Disruption of the Active Site Solvent Network in Carbonic Anhydrase II Decreases the Efficiency of Proton Transfer†

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ABSTRACT: The importance of maintaining the active site water network for efficient proton transfer was investigated by substituting amino acids of varying size at position 65 in carbonic anhydrase II (including four amino acids found in other CA isozymes, F, L, S, and T, and two amino acids that do not occur naturally at position 65, G and H) and measuring the rate constants for the proton transfer reactions in the variant carbonic anhydrases. Intramolecular proton transfer between zinc-bound water and H64 is significantly inhibited by the introduction of bulky residues at position 65; k_{cat} for CO_2 hydration decreases up to 26-fold, comparable to the observed decrease in intramolecular proton transfer caused by removal of H64 [Tu, C., Silverman, D. N., Forsman, C., Jonsson, B.-H., & Lindskog, S. (1989) *Biochemistry 28*, 7913−7918]. Intermolecular proton transfer between protonated H64 and external buffer is also inhibited, although to a lesser degree. Furthermore, an alternative proton transfer pathway, consisting of an active site solvent-mediated proton transfer from zinc−water to imidazole buffer, is inhibited in the A65F, A65L, and A65H CAII variants. Therefore, the active solvent bridge between zinc-bound water and H64 is disrupted by substitutions at position 65. The inhibition of proton transfer reactions correlates with the disruption of the crystallographically observed solvent network in the CA active site and rotation of the proton acceptor, H64 [Scolnick, L. R., & Christianson, D. W. (1996) *Biochemistry 35*, 16429−16434], suggesting that this solvent network, including water molecules 292, 264, and 369, or a structurally related network, forms the proton transfer pathway in CAII for both intramolecular proton transfer and stimulation of proton transfer in imidazole buffers.

Human carbonic anhydrase II (CAII;[1] EC 4.2.1.1) is a zinc metalloenzyme that catalyzes the reversible hydration of CO_2 to HCO_3^- and a proton (Heck et al., 1996; Silverman, 1995; Silverman & Lindskog, 1988). The structure of CAII has been determined and refined at 1.54 Å (Eriksson et al., 1988; Hakansson et al., 1992). The single zinc lies at the bottom of a conical cleft about 15 Å deep where it is coordinated in a tetrahedral fashion by the imidazole side chains of three histidine residues (H94, H96, and H119) and one solvent molecule (water 263). These zinc ligands are fully saturated by hydrogen bonds with second shell residues: H94 donates a hydrogen bond to the carboxamide side chain of Q92, H119 donates a hydrogen bond to the carboxylate side chain of E117, H96 donates a hydrogen bond to the backbone carbonyl oxygen of N244, and zinc-bound hydroxide donates a hydrogen bond to the hydroxyl side chain of T199.

CO_2 hydration catalyzed by CAII occurs in two distinct steps (Silverman & Lindskog, 1988). First, zinc-bound hydroxide attacks the carbonyl carbon of CO_2 to form a zinc-bound bicarbonate that is subsequently replaced by water, releasing product bicarbonate and leaving zinc-bound water in the enzyme active site (eq 1). In CAII, the second-order rate constant for CO_2 hydration (k_{cat}/K_M) approaches the diffusion-controlled limit. In the second step that regenerates the active zinc hydroxide species, a proton is transferred from zinc-bound water to a solvent buffer molecule; this is the rate-limiting step for CO_2 hydration at high substrate and buffer concentrations (Jonsson et al., 1976; Steiner et al., 1975). This takes place in two stages: first, the proton is transferred intramolecularly to a protein side chain with a pK_a of 7; then, an intermolecular proton transfer from this side chain to buffer completes the process (eq 2). The protein residue responsible for this proton shuttling activity in CAII has been identified as H64 (Steiner et al., 1975; Tu et al., 1989).

Seven mammalian isozymes of carbonic anhydrase have been characterized to date. These isozymes vary in cellular localization, tissue expression, physiological role, inhibitor sensitivity, and catalytic activity, from $k_{cat} = 1 \times 10^4$ s^{-1} for the relatively inefficient CAIII to $k_{cat} = 1 \times 10^6$ s^{-1} for the highly active CAII (Silverman & Lindskog, 1988). Nonetheless, the data indicate that all of the isozymes share

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[1] Abbreviations: CAI, human carbonic anhydrase I; CAII, human carbonic anhydrase II; A65F, CAII variant with phenylalanine substituted for alanine-65; A65H, CAII variant with histidine substituted for alanine-65, etc., using the one-letter amino acid codes; EDTA, (ethylenedinitrilo)tetraacetic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TAPS, *N*-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PNPA, *p*-nitrophenyl acetate; PMSF, phenylmethanesulfonyl fluoride; TAME, N^{α}-*p*-tosyl-L-arginine methyl ester; SHIE, solvent hydrogen isotope effect.

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$$\text{E-Zn-OH}^- + \text{CO}_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E-Zn-HCO}_3^- + \text{H}_2\text{O} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{E-Zn-OH}_2 + \text{HCO}_3^- \quad (1)$$

$$\begin{array}{l}\text{His 64}\\ |\\ \text{E-Zn-OH}_2\end{array} \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} \begin{array}{l}\text{His 64-H}^+\\ |\\ \text{E-Zn-OH}^-\end{array} + \text{B} \overset{k_4}{\underset{k_{-4}}{\rightleftharpoons}} \begin{array}{l}\text{His 64}\\ |\\ \text{E-Zn-OH}^-\end{array} + \text{BH}^+ \quad (2)$$

a common mechanism. The differences in turnover number can be partially attributed to the natural variations at positions 64 and 65 observed among the different isozymes and the resulting effects of these variations on proton transfer. In CAII, the alanine occurring naturally at position 65 is small and does not protrude into the active site cavity, allowing for efficient proton transfer between Zn-bound water and H64. Isozymes I, IV, VI, and VII also contain H64 which might similarly function as a proton shuttle group and a relatively small amino acid at position 65, either S (I,VII) or T (VI, VIII) (Hewett-Emmett & Tashian, 1996). In contrast, CAV contains Y64 and F65, and proton transfer does not occur through Y64, even at high pH (Heck et al., 1994); this is proposed to be at least partially due to a steric block of the direct proton transfer pathway between Zn−OH_2 and Y64 by the bulky side chain of adjacent F65 (Boriack-Sjodin et al., 1995; Heck et al., 1996). Instead, the three-dimensional structure of CAV suggests that a residue near Y131 may act as a proton shuttle group. CAIII, the lowest activity isozyme assayed so far, contains K64 and T65; K64 plays only a minor role in proton transfer, and no other residue capable of functioning as a proton shuttle has yet been identified (Jewell et al., 1991). These examples indicate that the magnitude of CA turnover depends on both the identity of the proton shuttle group, often found at position 64, and the residue occupying position 65.

The high-resolution crystal structures of CAII reveal that H64 is located ≈8 Å away from the zinc-bound water (Eriksson et al., 1988) and that this residue exhibits significant conformational mobility (Krebs et al., 1991; Nair et al., 1991; Taoka et al., 1994). This distance is too great for proton transfer between these two groups to occur directly; instead, the rate-limiting intramolecular proton transfer is postulated to occur via a highly ordered array of water molecules observed in the active site cavity (Eriksson et al., 1988; Hakansson et al., 1992; Merz et al., 1989; Venkatasubban & Silverman, 1980). Proton transfer can be modulated by changing the identity or position of the proton shuttling residue. Glutamic acid or lysine residues in place of H64 in CAII, or histidine at positions 62, 67, or 200, all can function as proton shuttle groups, albeit less efficiently than the native H64 (Engstrand et al., 1992; Liang et al., 1993). Also, mutagenesis of nearby residues can change the pK_a of either the proton donor or proton acceptor groups, thereby affecting the rate constant for intramolecular proton transfer in a manner that is successfully predicted by a Brönsted relationship describing the dependence of proton transfer on the relative pK_a of donor and acceptor groups (Ren et al., 1995; Silverman et al., 1993). Marcus theory analysis of the rate of intramolecular proton transfer as a function of ΔpK_a between donor and acceptor suggests that proton transfer occurs with a relatively small overall energy barrier ($\Delta G_o^{\ddagger}$ = 1.3 kcal/mol) but that the transfer is accompanied by substantial work functions that include energy required to orient the donor, acceptor, and intervening water structure for efficient proton transfer (Ren et al., 1995; Silverman, 1995; Silverman et al., 1993).

In this study we test the importance of orientation of the donor, acceptor, and crystallographically observed intervening water molecules for efficient proton transfer by substituting bulky residues (F, H, or L) for A65. These amino acids were predicted to protrude into the active site cavity and perturb the water structure in that region. Indeed, substitution of large amino acids (F, H, or L) but not small side chains (G, S, or T) decreases the rate constant for proton transfer up to 26-fold without affecting the pK_a of either the proton donor or acceptor group or altering the rate-determining step for CO_2 hydration. These results correlate with the perturbation of active site solvent structure observed in the high-resolution X-ray crystallographic structures of these variants (Scolnick & Christianson, 1996), indicating that this pathway may be a functionally relevant trajectory for proton transfer in CAII.

MATERIALS AND METHODS

Mutagenesis. Oligonucleotide-directed mutagenesis (Kunkel et al., 1987) of the cloned CAII gene in plasmid pCAM (Krebs & Fierke, 1993) was performed using a 26-base oligonucleotide in which the A65 codon (GCT) was replaced by codons specifying each of the following residues: F (TTC), H (CAC), L (CTC), G (GGT), S (TCT), and T (ACT). The resulting DNA was transformed into XL-1 Blue cells using the procedure of Hanahan (1983). The entire sequence of the CAII gene was determined for each variant by the method of Sanger et al. (1977) to confirm that mutations were introduced only at the codon for position 65.

Enzyme Induction and Purification. The mutant CAII plasmid was transformed into BL21(DE3) cells (Studier & Moffatt, 1986) and grown in induction media (Nair et al., 1991) to A_{600} = 1. At this point, CAII was induced by the addition of 0.25 mM isopropyl β-D-thiogalactopyranoside and 0.4 mM $ZnSO_4$, and the culture was incubated for 8 h at 30 °C. Five hours after induction, protease inhibitors (1 μg/mL TAME and 8 μg/mL PMSF) were added. The cells were pelleted and resuspended in an equal volume of lysis buffer (50 mM Tris−SO_4, pH 8.0, 50 mM NaCl, 10 mM EDTA, 1 mM 1,4-dithiothreitol, 10 μg/mL PMSF, 1 μg/mL TAME, 200 μM $ZnSO_4$) and frozen at −70. The cells were lysed by incubation with 0.2 mg/mL hen egg white lysozyme, and the cellular debris was removed by centrifugation (16000*g*, 45 min). The lysate was fractionated by a 10% streptomycin sulfate precipitation, and the precipitate was pelleted by centrifugation (10000*g*, 30 min). The CAII-containing supernatant was dialyzed extensively against 10 mM Tris−SO_4, pH 8.0, and 100 μM $ZnSO_4$ and then further fractionated by DEAE-Sephacel batch chromatography. The eluant was collected, dialyzed against 10 mM MES, pH 7.0, and applied to an S-Sepharose column (2.5 cm × 20 cm). CAII was then eluted from the column with a linear ammonium sulfate gradient (0−0.5 M) in 10 mM MES, pH 7.0. The CAII produced from these steps is ≥98% pure as assayed by SDS−PAGE (Laemmli, 1970), and the concentration of each

mutant CAII was determined by fluorescence titration of dansylamide-bound CAII with a competing inhibitor, acetazolamide (Krebs & Fierke, 1993; Krebs et al., 1991).

CO_2 Hydration Assays. Initial rates of CO_2 hydration catalyzed by CAII (20−400 nM) were measured at 25 °C in a KinTek stopped-flow apparatus by the changing pH-indicator method (Khalifah, 1971). Buffer/indicator pairs were as follows: TAPS/*m*-cresol purple (pH 8−9, 578 nm), MOPS/*p*-nitrophenol (pH 7−7.5, 400 nm), MES/chorophenol red (pH 6.5, 574 nm), and 1,2-dimethylimidazole/*m*-cresol purple (pH 7.5−8.9, 578 nm). The buffers contained 0.1 mM EDTA, and the ionic strength was maintained at 0.1 M with Na_2SO_4. The CO_2 concentration (2−24 mM) and buffer concentration (2−100 mM) were each varied separately. Solvent isotope effects were measured using solutions prepared with 94% D_2O. The pD of the solution was determined by adding 0.4 to the glass electrode pH meter reading (Glasoe & Long, 1960). The steady-state kinetic parameters and standard errors were determined by fitting the data to eq 3, using the Kaleidagraph (Synergy Software) curve-fitting program. The pH-independent rate constant (k_{cat} or k_{cat}/K_M) and observed pK_a were determined by a weighted fit of the pH dependence of the observed rate constants for CO_2 hydration using eq 4, where k is either k_{cat} or k_{cat}/K_M.

$$v_o = k_{cat}[E][S]/(K_M + [S]) \quad (3)$$

$$k_{obs} = k/(1 + 10^{pK_a - pH}) \quad (4)$$

Esterase Assay. The CAII-catalyzed hydrolysis of *p*-nitrophenyl acetate (PNPA) was measured at 25 °C in 50 mM buffer, either CHES (pH 9−9.5), TAPS (pH 8−8.5), MOPS (pH 7−7.5), or MES (pH 5.5−6.5), with the ionic strength maintained at 0.1 M by the addition of Na_2SO_4. The concentration of PNPA used was 0.45 mM (k_{cat}/K_M conditions), and the concentration of enzyme ranged from 0.3 to 0.5 μM. The change in absorbance per minute at 348 nm ($\Delta\epsilon_{348}$ = 5000 M^{-1} cm^{-1}) (Armstrong et al., 1966) was measured, and the pK_a and pH-independent k_{cat}/K_M were determined from these measurements by fitting the observed second-order rate constants to eq 4.

RESULTS

We have prepared variants of CAII at A65, the residue adjacent to the proton shuttle H64, in order to investigate how neighboring amino acids affect the orientation of the proton acceptor group and intervening water chain. We substituted A65 with three bulky amino acids (F, H, L), including two substitutions observed in CAV (L, F) where proton transfer occurs by an alternate pathway (Heck et al., 1994,1996), and three smaller residues (S, T, G), two of which have also been observed in natural variants (S, CAI and CAII; T, CAIII, CAVI, and CAVIII) (Hewett-Emmett & Tashian, 1996).

CO_2 Hydration. The pH dependence of the CO_2 hydrase activity of the A65 variants was measured using the pH indicator assay of Khalifah (1971) (Figure 1 and Table 1). Michaelis−Menten behavior was observed for all of the variants. The pH-independent k_{cat}/K_M, k_{cat}, and K_M for CO_2 hydration are all unaffected by small alterations at position 65, such as S, G, or T (Table 1). In contrast, when bulky amino acids, such as F, H, or L, are substituted at position

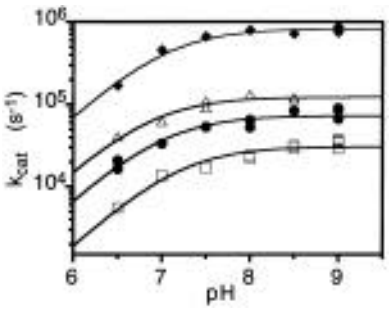

FIGURE 1: pH dependence of k_{cat} for CO_2 hydrase activity for CAII, wild type (◆), A65L (△), A65H (●), and A65F (□), measured using the pH-indicator assay (Khalifah, 1971) as a function of [CO_2] in 50 mM buffer, 25 °C, ionic strength = 0.1 with sodium sulfate. The data are fit to eq 4 with the Kaleidagraph (Synergy Software) curve-fitting program.

65, there is a large decrease (from 7- to 26-fold) in the turnover number, k_{cat} (Figure 1). The K_M for these variants (A65F, A65H, and A65L) is decreased to a value below the minimum concentration of CO_2 measurable by this technique (1 mM), allowing us to determine only a lower limit for k_{cat}/K_M. In all cases k_{cat}/K_M is similar to that observed in wild-type CAII (Table 1). Therefore, none of these substitutions (F, H, and L) significantly change the stability of the transition state for CO_2 hydration. Assuming that the rate-limiting step in k_{cat} is the same for the variants as for wild-type CAII, the observed decreases in k_{cat} suggest that these substitutions specifically impair intramolecular proton transfer.

For wild-type CAII, the pH dependence of k_{cat} for CO_2 hydration is consistent with ionization of a single group near pH 7, reflecting ionization of the proton acceptor, H64 (Campbell et al., 1975; Steiner et al., 1975). For the A65 variants, the observed dependence of k_{cat} on pH also reflects the titration of one ionizable group (Figure 1) with a pK_a comparable to that seen in wild-type CAII, presumably H64 (Table 1). Since the pK_a of H64 in the A65F, A65H, and A65L variants is unaffected by the substitutions at position 65 (Table 1, Figure 1), the decrease in proton transfer cannot be attributed to perturbations in the basicity of the proton acceptor.

Solvent Hydrogen Isotope Effects. To test whether the rate-limiting steps reflected in each of the steady-state kinetic parameters are the same for the A65 variants and wild-type CAII, we measured the solvent hydrogen isotope effects (SHIE) on the pH-independent k_{cat} and k_{cat}/K_M for CO_2 hydration at pH/pD 8.5 (Table 1). The large isotope effect on k_{cat}, observed with both wild-type CAII and all of the variants at position 65, indicates that the transition state for the rate-limiting step at high CO_2 concentrations contains a proton transfer, suggesting that intramolecular proton transfer is still the rate-limiting step under k_{cat} conditions with the A65 variants. Additionally, the isotope effect is significantly smaller under k_{cat}/K_M conditions, approaching a value of 1, indicating that the rate-limiting step at low concentrations of CO_2 does not involve a proton transfer.

Intermolecular Proton Transfer. Intermolecular proton transfer from H64 of CAII to the basic form of buffer in solution is rate limiting for wild-type CAII under conditions of high CO_2 and low buffer concentration (Jonsson et al., 1976; Rowlett & Silverman, 1982; Silverman et al., 1993). Therefore, to ascertain the effect of substitutions at position

Table 1: CO_2 Hydrase Activity of Ala 65 CAII Variants

variant	k_{cat} ($\times 10^{-5}$ s^{-1})	pK_a of His 64[a]	k_{cat}/K_M ($\times 10^{-7}$ M^{-1} s^{-1})	$(k_{cat}/K_M)_{buffer}$[b] ($\times 10^{-8}$ M^{-1} s^{-1})	k_{cat}^H/k_{cat}^D [c]	$(k_{cat}/K_M)^H/(k_{cat}/K_M)^D$ [c]
WT CAII	8.2 ± 0.2[d]	6.9 ± 0.1	9.2 ± 0.4[d]	7.5 ± 0.9	3.8 ± 0.2	0.99 ± 0.09
A65F	0.31 ± 0.1[d]	7.2 ± 0.1	≥ 5.4[e]	1.5 ± 0.8	4.5 ± 0.3	1.2 ± 0.3
A65H	0.73 ± 0.2[d]	7.0 ± 0.1	≥ 10[e]	4.0 ± 1.9	5.3 ± 0.4	2.3 ± 0.5
A65L	1.5 ± 0.1[d]	6.9 ± 0.1	≥ 10[e]	2.7 ± 0.5	6.0 ± 0.3	1.9 ± 0.3
A65G	6.5 ± 0.2[f]	ND	11 ± 0.6[f]	4.6 ± 1.1	6.7 ± 0.4	1.1 ± 0.2
A65S	8.9 ± 0.5[f]	ND	10 ± 0.9[f]	5.2 ± 1.2	2.7 ± 0.3	0.91 ± 0.3
A65T	10 ± 0.1[f]	ND	11 ± 0.5[f]	8.1 ± 1.6	ND	ND

[a] pK_a calculated from pH dependence of k_{cat} using eq 4. [b] Determined as a function of concentration of the basic form of buffer in TAPS, pH 8.0, ionic strength = 0.1 with sodium sulfate at 25 °C. [c] k^D measured in 94% D_2O, pD 8.5. [d] pH-independent value calculated using eq 4. [e] Lower limit for pH-independent k_{cat}/K_M, calculated from the pH dependence of k_{cat}/K_M using eq 4. [f] pH-independent value measured at pH 8.5.

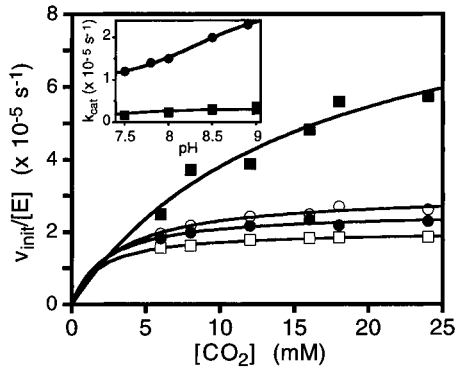

FIGURE 2: CO_2 hydrase activity of A65 variants, A65T (■), A65L (○), A65H (●), and A65F (□), measured by the changing pH-indicator method (Khalifah, 1970) in the presence of 100 mM 1,2-dimethylimidazole buffer, pH 8.5 at 25 °C, ionic strength = 0.1 with sodium sulfate. Inset: CO_2 hydrase activity catalyzed by A65F CAII measured as a function of pH in 100 mM 1,2-dimethylimidazole buffer (●) and 50 mM non-imidazole buffer (MOPS, pH 7.5; TAPS, pH 8−9) (■).

65 on the rate constant for intermolecular proton transfer, we measured the catalytic activity as a function of the concentration of the basic form of external buffer, $(k_{cat}/K_M)_{buffer}$ (Table 1), at pH 8.0. Substitution of a bulky residue (F, H, or L) at position 65 also decreases the rate constant for intermolecular proton transfer (up to 5-fold), while the smaller substitutions (G, S, or T) have little or no effect on this rate constant (Table 1). However, this reduction is not as striking as the decreased rate constant for intramolecular proton transfer exhibited by these variants, indicating that while intermolecular proton transfer is somewhat inhibited, bulky substituents at position 65 cause a more profound disruption in the proton transfer pathway between $Zn-OH_2$ and H64.

Proton Transfer in Imidazole Buffers. In H64A CAII, where intramolecular proton transfer has been entirely disrupted by removal of the proton shuttling residue H64, the rate constant for proton transfer can be restored to wild-type levels by assaying CO_2 hydrase activity in the presence of imidazole buffers (Ferscht, 1985; Taoka et al., 1994; Tu et al., 1989). To determine whether imidazole buffer could similarly rescue proton transfer in the A65 variants, CO_2 hydrase activity was measured in the presence of 50, 100, and 200 mM 1,2-dimethylimidazole at pH 8.5. With each of the proton transfer-deficient A65 variants, 1,2-dimethylimidazole buffer enhances k_{cat}; however, the proton transfer rate constant in all of the bulky A65 variants was still lower than that of wild-type CAII (Figure 2). Turnover increased when the buffer concentration doubled from 50 to 100 mM, indicating that this activation is dependent on the concentration of imidazole in the assay, as it is for H64A CAII (Ferscht, 1985; Taoka et al., 1994; Tu et al., 1989). However, no additional increase was observed at 200 mM 1,2-dimethylimidazole, perhaps due to inhibition by imidazole, as observed for wild-type CAII (Ferscht, 1985; Taoka et al., 1994). When the pH−rate profile of activity in the presence of 100 mM 1,2-dimethylimidazole was determined, an additional pK_a of ≈8.2 was observed for A65H, A65L, and A65F CAII (Figure 2, inset). This confirms that the basic form of 1,2-dimethylimidazole buffer (pK_a = 8.2) is participating in a catalytically relevant proton transfer to regenerate the active zinc-bound hydroxide, as proposed for H64A CAII (Taoka et al., 1994). Nonetheless, the 3−6-fold enhanced k_{cat} observed in 1,2-dimethylimidazole buffer is still lower than the wild-type proton transfer rate constant, indicating that this proton transfer is still limited by other factors in the A65F, A65L, and A65H CAII variants.

Table 2: Esterase Activity and pK_a of Zinc-Bound Water

variant	k_{cat}/K_M (M^{-1} s^{-1})[a]	pK_a($Zn-OH_2$)[b]
WT CAII	3640 ± 150	7.0 ± 0.1
A65F	7620 ± 240	7.2 ± 0.07
A65H	4150 ± 130	7.0 ± 0.08
A65L	10200 ± 310	7.1 ± 0.1
A65G	4750 ± 190	6.9 ± 0.03
A65S	2700 ± 100	6.8 ± 0.04
A65T	4510 ± 210	6.9 ± 0.04

[a] pH-independent value for second-order rate constant at 0.45 mM PNPA determined by fit of data to eq 4. Activity was measured in 25 mM buffer, ionic strength maintained at 0.1 M with sodium sulfate. [b] Calculated from pH dependence of k_{cat}/K_M fit to eq 4.

Esterase Activity and pK_a of the Zinc−Water. The pK_a of the $Zn-OH_2$ group can be determined from the pH dependence of the *p*-nitrophenyl acetate (PNPA) esterase activity of CAII (Figure 3). For CAII, esterase activity depends on the ionization state of the zinc-bound water; hence the pH dependence of esterase activity directly reflects ionization of this group (Silverman & Lindskog, 1988; Lindskog, 1966). The pK_a values for $Zn-OH_2$ derived from the pH dependence of k_{cat}/K_M for esterase activity are shown in Table 2 and Figure 3. These data indicate that substitutions at A65 have little effect on the pK_a of zinc−water, except for A65S where the zinc hydroxide form is slightly stabilized. Thus, the observed decreases in proton transfer are not due to changes in the acidity of the proton donor.

Interestingly, the pH-independent k_{cat}/K_M for ester hydrolysis (Table 2 and Figure 3) actually increases up to 3-fold for substitution of larger groups for A65. This increase roughly correlates with the hydrophobicity of the amino acid residue, as indicated by the transfer free energy of amino acids between cyclohexane and water (Radzicka & Wolfend-

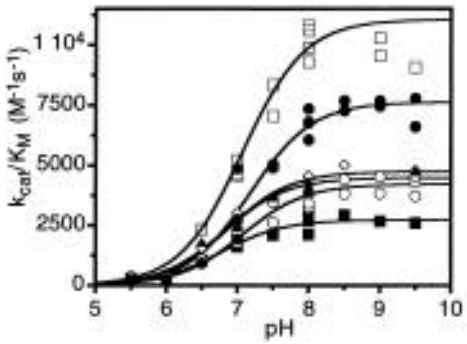

FIGURE 3: pH dependence of k_{cat}/K_M for PNPA esterase activity for A65 CAII variants, A65L (□), A65F (●), A65G (◇), A65T (▲), A65H (○), and A65S (■), measured at 0.45 mM PNPA and 50 mM buffer, 25 °C, with the ionic strength maintained at 0.1 with sodium sulfate. The data are fit using eq 4 with the Kaleidagraph (Synergy Software) curve-fitting program.

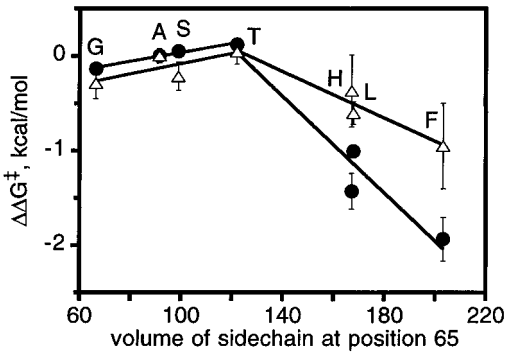

FIGURE 4: Plot of destabilization of the transition state for proton transfer ($\Delta\Delta G^{\ddagger}$) as a function of the side chain volume (Richards, 1977) of the substituted amino acid at position 65. $\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{mut} - \Delta G^{\ddagger}_{WT} = -RT \ln(k_{WT}/k_{mut})$ (Fersht, 1985), where k is k_{cat} (●) or $(k_{cat}/K_M)_{buffer}$ (△) for CO_2 hydration. The points corresponding to each amino acid substitution are identified according to their one-letter amino acid codes.

en, 1988; Sharp et al., 1991). The slope of a plot of log-$(k_{cat}/K_M)_{esterase}$ versus transfer free energy of the amino acid side chain is 0.05 ($R = 0.83$) (data not shown). Similar correlations between esterase activity and hydrophobicity have previously been observed for substitutions at V121 and L198 in the active site of CAII (Krebs & Fierke, 1993; Krebs et al., 1993; Nair et al., 1991), likely reflecting increased affinity of the CAII variant for the PNPA substrate.

DISCUSSION

We have taken an alternative approach to investigating the structural determinants of efficient proton transfer in CAII by studying the effects of substitutions at A65. Previous studies have observed decreased efficiency of intramolecular proton transfer, either by altering the position of the proton shuttling residue (Liang et al., 1993), removing the proton shuttle (Engstrand et al., 1992; Ferscht, 1985; Tu et al., 1989), or changing the respective pK_a's of the proton donor or acceptor groups (Ren et al., 1995; Silverman et al., 1993). However, in studying variants at A65, we have done none of these things: (1) H64 is intact and still able to act as the proton shuttle residue, and (2) pK_a measurements reveal that the pK_a's of both the donor zinc−water and the acceptor H64 are not perturbed by A65 substitutions (Tables 1 and 2). We propose instead that the introduction of large, bulky residues at position 65 alters the active site water structure required for efficient proton transfer. This hypothesis is supported by the high-resolution crystallographic studies of these variants showing that active site waters 292, 369, and 264 have been displaced by the larger amino acids at position 65 (Scolnick & Christianson, 1996).

Our choice of position 65 as the site for insertion of bulky amino acids to modulate proton transfer efficiency was guided by several observations. Computer modeling studies suggested that bulky residues at position 65, specifically phenylalanine, would disrupt the crystallographically observed water structure in the wild-type CAII active site. Furthermore, crystallographic studies of another carbonic anhydrase isozyme, CAV, suggest that a phenylalanine at position 65 protrudes into the active site and blocks efficient proton transfer between zinc-bound water and a tyrosinate anion at position 64 (Boriack-Sjodin et al., 1995). To test these proposals, we measured the specific effects of varying the side chain size at position 65 on intramolecular and intermolecular proton transfer, as well as on the "alternative" proton transfer pathway observable in imidazole buffers, and can now correlate these changes with observed alterations in active site solvent structure (Scolnick & Christianson, 1996).

The Active Site Water Network. When the CO_2 hydration activity of wild-type CAII is measured at saturating buffer concentrations, the rate-limiting step in the steady-state parameter k_{cat} reflects the intramolecular proton transfer from zinc-bound water to H64; $k_{cat} = k_2k_3/(k_2 + k_3)$ (see eqs 1 and 2) (when k_2, the rate constant for exchange between water and bicarbonate in the active site, is faster than k_3, the rate constant for proton transfer) (Silverman & Lindskog, 1988). Moreover, the large solvent isotope effects on k_{cat} observed with wild-type CAII and the A65 variants are consistent with a rate-determining intramolecular proton transfer. Therefore, the decreased k_{cat} values (Figure 1) indicate that this step is specifically inhibited by bulky substituents at position 65, while substitution of smaller amino acids has little effect on k_{cat} (Table 1). The observed decreases in k_{cat} are not likely to be due to increased hydrophobicity of the residue at position 65, as no correlation ($R \leq 0.7$) between log rate constant for proton transfer and hydrophobicity of the substituted amino acid (as indicated by the transfer free energy between either cyclohexane and water or octanol and water) can be discerned from these data (Fauchere & Pliska, 1983; Radzicka & Wolfenden, 1988). These results are consistent with our hypothesis that the large size of the residue at position 65 is the main factor responsible for the decreased efficiency of proton transfer seen in the A65F, A65H, and A65L variants. In fact, a plot of the difference free energy[2] for turnover of A65 variant CAII's as a function of the replacement side chain volume (Richards, 1977) demonstrates that increases in the size of amino acid 65 can be tolerated up to a critical volume of around 120 Å^3 with no effect on intramolecular proton transfer efficiency; beyond that point the rate of proton transfer falls off sharply with increasing side chain volume (Figure 4).

In contrast, the steady-state k_{cat}/K_M's for all of the variants at A65 were essentially unchanged from their wild-type values (Table 1). Since k_{cat}/K_M contains the rate constants for the catalytic steps up to, but not including, proton transfer $[k_{cat}/K_M = k_1k_2/(k_{-1} + k_2)]$, substitutions at position 65 do

[2] $\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{mut} - \Delta G^{\ddagger}_{WT} = -RT \ln(k_{WT}/K_{Mut})$ (Ferscht, 1985).

not significantly affect either the rate constant for chemical conversion of CO_2 to HCO_3^- or the subsequent release of product bicarbonate. Furthermore, substitution of A65 by smaller amino acids (G, S, and T) has little or no effect on either k_{cat} or k_{cat}/K_M for CO_2 hydration (Table 1).

In human CAIII, modulation of the pK_a difference between the proton donor and acceptor groups by mutagenesis of adjacent active site residues influences the observed rate constant for intramolecular proton transfer. These effects can be described by a Brönsted relationship similar to that observed for proton transfer between nitrogen and oxygen acids and bases with a maximum rate constant obtained at $\Delta pK_a \approx 1$ (Silverman et al., 1993). However, the decreased efficiency of intramolecular proton transfer that we have observed with the larger A65 variants cannot be ascribed to changes in the pK_a's that affect the relative ability of either H64 and Zn−OH_2 to accept or donate protons. In fact, the pK_a of the proton donor group H64, as determined from the pH dependence of k_{cat} (eq 4) is ≈7 (Table 1) in accordance with the wild-type pK_a. Similarly, the pK_a of the zinc-bound water molecule is unaffected by these substitutions, as indicated by the pK_a of ≈7 for PNPA esterase activity (Table 2). Therefore, the increased side chain size, as shown in Figure 4, is the main factor restricting the rate constant for intramolecular proton transfer.

This dependence on side chain volume can be understood in terms of changes in the active site solvent structure that occur upon introduction of the bulky residues at position 65. An organized solvent network in the CAII active site is apparent in the refined crystal structure of CAII; the Zn-bound water (number 263) is hydrogen bonded to water 318, which subsequently forms a hydrogen bond with water 292. Water 292 is then able to form hydrogen bonds with two waters, 264 and 369; all three of these are located 3.4−3.5 Å from H64 (Eriksson et al., 1988; Hakansson et al., 1992). These latter three waters have been displaced in the crystal structures of the three variants with decreased proton transfer efficiency, A65F, A65H, and A65L, but not in variants with smaller substitutions at position 65 (Scolnick & Christianson, 1996). Additionally, bulky substitutions at position 65 also cause the proton acceptor, H64, to rotate away from the active site to the "out" conformation. However, this conformational change has also been observed in variants that retain efficient proton transfer pathways (T200S, A65S, and A65T), indicating that rotation of H64 is not sufficient to compromise catalytic activity (Krebs et al., 1991; Scolnick & Christianson, 1996). Nonetheless, mobility of H64 may be essential for efficient proton transfer. Additionally, perturbation of the active site solvent structure between zinc-bound water and H64 correlates with decreased proton transfer efficiency, suggesting that waters 292, 264, and 369 comprise part of a functional proton transfer pathway in CAII.

The first proton transfer, i.e., transfer from H_2O 263 to H_2O 318 in the crystallographically observed pathway, has been proposed as the true rate-limiting step for proton transfer (Liang & Lipscomb, 1988; Venkatasubban & Silverman, 1980). Thus, one might predict that a decreased rate constant for proton transfer should be accompanied by disruptions in the position of the first water. If the crystallographically observed pathway is functional in proton transfer, the first water would be water 318, yet the displacement of this solvent molecule was not observed in the bulky A65 CAII variants. However, in a model proton transfer system consisting of zinc-bound water connected to ammonia by a bridging network of water molecules, Liang and Lipscomb (1988) demonstrated that removal of the terminal ammonia destabilized later proton transfer steps much more strongly than the transition state for the first proton transfer, such that the later steps become rate limiting. Therefore, the displacement of water molecules 292, 264, and 369 should substantially decrease the rate constant for intramolecular proton transfer by preventing the terminal acceptor, H64, from readily accepting protons, either by a decreased ability of H64 to move into the correct position for optimal proton transfer with these waters or by forcing the waters to occupy positions where they are no longer able to transfer the proton efficiently. The strong correlation between the efficiency of proton transfer in these variants and the crystallographically observed solvent network suggests that these water molecules are involved in forming the proton transfer pathway used by CAII in carrying out CO_2 hydration. In the active pathway the positions of these water molecules may be further modulated to a structure with more favorable hydrogen bonding to increase the efficiency of proton transfer (Hakansson et al., 1992). In either case, bulky substitutions at position 65 disrupt the bridging solvent network between zinc-bound water and H64.

Alternative Proton Transfer Pathway. Significantly, the intramolecular proton transfer rate constant in the A65F and A65H CAII variants (Table 1) is decreased nearly to the level of H64A CAII (1.5×10^4 s^{-1}; Tu et al., 1989), where intramolecular proton transfer via the native proton shuttle is impossible. This leads to speculation as to whether proton transfer in the variants with bulky side chains at position 65 is actually proceeding via a pathway using H64 as a proton shuttle (eqs 1 and 2) or whether these variants use an alternate proton transfer pathway from Zn-bound water directly to buffer, as has been proposed for the H64A variant (Taoka et al., 1994). Several pieces of data indicate that proton transfer in these A65 variants proceeds via H64, as it does with wild-type CAII. First, the observed pK_a of k_{cat} in non-imidazole buffer is not dependent on the amino acid at position 65 (Table 1), suggesting that k_{cat} reflects ionization of a common group, most likely the imidazole side chain of H64. Second, the K_M^{buffer} of the variants with a bulky amino acid at position 65 is comparable to or smaller than wild type, suggesting that the K_M reflects a change in rate-limiting step rather than the affinity of a buffer molecule. Finally, in the A65H CAII variant the new histidine at position 65 might be able to mediate proton transfer from the active site zinc-bound water to buffer. However, the work of Liang et al. (1993) demonstrated that when H64 is absent, histidines placed in the active site of CAII at positions 62, 67, and 200, but not at position 65, increased the rate constant for proton transfer; thus it is improbable that H65 functions as a proton transfer residue in the A65H variant. Therefore, H64 remains the most likely candidate for the proton acceptor involved in intramolecular proton transfer in the A65 CAII variants.

However, the stimulation of k_{cat} for CO_2 hydration in A65F, A65H, and A65L CAII by the addition of 1,2-dimethylimidazole (Figure 2, inset) indicates that an alternative proton transfer pathway, proton transfer from zinc−water to imidazole, is viable in these variants as previously observed for H64A CAII (Ferscht, 1985; Taoka et al., 1994;

Tu et al., 1989). Taoka et al. (1994) observed similar solvent isotope effects and Marcus energy barriers to proton transfer between (1) unmodified CAII, where the primary proton transfer pathway is the intramolecular one via H64, and (2) H64A CAII in the presence of imidazole buffers, where proton transfer must proceed via an alternate pathway from zinc−water, leading them to suggest that the imidazole-stimulated alternate pathway also proceeds through a network of active site water molecules. Thus, substitutions at position 65 in CAII that interfere with this active site water structure might impede both proton transfer via H64 and the alternate proton transfer pathway, in a manner that is not observed in the H64A variant where the water structure is presumably left intact. In fact, substitutions of F, H, or L at position 65 do inhibit this alternate proton transfer pathway 3−5-fold, whereas full activity can be restored to the H64A CAII variant under the same conditions (Figure 2). The decreased rate constant in the presence of imidazole buffer suggests that the active site waters (264, 292, and 369, see above) play common roles in both the "alternate" and the "normal" proton transfer pathways. The use of a "common" water network for both proton transfer pathways also explains the absence of stimulated proton transfer in wild-type CAII when assayed in the presence of imidazole buffers (Taoka et al., 1994; Tu et al., 1989). These data support previous hypotheses that the imidazole buffer does not bind to CAII at the position of H64 to activate proton transfer since H64 is intact in this experiment. The fact that proton transfer does increase somewhat in the presence of imidazole suggests that the orientation of the waters is not the only factor determining the efficacy of this pathway and that other components, such as steric crowding in the active site, might preferentially affect the alternate pathway, perhaps explaining why the normal H64 pathway is more efficient in the wild-type enzyme.

Proton Transfer to External Buffer. The steady-state rate equations for CO_2 hydration can be solved as a function of the concentration of the basic form of external buffer, yielding $(k_{cat}/K_M)_{buffer}$, with the intermolecular proton transfer from protonated H64 to buffer comprising the rate-limiting step under these conditions (Rowlett & Silverman, 1982). We have found that the $(k_{cat}/K_M)_{buffer}$ for the A65 variants is also dependent on the size of the amino acid side chain at position 65. Again, larger side chains (F, H, and L) increase the energy required for intermolecular proton transfer by up to 0.9 kcal/mol[2], while smaller side chains (Thr, Gly, and Ser) have no effect. A plot of $\Delta\Delta G^{\ddagger}$ for intermolecular proton transfer[2] in the A65 variants versus side chain volume of the variant exhibits the same pattern as that observed for intramolecular proton transfer, except that the rate constant for the intermolecular transfer drops off less sharply with increasing side chain volume beyond the previously described critical volume of threonine (Figure 4). Thus, bulky substitutions at position 65 decrease the rate constants for both intermolecular and intramolecular proton transfer; however, the primary effects of these mutations are on the intramolecular process. The similar dependence of these two processes on side chain volume is intriguing, suggesting that a common critical active site accessibility is necessary for both; however, the more dramatic effects on the intramolecular process suggest that the intermolecular transfer might be more flexible in nature and better able to tolerate changes in the active site solvent structure.

Hydrophobicity. The hydrophobic nature of the CAII active site enhances the catalysis of CO_2 hydration and PNPA hydrolysis. However, increased hydrophobicity at position 65 does not significantly affect the pK_a of H64, even though the pK_a might be predicted to decrease, reflecting the decreased stability of positive charges in a hydrophobic environment, as observed for CAII variants at L198 (Krebs et al., 1993). This suggests that the H64 side chain is solvent exposed, consistent with the crystal structure (Eriksson et al., 1988; Hakansson et al., 1992) and the role of H64 as a proton shuttle group. Furthermore, the hydrophobicity at position 65 also has little or no effect on the k_{cat}/K_M for CO_2 hydration, again in contrast with studies in which the hydrophobic nature of positions V121, V143, and L198 was found to be an important determinant of this aspect of CAII activity (Alexander et al., 1991; Fierke et al., 1991; Krebs et al., 1993; Nair et al., 1991). From this we conclude that position 65 is not a part of the CO_2 binding site, nor is it close enough to affect the energy of the transition state for chemical catalysis of CO_2 hydration. Modeling studies suggest the presence of a second CO_2 binding site formed by H64, H94, H96, A65, N244, Y7, F93, and T200 (Merz, 1991), and substitutions at position 65 are predicted to significantly perturb this binding site. Therefore, the lack of dependence of k_{cat}/K_M on the side chain at position 65 suggests that the proposed second CO_2 binding site is not crucial for catalysis.

However, the hydrophobicity of position 65 does affect the k_{cat}/K_M for PNPA esterase activity (Table 2, Figure 3); similar correlations between esterase activity and hydrophobicity have been observed for substitutions at V121, V143, and L198 in the active site of CAII (Alexander et al., 1991; Fierke et al., 1991; Krebs et al., 1993; Nair et al., 1991; Nair & Christianson, 1993). The transition state for PNPA hydrolysis is similar to the transition state for CO_2 hydration, except that the leaving group has a higher pK_a and therefore is more difficult to protonate. The increase in k_{cat}/K_M for ester hydrolysis is unlikely to be due to enhanced protonation of this transition state, since substitutions at position 65 decrease the rate constant for proton transfer through solvent molecules. Therefore, the increased hydrophobicity at position 65 likely only enhances the affinity of the enzyme for the nonphysiological substrate, PNPA, without significantly altering the transition state for ester hydrolysis.

Conclusions. The crystal structure of CAII reveals several waters that could function as the proton relay network for both intramolecular and intermolecular proton transfer; however, none of these has been positively identified as such. Here, we correlate inhibition of the rate constants for proton transfer by substitutions at position 65 with displacement of the active site solvent molecules 264, 292, and 364 (Scolnick & Christianson, 1996), suggesting that the crystallographically observed waters may be functional in proton transfer. We have also demonstrated that active site solvent structure is likely to be an important determinant in proton transfer via the alternate proton pathway that is operative in the absence of a functional intramolecular proton shuttle group. This work reaffirms that the correct location and orientation of active site solvent structure, in addition to the well-investigated significance of ΔpK_a, is an important determinant of the efficiency of proton transfer in carbonic anhydrase.

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