Amino acid substitutions at Thr199 of human carbonic anhydrase II (CAII) (Thr199 → Ser, Ala, Val, and Pro) were characterized to investigate the importance of a conserved hydrogen bonding network. The three-dimensional structures of azide-bound and sulfate-bound T199V CAIIIs were determined by x-ray crystallographic methods at 2.25 and 2.4 Å, respectively (final crystallographic R factors are 0.173 and 0.174, respectively). The CO₂ hydrase activities of T199S and T199P variants suggest that the side chain methyl and backbone amino functionalities stabilize the transition state by 0.4 and 0.8 kcal/mol, respectively. The side chain hydroxyl group causes: stabilization of zinc-hydroxide relative to zinc-water (pKₐ increases ≈ 2 units); stabilization of the CAII-HCO₃⁻ complex (≈ 5 kcal/mol); and destabilization of the CAII-HCO₃⁻ complex (≈ 0.8 kcal/mol). An inverse correlation between log(kₚ/H₂O) and the pKₐ of zinc-water (r = 0.95, slope = -1) indicates that the hydrogen bonding network stabilizes the chemical transition state and zinc-hydroxide similarly. These data are consistent with the hydroxyl group of Thr199 forming a hydrogen bond with the transition state and a non-hydrogen-bonded van der Waals contact with CAII-HCO₃⁻.

Carbonic anhydrase is a zinc metalloenzyme which efficiently catalyzes the hydration of CO₂ to bicarbonate and a proton. One isoform, human carbonic anhydrase II (CAII), is one of the fastest enzymes known, having a maximum turnover rate for CO₂ hydration of more than 10⁶ s⁻¹ (for review, see Refs. 1 and 2). CAII also catalyzes the hydrolysis of aromatic esters and is potently inhibited by sulfonamide compounds and monovalent anions (3). A crystal structure of CAII has been solved and refined to 1.54-Å resolution (4, 5), providing insights into structure-function relationships within the enzyme. The zinc cofactor is seated at the base of the conical active-site cleft where it is coordinated to 3 histidine residues in a tetrahedral geometry. At physiological pH the fourth zinc ligand is a hydroxide molecule which acts as a nucleophile to catalyze the hydrolysis of CO₂ and esters. There is considerable evidence that CAII-catalyzed CO₂ hydration occurs in two steps (2): conversion of CO₂ to HCO₃⁻, leaving water as a ligand on zinc (Equation 1), followed by proton transfer to solvent involving His₁⁴⁴ as a proton shuttle and containing a buffer dependent reaction (Equation 2) (2, 6). CO₂ binding is not included in this kinetic scheme since it is weak and rapid (2, 7).

$$
\begin{align*}
\text{CO}_2 + \text{H}_2\text{O} & \rightarrow \text{HCO}_3^- + \text{H}^+ \\
\text{HCO}_3^- + \text{H}_2\text{O} & \rightarrow \text{CO}_3^{2-} + \text{H}_2\text{O}
\end{align*}
$$

(Equation 1)

The active-site cleft is split into hydrophobic and hydrophilic regions. A genetic-structural analysis of the hydrophobic pocket adjacent to the zinc-bound hydroxide indicates that this region is the site of CO₂ association and that the hydrophobicity modulates the catalytic activity (7–11). Of the polar active-site residues, the hydroxyl group of the Thr199 side chain forms hydrogen bonds with the zinc-hydroxide group and the Glu₁⁰⁶ side chain to form a Zn-OH/Thr¹⁹⁹/Glu₁⁰⁶ hydrogen bond network (4, 5) which is absolutely conserved in all non-plant carbonic anhydrase isozymes (12). The hydrogen bond between Thr¹⁹⁹ and zinc-hydroxide has been postulated to be important for maintaining the orientation and reactivity of the zinc-hydroxide group (13), catalyzing proton transfer (14), and discriminating between protic and aprotic anions (5). A previous study on a Thr¹⁹⁹ → Ala (T199A) CAII variant indicated that the catalytic activity is decreased 100-fold (15) implying a role for this hydrogen bond in catalysis. In addition to the apparent functional importance of the side chain hydroxyl group, molecular dynamics studies have suggested that the methyl group of Thr¹⁹⁹ plays a role in CO₂ binding (16, 17). Also, crystallographic analysis of the CAII-anion complexes has revealed that anions, and possibly CO₂ as well, interact with the backbone amide group of Thr¹⁹⁹ (5, 18). Finally, random mutagenesis of the Asp¹⁹⁰-Ile²¹⁰ region of CAII suggests that Thr¹⁹⁹ is important for both catalysis and inhibitor binding (19).

To further delineate the role of Thr¹⁹⁹ in catalysis, four variants (Thr¹⁹⁹ → Ser, Val, Ala, and Pro) were characterized using steady-state kinetic analysis, and the three-dimensional structures of azide-bound and sulfate-bound T199V CAII were studied by x-ray crystallographic methods. These studies clearly indicate that the Thr¹⁹⁹ hydroxyl group stabilizes (3–5 kcal/mol) both the ground-state zinc-hydroxyl and the transition-state species and that the side chain methyl and backbone...
amide groups modulate the activity for high catalytic efficiency. The increased binding of bicarbonate to the T199A and T199V variants indicates that the hydroxyl moiety of Thr199 does not form an energetically favorable hydrogen bond with CAI-bound bicarbonate.

MATERIALS AND METHODS

Mutagenesis—Oligonucleotide-directed mutagenesis (20) of the cloned CAII gene in pCMV-1 (19) was performed using a 26-base oligonucleotide in which the Thr199 codon was replaced by a degenerate codon (an equimolar mixture of all four bases). The resulting DNA was first transformed into WKE6 mut35 and then into BL21(DE3) pLysS using the procedure described by Ang et al. (21). Colony plating of the plasmids encoding CAII variants were identified by screening for esterase activity and sulfonamide binding (19). The entire DNA sequence of the CAII gene of each variant was determined using the method of Sanger et al. (22).

Enzyme Induction and Purification—BL21(DE3) cells containing a plasmid encoding mutant CAII were grown, CAII was induced with 1 mm isopropyl-$\beta$-D-thiogalactopyranoside, and a crude lysate prepared as described (23). T199A, T199V, and T199P variants were purified to homogeneity by chromatography on an S-Sepharose fast flow column (2.5 cm x 20 cm) in 15 mM MESS, pH 7.0, eluting with a linear sodium sulfate gradient (0–5 M) followed by a Sephacryl S-100 HR column (2.5 cm x 14 cm). The flow was Tris-SO4 (8.0, 100 mM sodium sulfate). Wild-type and T199S CAIIs were purified using sulfonamide affinity chromatography (24). The concentration of CAII was determined by measuring the absorbance using $\varepsilon_{280}$ = 5.4 x 10$^3$ M$^{-1}$ cm$^{-1}$ (25).

Esterase Activity—The specific activity for p-styrenophenol acetate (PSPA) hydrolysis was determined at 25 °C in 0.5 mM PNPA by measuring the change in $A_{340}$ per min ($A_{340}$ = 5000 x $10^{-3}$ cm$^{-1}$ (26)). The pH dependence of esterase activity was measured in 50 mM Na-MES (pH 5.5–7.0), Tris-SO4 (7.5–9.0) or Na-CHES (9.0–10.0) buffer with a protein concentration of 0.5 μM (wild-type) to 20 μM (T199P). The apparent second-order rate constants, $k_{cat}/K_M^{obs}$, were calculated from initial rates after subtraction of the acetazolamide-inhibited rates. The $k_F$ and $k_{obs}$, and pH-independent $k_{cat}/K_M$ for esterase activity were determined by fitting the $p$F dependence of esterase activity to Equation 3 using the statistical program SYSTAT (Satyst, Inc.).

$$k_{cat}/K_M^{obs} = \frac{v_{max}}{[S]} = \frac{k_F}{K_M^{obs}} \times \frac{[S]}{1 + [S]/K_M}$$

(CO$_2$ Hydration and Bicarbonate Dehydration—Initial rates of CO$_2$ hydration and bicarbonate dehydration were measured in a Kin-Tec stopped-flow apparatus at 25 °C by the changing pH indicator method (27). Buffer-indicator pairs (with the wavelengths observed) were: MES/ chloroform buffer (pH 6.1, 574 nm); MOESP-nitrophosphol (pH 6.7–7.7, 407 nm); and TAPS/m-cresol purple (pH 7.5–8.9, 578 nm). The buffer concentration varied from 50 to 150 mM for CO$_2$ hydration and fixed at 100 mM for bicarbonate dehydration. The ionic strength was adjusted to 0.1 M with Na$_2$SO$_4$ (7.5–9.0) or Na-CHES (9.0–10.0) buffer. The pH was measured at 25 °C using a Radiometer pH meter (Model 632, Copenhagen, Denmark) and the pH was adjusted to 7.01, Tris-SO$_4$ (7.5–9.0) or Na-CHES (9.0–10.0) buffer with the ionic strength adjusted to 0.1 M using sodium sulfate and CAII concentrations varied from 0.5 μM (wild-type) to 20 μM (T199P). The apparent second-order rate constants, $k_{cat}/K_M^{obs}$, were calculated from initial rates after subtraction of the acetazolamide-inhibited rates. The $k_F$, $k_{obs}$, and pH-independent $k_{cat}/K_M$ for esterase activity were determined by fitting the $p$F dependence of esterase activity to Equation 3 using the statistical program SYSTAT (Satyst, Inc.).

RESULTS

Esterase Activity—To examine the function of a conserved hydrogen bond network in the active site of CAII, the catalytic properties of a series of four 199 variants (Thr199, Ser, Ala, Val, and Pro) were determined in detail. The pH dependence of $k_{cat}/K_M$ for PNPA hydrolysis was measured at 25 °C (Fig. 1) for all the Thr199 variants. The pH dependence of esterase activity is consistent with the ionization of a single enzyme group, the pH-independent rate constant, $k_{cat}/K_M$, and the calculated $pK_a$ are listed in Table II for each variant. These data indicate that removal of the γ-methyl group (Thr199S) results in a decrease (60-fold), consistent with the results obtained by Liang et al. (15). Therefore, the hydroxyl moiety is crucial for stabilization of the transition state for ester hydrolysis relative to the unbound ground states. However, the isosteric substitution of a methyl group for the hydroxyl group in Thr199 causes
When Thr199 is also dramatically increase the observed pKs for ester hydrolysis; this pKs likely reflects ionization of the zinc-water ligand as observed for wild-type (2). The observed pKs is 1.5-2.5 units higher for variants with aliphatic side chains at position 199 (Ala, Val, Pro) than for variants possessing side chains capable of forming hydrogen bonds with the zinc-bound solvent molecule (Thr and Ser), clearly indicating that the hydrogen bond with Thr199 lowers the pKs of the zinc-water moiety. In the isosteric substitution of Val for Thr199, indicating that the rate-limiting step in this direction as well. Therefore, a step involving CO2/H/O interconversion becomes the rate-limiting step in this direction as well. However, the substitution

only a 5-fold decrease in catalysis suggesting that the inserted methyl group makes additional favorable interactions with the transition state.

Substitutions at Thr199 also dramatically increase the observed pKs for ester hydrolysis; this pKs likely reflects ionization of the zinc-water ligand as observed for wild-type (2). The observed pKs is 1.5-2.5 units higher for variants with aliphatic side chains at position 199 (Ala, Val, Pro) than for variants possessing side chains capable of forming hydrogen bonds with the zinc-bound solvent molecule (Thr and Ser), clearly indicating that the hydrogen bond with Thr199 lowers the pKs of the zinc-water moiety. In the isosteric substitution of Val for Thr199, the zinc-hydroxide is destabilized relative to zinc-water by 2.4 kcal/mol at 25 °C. The pKs of the T199P variant (9.2) is even higher than that of T199V, which may suggest that the backbone amide group also lowers the pKs of the enzyme. Collectively, these results indicate that the methyl, hydroxyl and amide groups make significant (albeit varying) contributions to the stability of the zinc-hydroxide group.

**CO2 Hydration and HCO3 Dehydration**—The CO2 hydrase activity of CAII variants at position 199 was measured at various pH, solvent, and buffer conditions (Table III) using the pH indicator method of Khalilah (27). For all variants, kcat/KM for CO2 hydration increases with pH consistent with the observed pKs values for esterase activity (Tables II and III). The pH-independent kcat/KM (Table III) is very similar to the value measured at pH 8.9 except for the T199P variant where it is increased due to the high pK of this variant. The invariance in kcat/KM as the buffer concentration increases, and the sodium sulfate concentration decreases to maintain constant ionic strength, suggests that sulfate does not significantly inhibit any of the CAII variants under these conditions (Table III). The 3- and 86-fold decreases, respectively, in kcat/KM caused by removing the y-methyl group (T199S) and both the y-methyl and the hydroxyl groups (T199A) parallel the effects on esterase activity. This suggests that the hydroxyl moiety stabilizes the transition states for CO2 hydration and ester hydration similarly. However, the substitution of alkylic groups for the hydroxyl group in T199V and the T199P substitutions cause larger decreases in the CO2 hydrase activity (30- and 170-fold, respectively) than the esterase activity. Additionally, substitutions at position 199, except for T199S CAII, cause substantial reductions (>100-fold) in kcat.

The kinetic parameters for HCO3 dehydorization catalyzed by Thr199 variants were measured at pH 6.1 as shown in Table IV. Since this pH is well below the observed pKs (Table II), the majority of the enzyme should be in the catalytically active form. Variants lacking a hydroxyl group (Ala, Val, and Pro) cause large (108-109-fold) decreases in both kcat/KM and kcat for bicarbonate dehydration. These decreases are larger than those observed for CO2 hydration, as indicated by the increased enzyme pKs and the overall equilibrium for this reaction described by the Haldane relation (Equation 4; the apparent acid dissociation constant for H2CO3/KHCO3, approximately 6.3 x 10-7 m) (35, 36). For all variants, the pKs for zinc-water (pK2, H2O) calculated from Equation 4 agrees within experimental error with the pKs measured from the pH dependence of esterase activity (Table II).

\[
K_{Zn-H_2O} = (P_{H_2O})/(K_{H_2O}/(K_{CO2}/K_{HCO3})) \quad (Eq. 4)
\]

To indicate whether a proton transfer is occurring in the rate-limiting step, the solvent deuterium isotope effects on the steady-state kinetic parameters for CO2 hydration at pH 6.9 and HCO3 dehydrolysis at pH 6.1 were measured for substitution of Ser and Ala at position 199 (Tables III and IV). No large isotope effect is observed in kcat/KM for the majority of amino acids at position 199, indicating that the rate-limiting step under these conditions does not involve a proton transfer. Additionally, the isotope effect observed in kcat/KM catalyzed by the T199A variant is probably derived primarily from an equilibrium rather than a kinetic isotope effect (36, 37).

A kinetic isotope effect is observed on kcat for both CO2 hydration and HCO3 dehydorization for wild-type and the majority of Thr199 variants, which is indicative of rate-limiting proton transfer from the zinc-water molecule to solvent (36). Since kcat for wild-type and T199S are not dependent on the concentration of TAPS base under these conditions (Table III; 23), proton transfer to buffer is not rate-limiting (k3, Equation 2); however, an intramolecular proton transfer to His64 is slow (k3, Equation 2) (2, 6). Conversely, kcat for both T199A and T199V CAII is linearly dependent on the concentration of TAPS base (Table III) indicating that intermolecular proton transfer to buffer is rate limiting for these variants, as observed for wild-type at low buffer concentrations (23, 38). The Kf for buffer (calculated from a plot of kcat versus [buffer]) has increased from ∼5 mm for wild-type to >0.15 mm for T199A and T199V CAII. In the reverse direction, a primary solvent isotope effect (Table IV) but no buffer dependence is observed for kHCO3 for T199S and wild-type CAII suggesting that intramolecular proton transfer is the rate-limiting step in this direction as well. However, no isotope effect is observed on kHCO3 for T199A CAII; therefore, a step involving CO2/H2O interconversion becomes rate-limiting.

**X-Ray Crystallography**—A difference electron density map of azide-bound T199V CAII is found in Fig. 2, which reveals an unambiguous outline of the Val199 side chain. Overall, the structure of the variant is similar to that of wild-type CAII (10),
Hydrogen Bond Network in CAII

Activity measured as a function of [CO$_2$] in MES (pH 6.1), MOPS (pH 7.7) or TAPS (pH 8.9) buffer, 25 °C, I = 0.1 with sodium sulfate, using a pH indicator assay (27).

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer</th>
<th>$k_{cat}/K_M$, μM$^{-1}$ s$^{-1}$</th>
<th>Amino acid at position 199</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>50</td>
<td>67 ± 3</td>
<td>Thr, 25 ± 1, 0.12 ± 0.02, 0.1 ± 0.02</td>
</tr>
<tr>
<td>7.7</td>
<td>50</td>
<td>80 ± 6</td>
<td>Ser, 46 ± 3, 1.1 ± 0.05, 2.4 ± 0.6</td>
</tr>
<tr>
<td>8.9</td>
<td>50</td>
<td>120 ± 20</td>
<td>Ala, 0.37 ± 0.05, 0.16 ± 0.01</td>
</tr>
<tr>
<td>8.9</td>
<td>150</td>
<td></td>
<td>Val, 0.37 ± 0.05, 0.16 ± 0.01</td>
</tr>
<tr>
<td>pH independent</td>
<td>50</td>
<td>110</td>
<td>54</td>
</tr>
</tbody>
</table>

*a pH-independent $k_{cat}/K_M$ is estimated from this data using Equation 1 and the $pK_a$ for E$\cdot$ZnOH$_2$ listed in Table II.

*b Activity measured at 100 mM TAPS buffer.

c $k_D$ was determined in 99.9% D$_2$O.

TABLE IV

HCO$_3^-$ hydration for CAI variants at Thr$^{199}$, pH 6.1

Activity measured as a function of HCO$_3^-$ concentration (4-200 μM) in 50 mM MES, pH 6.1, 25 °C, I = 0.1 with sodium sulfate, using a pH indicator assay (27).

<table>
<thead>
<tr>
<th>Variant</th>
<th>$k_{cat}/K_M$, μM$^{-1}$ s$^{-1}$</th>
<th>$k_{cat}/K_M$</th>
<th>$k_{cat}$, ms$^{-1}$</th>
<th>$k_{cat}/k_D$, ms$^{-1}$</th>
<th>$K_M$, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>9.4 ± 1.0</td>
<td>1.0 ± 0.0</td>
<td>720 ± 80</td>
<td>3.9 ± 0.5</td>
<td>82 ± 15</td>
</tr>
<tr>
<td>T199G</td>
<td>4.7 ± 0.6</td>
<td>1.2 ± 0.6</td>
<td>380 ± 60</td>
<td>4.2 ± 0.5</td>
<td>76 ± 21</td>
</tr>
<tr>
<td>T199A</td>
<td>0.009 ± 0.002</td>
<td>1.1 ± 0.4</td>
<td>0.25 ± 0.02</td>
<td>1.2 ± 0.2</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>T199V</td>
<td>0.006 ± 0.001</td>
<td></td>
<td>0.15 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T199P</td>
<td>≤0.0003</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a $k_D$ measured in 99.9% D$_2$O.

*b No activity over background was observed at 21 μM T199P CAII with 9, 45, or 90 mM HCO$_3^-$.

and the rms deviation of Cα atoms between the two structures is 0.2 Å. However, structural changes in the immediate active site accommodate the Thr$\rightarrow$Val substitution, and these changes involve the conformation of Glu$^{106}$ as well as the identity and coordination mode of the non-protein zinc ligand. Despite these surrounding structural changes, the Val$^{199}$ side chain remains in a location quite close to that of Thr$^{199}$ in the wild-type enzyme (Fig. 3).

In wild-type CAII, the side chain of Glu$^{106}$ forms an energetically favorable, syn-oriented hydrogen bond with the hydroxyl group of Thr$^{199}$ (see Gandour (39) for a review of syn/anti nomenclature). Glu$^{106}$ also hydrogen bonds to the backbone nitrogen of Arg$^{246}$ (anti) and a solvent molecule (syn). In T199V CAII, Glu$^{106}$ is substantially displaced from its wild-type position in order to avoid unfavorable interactions with the hydrophobic side chain of Val$^{199}$ (Fig. 3). The Glu$^{106}$ side chain torsion angles $\chi_2$ and $\chi_3$ rotate by 78° and 68°, respectively (in azide-bound T199V CAII), from their wild-type values (Table V). This conformational change results in a different array of hydrogen bond interactions (Table VI). The most notable of these interactions occurs between Glu$^{106}$ Oe2 and His$^{96}$ N81. His$^{96}$ is coordinated to the active-site zinc ion; its interaction with the negatively charged carboxylate group of Glu$^{106}$ probably enhances its coordination to zinc. In turn, the increased

Fig. 2. Difference electron density map contoured at 3.0σ of the active site of azide-bound T199V CAII, calculated with Fourier coefficients ($|F_C| - |F_E|$) and phases calculated from the final model minus the atoms of the Val$^{199}$ side chain and azide. Refined atomic coordinates are superimposed; Val$^{199}$, Glu$^{106}$, azide (AZD), and zinc are indicated. The zinc-nitrogen separation is 2.4 Å.
It is not too surprising, given the substitution of the hydrophobic valine sidechain, such that the location of the zinc-coordinated nitrogen is more, the anion is slightly displaced away from the Val1'' side chain in the variant.

The effects of carboxylate-histidine-zinc interactions in modulating the reactivity of zinc-bound solvent is altered in the variant (Table 11). The pK, of zinc-bound solvent in T199V CAII relative to the wild-type enzyme (pK, 9; 44) but can be modeled by tetracoordinate zinc complexes (45, 46). In T199C CAII, the plastic Ser197-Cys206 loop contorts considerably to permit the coordination of Cys197 to zinc. However, in T199V CAII, neither the side chain of residue 199 nor the Ser197-Cys206 loop moves from its wild-type position. Instead, the non-protein zinc ligand and the side chain of Glu106 undergo conformational changes while the position of residue-199 remains relatively constant. Clearly, the chemical nature of the substituted side chain dictates the precise nature of accommodating structural changes.

**DISCUSSION**

Thr199 is a conserved residue in CAII which forms part of an active-site hydrogen bond network; the Thr199 hydroxyl group accepts a hydrogen bond from the zinc-water group and in turn donates a hydrogen bond to the Glu106 side chain (4, 5). This network is proposed to be important for orientation of the zinc-hydroxide group (10), specificity of anion binding (5), and proton transfer reactions (14). Additionally, the backbone amide and side chain methyl moieties of Thr199 have also been implicated in the binding of substrates and inhibitors (5, 16–18). Our kinetic and structural studies of a series of Thr199 variants permit the delineation of the functional importance of the hydroxyl, methyl and amide groups of this residue.

**Enzyme pK, —** It is generally accepted that the pH dependence of the esterase activity for wild-type CAII (pK, 6.8) directly reflects the ionization of the zinc-water moiety (2). This value is lower than the pK, of water in many zinc complexes (pK, 9; 44) but can be modeled by tetracoordinate zinc complexes (45, 46). In CAII this pK, is relatively insensitive to substitutions in a nearby hydrophobic pocket (7–9). However, disruption of the hydrogen bond network in CAII by substitution of alanine, proline, or valine at position 199 causes the pK, of the zinc-bound water to increase substantially, at least partially due to destabilization of the zinc-bound hydroxide by the introduction of nearby hydrophobic groups. Furthermore, these increases in pK, are likely mediated by interactions between the zinc-solvent group and active-site solvent molecules. It is reasonable to assume that in the T199V, T199A, and T199P variants a water molecule serves as a hydrogen bond acceptor for the zinc-bound solvent molecule. (However, an additional fixed water molecule is not observed in either the azide- or sulfate-bound T199V CAII structures.) The stability of hydrogen bonds with solvent compared to protein side chains is de-
increased mainly due to the entropic cost of sequestering a water molecule into the hydrophobic active site (47). Because the basic zinc-hydroxide species is much more tightly hydrated than the zinc-water species (48), the entropic penalty for the formation of hydrogen bonds between solvent and zinc-bound hydroxide is probably larger than similar bonding to the zinc-OH$_2$ species. This produces a net destabilization of the zinc-hydroxide moiety and a higher pK$_a$. Mutations which disrupt hydrogen bonding networks at the active sites of α-lytic protease (49) and dihydrofolate reductase (50) also cause increases in the pK$_a$ of active-site residues (His$_{57}$ and Asp$_{71}$, respectively), although the magnitude is smaller. Additionally, the crystal structure of T199V CAII indicates that G1u$_{106}$ forms a novel hydrogen bond to the zinc ligand, His$_{86}$, which increases the electron density on this histidine. This increased density should decrease the ability of the Zn$^{2+}$ to stabilize the negative charge on the zinc-bound hydroxide leading to an increase in the pK$_a$ (40). A maximal value for this effect can be estimated by the increase in the pK$_a$ of 9–10 caused by substitution of one of the zinc ligands, His$_{84}$, with either a negatively charged carboxylate (H94D) or thiolate (H94C) (51). Therefore, this new hydrogen bond with His$_{86}$ is unlikely to be the source of the entire increase in pK$_a$ but may magnify the effect.

Esterase Activity—In addition to increasing the pK$_a$ of the zinc-bound solvent, substitutions at position 199 also decrease the pH-independent rate constant. The individual contributions of the methyl and hydroxyl groups of Thr$_{199}$ toward stabilization of the transition state for ester hydrolysis relative to the unbound ground state can be estimated from a comparison of the esterase activity of T199S to wild-type, AG*Mutant = 1.7 kcal/mol, and T199A to T199S CAII, ΔΔG$^T$ = 1.7 kcal/mol, respectively. However, substitution of a methyl group for the hydroxyl group in T199V destabilizes the transition state less, ΔΔG$^T$ = 1.0 kcal/mol, suggesting that the inserted methyl group provides ~0.7 kcal/mol of additional favorable interactions. This increased activity of T199V compared to T199A could be mediated by the movement of the G1u$_{106}$ side chain; it is proposed to form a hydrogen bond with the zinc-solvent molecule in T199A (15, 52) rather than with His$_{86}$ as observed in T199V. Overall, the contribution of the hydroxyl group to transition state stabilization is consistent with the 0.5–1.8 kcal/mol observed by Fersht (47) for loss of a single hydrogen bond to an uncharged donor. However, the non-local structural changes observed in the G1u$_{106}$ side chain and the non-protein zinc ligand likely affect the stability of the transition state as well.

CO$_2$ and k$^{\text{cat}}$ Interconversion—Substitutions at Thr$_{199}$ also cause significant decreases in k$^{\text{cat}}$ and k$^{\text{cat}}$/K$_M$ for both CO$_2$ hydration and HCO$_3^-$ dehydration. These effects may be best interpreted in the context of the accepted catalytic mechanism of CAII (2) as shown in Equations 1 and 2. The second-order rate constant, k$_{\text{cat}}$/K$_M$, is only a function of rate constants up to and including product release (Equation 1) but not proton transfer (Equation 2). The pH-independent k$^{\text{cat}}$/K$_M$ = k$_1$k$_2$(k$_{-1}$ + k$_{2}$) = k$_1$ and k$^{\text{cat}}$/K$_M$ = k$_{-1}$k$_{-2}$(k$_{-1}$ + k$_{2}$) = k$_{-1}$/K$_2$ where the simplifications assume that k$_2$ > k$_{-1}$. This assumption is true for wild-type (53); however, the decreased K$_M$ (Table IV) and K$_D$ (15) for bicarbonate for the variants indicates that bicarbonate binds more tightly, and, therefore, the rate constant for bicarbonate dissociation (k$_D$) might decrease. Despite this, the linear correlation of log(k$^{\text{cat}}$/K$_M$) and log(k$^{\text{cat}}$/K$_M$) with log k$^{\text{cat}}$/K$_M$ for ester hydrolysis (correlation coefficient of r = 0.9 and r = 0.8, with slopes of −1 and 2, respectively), where nucleophilic attack on the ester is much slower, is convincing evidence that chemical interconversion is the rate-limiting step in k$^{\text{cat}}$/K$_M$ for all three reactions.

The roles of the hydroxyl, methyl and backbone amino groups of Thr$_{199}$ in catalysis can be delineated by kinetic characterization of this series of CAII variants. The energetic contribution of the methyl and hydroxyl groups to stabilization of the transition state for CO$_2$ hydration can be estimated at 0.4 and 2.2 kcal/mol, respectively, from the decrease in k$^{\text{cat}}$/K$_M$ for the substitution of Thr → Ser and Ser → Ala, paralleling the effects on esterase activity. However, no additional energy is observed for the substitution of a methyl group for the hydroxyl group, Thr → Val, ΔΔG$^T$ = 2 kcal/mol. Calculations by Merz (13) suggesting that the zinc-hydroxide/Thr$_{199}$/Glu$_{106}$ hydrogen bond network orients the hydroxide into an optimal conformation for nucleophilic attack is consistent with our data since this hydrogen bond might both stabilize the zinc-hydroxide and increase its reactivity by decreasing its conformational entropy. Finally, substitution of proline for threonine at position 199 causes even larger decreases in k$^{\text{cat}}$/K$_M$, ΔΔG$^T$ = 3.4 kcal/mol. If we assume that there are no major structural rearrangements in this proline variant, a comparison of the activity of the T199P and T199A variants suggests that removal of the backbone amide destabilizes the transition state by ~0.8 kcal/mol.
The energetic contributions of both the side chain hydroxyl and the backbone amide groups are consistent with the loss of a single hydrogen bond to the transition state compared to the unbound ground states (47). This latter result is consistent with crystallographic data indicating that the backbone amide forms a hydrogen bond with active-site inhibitors such as cyanate (5, 18). However, in all cases the second-order rate constant for bicarbonate dehydration is decreased significantly more than $k_{\text{cat}}/K_M$ reflecting the increased stability of the zinc-bound water in the ground state as well as the decreased stability of the transition state.

In bicarbonate dehydration catalyzed by T199A, no isotope effect is observed in $k_{\text{cat}}^{\text{inc}}$, indicating that the formation of CO$_2$ ($k_1$) is the main rate-contributing step and that $K_M^{\text{inc}}$ approximates the dissociation constant for HCO$_3^-$ (41). Therefore, the effect of substitutions at position 199 on these parameters delineates the role of the Thr$_{199}$ side chain in specific transition state stabilization and stabilization of the bound complex, respectively. The decreased $K_M^{\text{inc}}$ (Table IV) and $K_M^{\text{inc}}$ (15) for variants at position 199 indicates that interaction with the hydroxyl group of Thr$_{199}$ actually destabilizes the E-HCO$_3^-$ complex by at least 0.8 kcal/mol, thereby decreasing the rate constant for HCO$_3^-$ dissociation and increasing the efficiency of CO$_2$ hydration. Therefore, hydrogen bonding interactions between bound bicarbonate and Thr$_{199}$ are not major energetic determinants of bicarbonate binding. Two alternative binding modes for bicarbonate have been suggested by x-ray crystallographic studies of CAII-anion complexes (Fig. 5). A variety of anions, including sulfide, bisulfite, and bicarbonate under certain conditions (5, 54, 56), displace the zinc-solvent ligand to form a tetracoordinate complex with zinc and donate a hydrogen bond to the hydroxyl of Thr$_{199}$. However, azide, an anion which cannot donate a hydrogen bond to Thr$_{199}$, makes a non-hydrogen-bonded van der Waals contact with the hydroxyl of Thr$_{199}$ (41). Our data are more consistent with a bicarbonate binding mode similar to that of azide in which the negatively charged carboxylate interacts with the metal to obtain optimum stereochemistry while sacrificing the hydrogen bond interaction with Thr$_{199}$ (41). Additionally, this binding mode predicts that electrostatic repulsion between the hydroxyl of Thr$_{199}$ and the zinc-bound bicarbonate could destabilize the bound complex, as observed.

Finally, the 104-fold decrease in the rate constant for bicarbonate dehydration starting from the bound complex ($k_{\text{cat}}^{\text{inc}} = k_1$) for variants lacking a side chain hydroxyl group compared to wild-type ($k_1 = 3 \times 10^6$ s$^{-1}$; 85) indicates that the transition state for bicarbonate dehydration is destabilized by 5.2 kcal/mol relative to the bound bicarbonate complex. This destabilization is likely due to a combination of two effects: 1) the loss of a stabilizing hydrogen bond between the hydroxyl group of Thr$_{199}$ and the transition state, which can be estimated at $\sim 2.5$ kcal/mol from the observed destabilization of the transition state for CO$_2$ hydration; and 2) stabilization of the bound bicarbonate complex in either a productive or non-productive binding mode (as perhaps suggested by the altered position of zinc-bound azide in T199V CAII). A model consistent with these data (see Fig. 5) is that in the transition state for CO$_2$ hydration the zinc-bound bicarbonate group remains coordinated to the zinc and donates a hydrogen bond to the hydroxyl group of Thr$_{199}$. Subsequently, the bicarbonate product undergoes tautomerization either by proton transfer (57) or simple rotation (58, 59) to form the ground state bicarbonate complex with a non-hydrogen-bonded van der Waals interaction with Thr$_{199}$ (as discussed above).

**Structure-Function Correlations**—In contrast with the result of structure-function studies in the hydrophobic pocket of CAII (7, 9), there is little correlation between the hydrophobicity of the amino acid at position 199 and either the esterase or CO$_2$ hydrase activity; the linear correlation coefficient is 0.35 and 0.71, respectively, for a plot of log($k_{\text{cat}}/K_M$) versus $\Delta G_R$ (amino acid solvation energy (61)) (plot not shown). Similarly, the observed pK$_\alpha$ shows low correlation with the hydrophobicity of the substituted amino acid at position 199, as indicated by either 1.367 (r = 0.72, slope = 1.0) or $\Delta G_R$ (r = 0.8, slope = 1.3). However, there is an inverse correlation between log($k_{\text{cat}}/K_M$) and the pK$_\alpha$ of zinc-bound water as shown in Fig. 6. This Bronsted plot shows a linear correlation (r = 0.74, 0.95, and 0.99, respectively) between pK$_\alpha$ and log($k_{\text{cat}}/K_M$) for the esterase, hydrase, and dehydrase activities of a series of variants at position 199, with a slope of -0.5, -1.0, and -2.0, respectively. This is a very unusual result. Bronsted correlations with positive slopes have been observed for many reactions, including nucleophilic attack on a carbonyl (62), hydration of CO$_2$ by metal-bound hydroxides in inorganic complexes (63) and hydration of CO$_2$ catalyzed by a series of CAII variants at position 198 (64). In this latter case, $k_{\text{cat}}/K_M$ likely reflects rate-contributing dissociation of product HCO$_3^-$. However, small decreases in reactivity ($\beta = -0.1$) with increasing basicity of the nucleophile have only been observed in a few cases, including the reaction of quinuclidines with phosphorylated pyridines, which have been attributed to rate-contributing desolvation (48, 65). Rate-contributing desolvation of the zinc-hydroxide may contribute to the observed Bronsted correlation in CAII but it is unlikely to account for the magnitude of this effect. The observed slope of -1 for CO$_2$ hydration indicates that interactions which destabilize zinc-hydroxide relative to...
zinc-water similarly destabilize the transition state for CO₂ hydration relative to zinc-hydroxide. These data suggest that the negative charge on oxygen in the transition state retains characteristics of the zinc-hydroxide. Factors which destabilize zinc-hydroxide (discussed previously), and likely the transition state for CO₂ hydration as well, include: disruption of the hydrogen bond network which increases the electron density on the zinc-bound transition state; increased entropic costs for salvation; and movement of the Glu¹⁰⁶ side chain which decreases the ability of the zinc to stabilize the negative charge on the transition state.

Proton Transfer—The observed rate constant at saturating substrate concentrations, $k_{\text{cat}}$, contains rate constants from the enzyme-substrate-bound complex through regeneration of the active enzyme species (Equations 1 and 2). A significant solvent isotope effect is observed on $k_{\text{cat}}$ of both variants (Table II) indicating that proton transfer is a significant rate-contributing step ($k_2 > k_1$). The rate constants indicated in the pH-independent $k_{\text{cat}}$ are shown in Equation 5. These rate constants have been measured for wild-type CAII as: $k_3 = k_3 \approx 10^{-5}$ s⁻¹ and $k_4 = 2 \times 10^6$ M⁻¹ s⁻¹ (38, 66). A plot of $k_{\text{cat}}$ versus buffer concentration is hyperbolic; at low buffer

$$k_{\text{cat}} = k_1 k_2 (B)/(k_1 + k_1 + k_2 + k_1(B)) = k_0(B)(1 + k_1 + k_2)/(k_1 + k_2 + k_0(B)) \quad (Eq. 5)$$

concentrations (< 5 mM) the observed rate constant is linearly dependent on the buffer concentration (Equation 5) while at high buffer concentrations the rate constant is buffer-independent and equal to $k_0$, the rate constant for intramolecular proton transfer from zinc-H₂O to His⁶⁴ (2, 38, 66). However, $k_{\text{cat}}^0$ for T199V and T199V is linearly dependent on buffer at much higher concentrations (up to 150 mM total buffer) indicating that intramolecular proton transfer is the main rate-controlling step under these conditions. Using the $k_{\text{cat}}^0$ of zinc-H₂O from Table II, the observed $k_{\text{cat}}^0$ for both T199V and T199V CAI can be calculated using Equation 5 assuming either that $k_4$ is decreased to $\approx 7 \times 10^6$ M⁻¹ s⁻¹ (25-fold smaller than the wild-type value), or that the $k_{\text{cat}}^0$ of His⁶⁴ is lowered to 5. Either of these effects would be somewhat surprising since: (i) the side chain of Thr¹⁹⁹ is located 6 Å away from the His⁶⁴ so the effect would have to be mediated by the active-site water molecules (4, 5); (ii) structure-function studies of variants at active-site residues Val¹⁵¹, Val¹⁴⁵, and Leu¹⁰⁶ indicate that the $k_{\text{cat}}$ and rate constant for proton transfer are not lowered greatly by substitutions at these positions (7–9); and (iii) studies of the T202S variant indicate that $k_4$ is unaffected by changes in the configuration of His⁶⁴ and surrounding water molecules (23). Another explanation for the observed buffer dependence of $k_{\text{cat}}^0$ is that proton transfer occurs from zinc-water to buffer, bypassing the proton shuttle group, with a rate constant of $\approx 1 \times 10^6$ s⁻¹. In this mechanism the proton could be transferred to buffer directly or via active-site water molecules. To observe this pathway, the rate constant for intramolecular proton transfer ($k_3$) must be decreased by $\approx 10^{5}$-fold, similar to the observed rate constants in the absence of the His⁶⁴ proton shuttle group (6). Direct catalysis of proton transfer by buffers has been observed in CAIII (67). Since the observed decrease in the rate of intramolecular proton transfer ($k_4 < 4 \times 10^6$ s⁻¹) greatly exceeds the expected decrease caused by the increase in the zinc-water $pK_\alpha$ ($k_3 \approx 2 \times 10^4$ s⁻¹), the data suggest that the removal of the hydroxyl group of Thr¹⁹⁹ disrupts facile proton exchange between acidic and basic groups via active-site water molecules.

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Hydrogen Bond Network in CAII