration of CO_2 than does wild-type HCA III.

The relatively small changes in k_{cat}/K_m for hydration of CO_2 (no change in k_{cat}) with the replacement Thr-199 → Ser (Figure 3) demonstrate that the replacement of threonine-199 with another residue containing a hydroxyl side chain, serine, causes little change in catalysis of CO_2 hydration in isozyme III. Again, it is of interest that the mutation Thr-199 → Ser in HCA II shows different effects, a 5-fold decrease in CO_2 hydration activity (k_{cat} conditions; Krebs & Fierke, 1993).

Hydration of CO_2 by metal-bound hydroxides in inorganic complexes (Martin, 1976) and nucleophilic attack on carbonyl groups (Hudson & Klopman, 1974) are known to follow the Brønsted relation, in which the rate of hydration increases with the increasing pK_a of the attacking hydroxide or nucleophile. Such a Brønsted correlation (Brønsted slope 0.44) was found in a series of variants of HCA III in which Phe-198 was replaced by seven other amino acids (LoGrasso et al., 1993). Adherence to such a Brønsted plot was not observed with the replacements Thr-199 → Ala in HCA II (Liang et al., 1993; Lindskog et al., 1991) and HCA III; in both cases, the k_{cat}/K_m for catalytic hydration decreased when the pK_a of the zinc-bound water increased (Figure 3). This result suggests that although Thr-199 enhances nucleophilicity it does not enhance the basicity of zinc-bound hydroxide. This conclusion lead us to examine the interaction of Thr-199 with a nearby residue, Phe-198.

Residue 198, Phe in HCA III and Leu in HCA II, is key in understanding the substantial differences in catalytic properties of HCA II and III, as shown by LoGrasso et al. (1991). The 25-fold enhancement in k_{cat}/K_m for hydration of CO_2 caused by the replacement Phe-198 → Leu in HCA III is hypothesized to be due to a transmitted change in the interaction of the hydroxyl side chain of Thr-199 with the zinc-bound hydroxide in the free enzyme or the transition states determining the rate of interconversion of CO_2 and HCO_3^- (eq 1). We used double mutations at residues 198 and 199 to construct double mutant cycles as described by Carter et al. (1984) and tested this hypothesis (Figure 3). Upon making the replacement Phe-198 → Leu in variants containing Thr or Ser at position 199, the activation energies for maximal k_{cat}/K_m were both decreased 1.9 kcal/mol (Figure 3a). With the mutant containing Ala at 199, making the additional replacement Phe-198 → Leu caused almost no change (–0.1 kcal/mol) in the activation energy for maximal k_{cat}/K_m (Figure 3a). This is consistent with the hypothesis that the enhancement in catalysis observed with the replacement Phe-198 → Leu in HCA III is related to the interaction of the adjacent Thr-199 with the zinc-bound hydroxide or transition states for catalysis since the enhancement is abolished by the removal of the hydroxyl side chain at 199. This interaction probably occurs through a conformational change in the backbone. Such an enhancement is not easily explained by a mechanism involving the hydrophobicity or polarizability of residue 198 without a backbone conformational change because such mechanisms would apply to both wild-type and T199A HCA III, contrary to observations. It is pertinent that the most significant change in conformation of HCA II upon the replacement Thr-199 → Ala is a shift of the side chain of Leu-198 (Xue et al., 1993).

Another indication of changes in catalysis caused by the replacement Thr-199 → Ala comes from Figure 4 which indicates that the effective binding constant for all substrates, K_{eff}^S, for T199A HCA III at 38 mM is low compared with that of the wild-type HCA III (and T199S HCA III, data not shown), for which K_{eff}^S is too large to measure from the data in Figure 4. This indicates that the enzyme–bicarbonate complex is more stable for T199A than for wild-type HCA III, suggesting that a thermodynamic well for the enzyme–product complex in T199A HCA III may be a source of its lower activity. Another clue comes from the solvent hydrogen isotope effect (SHIE), which was near unity for maximal k_{cat}/K_m catalyzed by T199A HCA III. An SHIE of this magnitude for k_{cat}/K_m is usual for carbonic anhydrase; a value near unity is observed for HCA II (Steiner et al., 1975) and bovine CA III (Ren et al., 1988b). An SHIE near unity in k_{cat}/K_m for T199A HCA III indicates no rate-contributing proton transfer in the interconversion of CO_2 and HCO_3^- (eq 1). This is consistent with a direct nucleophilic attack of zinc-bound hydroxide on CO_2, as suggested for HCA II (Simonsson et al., 1979; Silverman & Lindskog, 1988); it is also consistent with dissociation of product HCO_3^- as a rate-contributing step for HCA III.

The residues we studied at 198 and 199 also interacted in their influence on the values of the apparent pK_a for catalysis (determined from k_{cat}/K_m for hydration, Figure 3c), which can be taken as equivalent to the pK_a of the zinc-bound water (Simonsson & Lindskog, 1982; Silverman & Lindskog, 1988). It is notable that the increase in this pK_a is much greater for the replacement Thr-199 → Ala in wild-type HCA III (1.8 kcal/mol) than it is in the mutant containing Leu-198 (–0.3 kcal/mol). This effect is consistent with a weakening of the

hydrogen bond, by changing its length or orientation, between Thr-199 and the zinc-bound water transmitted through a conformational change in variants containing Leu-198. It is significant that the direction of these changes in pK_a and activity is different: the replacement Phe-198 → Leu caused an increase in k_{cat}/K_m and an increase in the apparent pK_a for catalysis; the replacement Thr-199 → Ala caused a decrease in k_{cat}/K_m and an increase in the pK_a for catalysis (Figure 3). This may reflect dissociation of HCO_3^- as the major rate-contributing step in catalysis by HCA III and these mutants [in agreement with the suggestions of LoGrasso et al. (1993) based on replacements at residue 198], with the pK_a of the zinc-bound water and the nucleophilicity of its conjugate base having a lesser effect on activity.

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