Structure-Based Design of an Intramolecular Proton Transfer Site in Murine Carbonic Anhydrase V[†]

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ABSTRACT: Carbonic anhydrase V (CA V) is a mitochondrial enzyme that catalyzes the hydration of CO_2 to produce bicarbonate and a proton. The catalytic properties of wild-type murine CA V suggest the presence of a proton shuttle residue having pK_a = 9.2, the role of which is to transfer a proton from zinc-bound water to solution in the hydration direction to regenerate the zinc hydroxide form of the enzyme. Two likely candidates for shuttle residues are the tyrosines at positions 64 and 131 in the active site cavity. The crystal structure of wild-type carbonic anhydrase V [Boriack-Sjodin et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10949−10953] shows that the side chain of Tyr 64 is forced into an orientation pointing away from the zinc by Phe 65, although Tyr 131 is oriented toward the zinc. We have prepared mutants of murine CA V replacing both Tyr 64 and Tyr 131 with His and Ala and investigated the proton shuttle mechanism using stopped-flow spectrophotometry and the depletion of ^{18}O from CO_2 measured by mass spectrometry. Experiments with both single and double mutations showed that neither position 64 nor position 131 was a prominent site for proton transfer. However, a double mutant of CA V containing the two replacements, Tyr 64 → His and Phe 65 → Ala, demonstrated enhanced proton transfer with an apparent pK_a of 6.8 and maximal contribution to k_{cat} of 2.2×10^5 s^{-1}. In addition to the altered catalytic properties, the crystal structure of the His 64/Ala 65 double mutant strongly suggested proton transfer by His 64 after removal of the steric hindrance of Phe 65. This is the first structure-based design of an efficient proton transfer site in an enzyme.

Carbonic anhydrases are zinc-containing metalloenzymes which catalyze the hydration of CO_2:

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

To date, there are seven different isozymes of carbonic anhydrase (CA) known to exist in mammals (Tashian, 1989). Carbonic anhydrase V, located primarily in liver mitochondria, is similar to the other isozymes of carbonic anhydrase in having a molecular mass near 30 kDa with one zinc ion per molecule. Initial protein and cDNA sequences from guinea pig and mouse liver (Hewett-Emmett et al., 1986; Amor-Gueret & Levi-Strauss, 1990) led to the cloning and expression of carbonic anhydrase V (Heck et al., 1994; Nagao et al., 1994). The crystal structure of murine CA V has a backbone conformation similar to isozyme II (Boriack-Sjodin et al., 1995), and it shares about 50% amino acid sequence identity with isozyme II. Studies [reviewed by Dodgson (1991)] suggest that the mitochondrial carbonic anhydrase activity participates in the pathways of ureagenesis and gluconeogenesis, both of which require bicarbonate as substrate for enzymes located in mitochondria.

Like isozyme II of carbonic anhydrase, isozyme V displays kinetics consistent with two stages in catalysis of the hydration of CO_2 (Heck et al., 1994). The first is the interconversion of CO_2 and HCO_3^- shown in eq 2. The

$$CO_2 + EZnOH^- \rightleftarrows EZnHCO_3^- \overset{+H_2O}{\rightleftharpoons} EZnH_2O + HCO_3^- \quad (2)$$

second stage is the regeneration of the zinc-bound hydroxide by a series of proton transfer steps shown in eq 3. X is a

$$X{-}EZnH_2O + B \rightleftarrows XH^+\text{-}EZnOH^- + B \rightleftarrows X{-}EZnOH^- + BH^+ \quad (3)$$

residue of the enzyme capable of accepting a proton from the zinc-bound water and transferring it to buffer B in solution. Histidine at position 64 is capable of carrying out this function in both HCA II (Tu et al., 1989; Steiner et al., 1975) and in the mutant K64H HCA III (Jewell et al., 1991).

Heck et al. (1994) prepared expression vectors containing the full coding sequence of carbonic anhydrase V (Amor-Gueret & Levi-Strauss, 1990) as well as two different amino-terminal truncated genes, each of which expressed active carbonic anhydrases in *Escherichia coli*. The two truncated forms deleted (i) the 29 amino acid putative targeting sequence and (ii) 51 amino acids giving a protein equivalent

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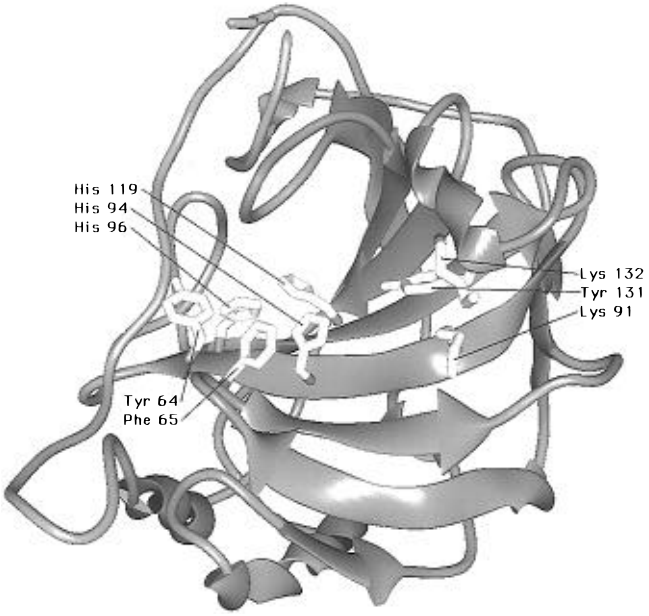

FIGURE 1: Structure near the active site of mouse carbonic anhydrase V as determined by Boriack-Sjodin et al. (1995). The zinc ligands are His 94, His 96, and His 119 with a water molecule as the fourth ligand.

to a CA V isolated from mouse liver mitochondria (Heck, 1996) and similar to CA V isolated from guinea pig (Hewett-Emmett et al., 1986) and rat liver mitochondria (Ohliger et al., 1993). These latter two forms of CA V had identical steady-state constants for the hydration of CO_2 with maximal values of k_{cat} and k_{cat}/K_m about 20% that of the most efficient of the CA isozymes, CA II (Heck et al., 1994). The pH profile of k_{cat} for hydration appeared to depend on the ionization of a single group with a pK_a of 9.2, suggesting a basic residue such as tyrosine or lysine as proton acceptor in catalysis.

We report here site-directed mutagenesis studies to identity the proton shuttle residues in murine mitochondrial CA V. Two possible candidates for shuttle residues are Tyr 64 and Tyr 131, located in the active site cavity (Figure 1). Heck et al. (1994) found that Tyr 64 is not necessary as a proton shuttle in the catalysis. The crystal structure of wild-type CA V (Boriack-Sjodin et al., 1995) explained this result by showing that the side chain of Tyr 64 is forced into an orientation pointing away from the zinc by Phe 65. On the other hand, Tyr 131 is oriented toward the zinc. We report here that replacements of Tyr 131 by site-directed mutagenesis and subsequent catalytic characterization showed that proton transfer in the wild-type enzyme does not require Tyr 131 as a proton shuttle residue despite its location with its side chain extending into the active site cavity. However, we have introduced an efficient proton transfer site into murine CA V by preparing a double mutant with His at position 64 and with Ala at position 65 replacing the bulky side chain of Phe 65. This double mutant Y64H/F65A gives a maximal rate of hydration of CO_2 which is up to 10-fold greater than for wild-type mCA V, consistent with enhanced proton transfer in the mutant. This is the first example of structure-based design of a proton transfer site in an enzyme and shows the influence of a neighboring group on intramolecular proton transfer.

METHODS

Mutagenesis and Expression of Mouse CA V. The coding sequence of mouse CA V was derived from BALB/C mouse liver mRNA by reverse transcription and PCR (Heck et al., 1994; Heck, 1996; S. M. Tanhauser, R. Manda, R. W. Heck, and P. J. Laipis, manuscript in preparation). Mutant forms of CA V were created using a mutating oligonucleotide (Kunkel, 1985) in the pET31 expression vector system (Tanhauser et al., 1992); alterations were verified by DNA sequencing. Wild-type and mutant forms of the enzyme were expressed in the same pET vector, transformed into *E. coli* BL21(DE3)pLysS (Studier et al., 1990). Protein expression levels ranged from 0.5 to 10 mg/L, depending on the mutant.

All of the expressed enzymes were truncated forms lacking the first 51 amino-terminal residues. In a sequence numbering scheme consistent with CA II, the expressed CA V variants began at residue 22, Ser. This truncated form of CA V [denoted CA Vc by Heck et al. (1994)] has been shown to have catalytic properties identical to those of CA V expressed from both a full-length coding sequence and a 30-residue truncation of CA V (Heck et al., 1994).

Purification. Purification of CA V and mutants was performed according to the procedures of Heck et al. (1994). Frozen BL21(DE3)pLysS cells expressing one of the mouse CA V variants were thawed in a solution of 100 mM Tris, 200 mM Na_2SO_4, 1 mM mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 2 μM leupeptin at pH 9. This solution was stirred at 4 °C in the presence of 0.1 mg/mL deoxyribonuclease I for 1 h to allow the cells to lyse and to degrade the bacterial DNA. After centrifugation to remove cellular debris, recombinant CA V was purified from the resulting supernatant by affinity chromatography using *p*-(aminomethyl)benzenesulfonamide coupled to agarose beads (Khalifah et al., 1977). The purity of the isolated enzymes was estimated at greater than 95% as determined by electrophoresis on a 10% polyacrylamide gel stained with Coomassie Blue. Concentrations of each enzyme were determined by titrating the active site with ethoxazolamide using a Henderson plot (Segel, 1975).

Crystallography. The double mutant Y64H/F65A mCA V was further purified prior to crystallization with a Mono Q HR 5/5 anion-exchange column (Pharmacia Biotech) on an automated FPLC system using 1 M sodium chloride as the eluant. Subsequently, protein was transferred into 50 mM Tris (pH = 8.0 at room temperature) and concentrated to 3 mg/mL. Crystals were obtained at room temperature by the vapor diffusion method, in which a hanging drop consisting of 4−4.5 μL of protein solution, 0.5 μL of 2.0 mM sucrose monolaurate, and 5 μL of precipitant buffer (16−12% PEG 8000, 0.2 M sodium acetate, and 0.1 M sodium cacodylate, pH = 6.5 at room temperature) was suspended over a reservoir of 1 mL of precipitant buffer. Platelike crystals typically appeared within 2−3 days and grew to full size within 2 weeks.

Data were collected with an R-AXIS IIc image plate detector mounted on an RU-200HB rotating anode generator with double focusing mirrors operating at 50 kV and 100 mA. Data were collected in successive 30 min, 2° oscillations about ϕ for a total scan of 110°; the crystal to detector distance was set at 105 mm. The crystal orientation was established with REFIX (Kabsch, 1993), and data were reduced using MOSFLM (Nyborg & Wonacott, 1977).

Crystals of Y64H/F65A mCA V were isomorphous with those of the wild-type enzyme and were indexed in the monoclinic space group *C*2 ($a = 101.1$ Å, $b = 67.5$ Å, $c = 48.9$ Å, $\beta = 107.6°$).

In the structure determination of the Y64H/F65A variant, structure factors obtained from the corrected intensity data were used to generate difference electron density maps using Fourier coefficients $|F_o| - |F_c|$ or $2|F_o| - |F_c|$ with phases calculated from the refined structure of murine CA V with residues 64 and 65 truncated to alanine. Atomic coordinates were then refined against the observed data using X-PLOR (Brünger et al., 1987). After the crystallographic *R*-factor dropped below 0.20, the side chain of His 64 and solvent molecules were modeled into electron density using the graphics software CHAIN (Sack, 1988) installed on a Silicon Graphics Indigo workstation. Refinement with simulated annealing was then completed with X-PLOR (Brünger, 1990). The final crystallographic *R*-factor for data between 6.5 and 2.8 Å resolution is 0.137 [$R_{free} = 0.241$ (Brünger, 1992)], with excellent stereochemistry; pertinent data collection and refinement statistics are reported in Table 2. Atomic coordinates have been deposited in the Brookhaven Protein Data Bank with accession code 1URT (Bernstein et al., 1977).

Measurement of Catalysis by Stopped Flow. Initial velocities of CO_2 hydration were measured by stopped-flow spectrophotometry (Applied Photophysics Model SF.17MV) following the change in absorbance of a pH indicator at 25 °C (Khalifah, 1971). Saturated solutions of CO_2 were prepared by bubbling CO_2 into water, and dilutions were made using two coupled syringes with a gas-tight connection. Final concentrations of CO_2 were varied from 0.5 to 17 mM. The buffer−indicator pairs, their values of pK_a, and the wavelength observed were Mes[1] (pK_a 6.1) with chlorophenol red (pK_a 6.3, 574 nm), Mops (pK_a 7.2) with *p*-nitrophenol (pK_a 7.1, 400 nm), Hepes (pK_a 7.5) with phenol red (pK_a 7.5, 557 nm), Taps (pK_a 8.4) with *m*-cresol purple (pK_a 8.3, 578 nm), and Ches (pK_a 9.3) with thymol blue (pK_a 8.9, 590 nm). Solutions were maintained at a constant total ionic strength of 0.2 M by addition of the appropriate amount of Na_2SO_4. The mean initial rate in each case was determined from at least six reaction traces comprising the initial 5−10% of the reaction. Uncatalyzed rates were subtracted, and determination of kinetic constants k_{cat} and k_{cat}/K_m was by nonlinear least squares methods (Enzfitter, Elsevier-Biosoft).

Oxygen-18 Exchange Kinetics. Mass spectrometry was used to measure the rate of exchange of ^{18}O between CO_2 and water and the rate of exchange of ^{18}O from $^{12}CO_2$ to $^{13}CO_2$ caused by the transitory labeling of the active site of carbonic anhydrase, as described by Silverman (1982). The reaction solution was in contact with a membrane permeable to gases; CO_2 passing across the membrane entered a mass spectrometer (Extrel EXM-200) providing a continuous measure of isotopic content of CO_2. Since this technique is carried out at chemical equilibrium, no buffers are needed to maintain pH, avoiding the difficulty of any possible effect of the buffer itself on the catalysis. Solutions were maintained at a constant total ionic strength of 0.2 M by addition of the appropriate amount of Na_2SO_4.

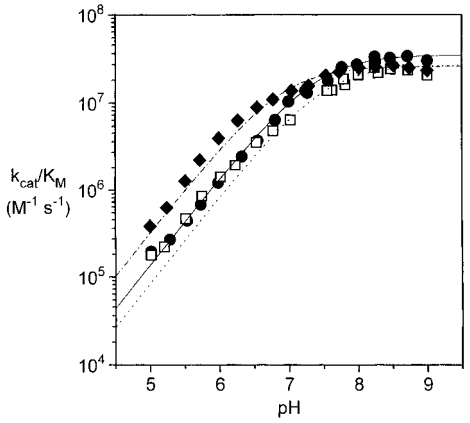

FIGURE 2: k_{cat}/K_m for the hydration of CO_2 catalyzed by wild-type mouse CA V (●), the mutant mouse Y64H CA V (□), and the mutant mouse Y64H/F65A CA V (◆). Data were obtained at 25 °C using ^{18}O exchange measured in the absence of buffers. Solutions were maintained at a constant total ionic strength of 0.2 M by addition of the appropriate amount of Na_2SO_4. The lines are least squares fit to one ionization with values given in Table 1.

The ^{18}O method measures the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium, R_1, as shown in eq 4. The substrate dependence of R_1 is given by $R_1/[E] =$

$$HCOO^{18}O^- + EZnH_2O \rightleftarrows EZn^{18}OH^- + CO_2 + H_2O \quad (4)$$

$k_{cat}^{ex}[S]/(K_{eff}^S + [S])$ 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 CO_2 or HCO_3^- or both (Simonsson et al., 1979). Values of k_{cat}^{ex}/K_{eff}^S for the enzymes were determined by nonlinear least squares fit of the above expression for R_1 to the data for varying substrate concentration or by measurement of R_1 at values of [S] much smaller than K_{eff}^S. In theory and in practice, $k_{cat}^{ex}/K_{eff}^{CO2}$ is equal to k_{cat}/K_m for hydration of CO_2 obtained by steady-state methods (Simonsson et al., 1979; Silverman, 1982). In this text we refer to this rate constant obtained from ^{18}O exchange as k_{cat}/K_m.

RESULTS

Catalysis. We have determined the catalytic constants for wild-type mouse carbonic anhydrase V and a series of mutants with amino acid replacements at two sites, 64 and 131, considered as possible proton shuttle sites and at an additional site, 65, adjacent to such a site. The catalytic constant k_{cat}/K_m contains rate constants for the interconversion of CO_2 and HCO_3^-, as in eq 2, and was determined both by ^{18}O exchange (Figure 2, Table 1) and by stopped-flow spectrophotometry (data not shown) with similar results.[2] The maximal values of k_{cat}/K_m for the hydration of CO_2 catalyzed by wild-type CA V and all the site-specific mutants studied here were very similar, varying from 1.9 to 3.7×10^7 M^{-1} s^{-1} (Table 1, with typical data shown in Figure 2). Moreover, the pH dependence of k_{cat}/K_m for hydration could be described as dependent on the basic form of a single ionization, with values of pK_a varying from 6.8 to 7.9 (Table 1). Hence, the values of k_{cat}/K_m and pK_a are rather insensitive

[1] Abbreviations: mCA V, murine carbonic anhydrase V; Y64H CA V, the mutant of CA V containing the replacement Tyr 64 → His; 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, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid.

Table 1: Maximal Values of k_{cat}/K_m and k_{cat} and Apparent Values of pK_a Determined from pH Profiles for Catalysis of CO_2 Hydration Catalyzed by Murine Carbonic Anhydrase V and Mutants at 25 °C[a]

	k_{cat}/K_m ($\times 10^7$ M^{-1} s^{-1})	p$K_{a(k_{cat}/K_m)}$[b]	k_{cat} ($\times 10^5$ s^{-1})	p$K_{a(k_{cat})}$[b]
wild type	3.5 ± 0.1	7.4 ± 0.1	3.2 ± 0.2	9.2 ± 0.1
			0.04 ± 0.01	6.9 ± 0.2
Y64A	1.9 ± 0.1	7.8 ± 0.1	2.5 ± 0.5	9.2 ± 0.1
Y131A	3.7 ± 0.1	7.1 ± 0.1	1.8 ± 0.2	9.2 ± 0.1
Y64A/Y131A	2.2 ± 0.1	7.4 ± 0.1	0.8 ± 0.1	8.8 ± 0.1
Y64H[c]	2.7 ± 0.1	7.5 ± 0.1	3.8 ± 0.6	9.2 ± 0.1
			0.05 ± 0.01	6.3 ± 0.1
Y131H	2.5 ± 0.1	7.9 ± 0.1	0.6 ± 0.1	9.2 ± 0.1
Y64H/F65A[c]	2.6 ± 0.1	6.9 ± 0.1	2.2 ± 0.1	6.8 ± 0.2
			5.3 ± 0.1	9.0 ± 0.1
F65A	2.0 ± 0.1	6.8 ± 0.1	3.0 ± 0.2	9.0 ± 0.02

[a] The values of k_{cat}/K_m were determined from ^{18}O exchange and the values of k_{cat} from stopped-flow spectrophotometry. These maxima and apparent values of pK_a were calculated from the best fit to a single ionization or to the sum of two ionizations by nonlinear regression analysis. [b] These are apparent pK_a values determined from k_{cat}/K_m and k_{cat}. [c] The data for k_{cat} were fit to a line describing two ionizations. For example, k_{cat} for Y64H/F65A was consistent with two components which were additive in their summed contribution to a maximal value of k_{cat} of 7.5×10^5.

Table 2: Data Collection and Refinement Statistics for Y64H/F65A mCA V

no. of crystals	1
no. of measured reflections	19846
no. of unique reflections	6760
maximum resolution (Å)	2.8
completeness of data (%)	87
R_{sym}[a]	0.088
no. of water molecules in final cycle of refinement	32
no. of reflections used in refinement (6.5−2.8 Å)	6174
crystallographic *R*-factor[b]	0.132
free *R*-factor[c]	0.241
RMS deviation from ideal bond lengths (Å)	0.010
RMS deviation from ideal bond angles (deg)	1.6
RMS deviation from ideal dihedral angles (deg)	25.6
RMS deviation from ideal improper dihedral angles (deg)	1.3

[a] R_{sym} for replicate reflections: $R = \sum|I_{hi} - \langle I_h\rangle|/\sum\langle I_h\rangle$; I_{hi} = intensity measured for reflection *h* in data set *i*; $\langle I_h\rangle$ = average intensity for reflection *h* calculated from replicate data. [b] Crystallographic *R*-factor: $R = \sum||F_o| - |F_c||/\sum|F_o|$; $|F_o|$ and $|F_c|$ are the observed and calculated structure factors, respectively. [c] Brünger, 1992.

to the replacements we made at positions 64, 65, and 131 (Table 1). However, in the case of k_{cat}/K_m for Y64H CA V there was a positive deviation from the line representing a single ionization (Figure 2) that is consistent with a very weak effect of His 64 with a pK_a of 6.0 ± 0.2 on this stage of the catalysis. The solvent hydrogen isotope effect on k_{cat}/K_m for wild-type CA V was 0.9 ± 0.1, consistent with no rate-contributing proton transfer in the steps of the interconversion of CO_2 and HCO_3^- (eq 2).

The maximal turnover number k_{cat} for CO_2 hydration contains rate constants from the enzyme−substrate complex

[2] The values of k_{cat}/K_m for these mutants determined by stopped-flow spectrophotometry were less precise than those determined by ^{18}O exchange. Although k_{cat}/K_m measured by stopped-flow was very similar to k_{cat}/K_m determined by ^{18}O exchange, there were some significant differences for certain mutants. For Y64H and Y64H/F65A CA V, for example, the values of k_{cat}/K_m determined by stopped flow were larger than those determined by ^{18}O exchange (with values of 4×10^7 M^{-1} s^{-1} for Y64H and 6×10^7 M^{-1} s^{-1} for Y64H/F65A), and the values of pK_a determined from the pH profiles were larger by about one pK_a unit.

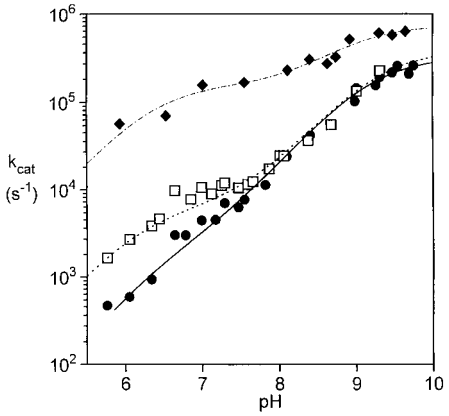

FIGURE 3: k_{cat} for the hydration of CO_2 catalyzed by wild-type mouse CA V (●), the mutant mouse Y64H CA V (□), and the mutant mouse Y64H/F65A CA V (◆). Data were obtained at 25 °C by stopped-flow spectrophotometry using solutions containing the following buffers at 25 mM: pH 5.8−6.9, Mes; pH 6.6−pH 7.3, Mops; pH 7.1−7.9, Hepes; pH 8.1−8.7, Taps; pH >8.9, Ches. The lines are least squares fits of the data to two ionizations with values given in Table 1.

to the completion of catalysis including the proton transfer steps of eq 3. We observed that wild-type CA V and each variant also had a pH profile indicating dependence on the ionization of a residue with apparent pK_a between 8.8 and 9.2 (Table 1, Figure 3); this pK_a was not sensitive to the replacements we made at positions 64, 65, and 131 (Table 1). However, two mutants containing the replacement Tyr 64 → His (Y64H and Y64H/F65A) showed evidence of a second ionization affecting k_{cat} (Figure 3); the apparent values of the pK_a for this second ionization were 6.3 and 6.8 (Table 1), consistent with an effect of His 64 on the catalysis. Although difficult to discern because of experimental error in k_{cat} at low pH, there was weak evidence for a small effect of an ionization near pK_a 6.9 on the wild-type CA V (Figure 3) not due to His 64 but perhaps to some unidentified residue or buffer. The maximal values of k_{cat} were more affected than k_{cat}/K_m by the replacements we made at positions 64, 65, and 131. With two exceptions, these replacements decreased k_{cat} slightly with respect to the wild type (Table 1); the exceptions were the mutants that contained His 64 which showed enhanced values of k_{cat}. The solvent hydrogen isotope effect on R_{H_2O}/[E] for wild-type carbonic anhydrase V describing the rate of proton transfer dependent release of $H_2{}^{18}O$ from the enzyme (Silverman, 1982) was 2.8 ± 0.3. This value is consistent with a rate-contributing proton transfer in the steps of the catalysis shown in eq 3.

Structure. With the exception of the residue 64 region, a structural comparison of Y64H/F65A mCA V reveals only minor differences in surface residues when compared to the wild-type enzyme. A loop region containing residues 73−76 is poorly defined, and the occupancies of these atoms have been set to zero. The overall RMS deviation of Cα atoms between the wild-type and variant enzymes is 0.40 Å. Clear electron density is seen for the backbone and side chains of residues 64 and 65 (Figure 5). Intriguingly, the side chain conformation of His 64 resembles that of Tyr 64 in wild-type CA V more than that of His 64 in CA II (Figure 6) and forms a hydrogen bond with the carbonyl oxygen of Asn 244. Smeared electron density adjacent to the His 64 side chain is interpreted as a hydrogen-bonded water molecule, but it is possible that this density corresponds to a low-occupancy conformer as observed in CA II (Krebs et

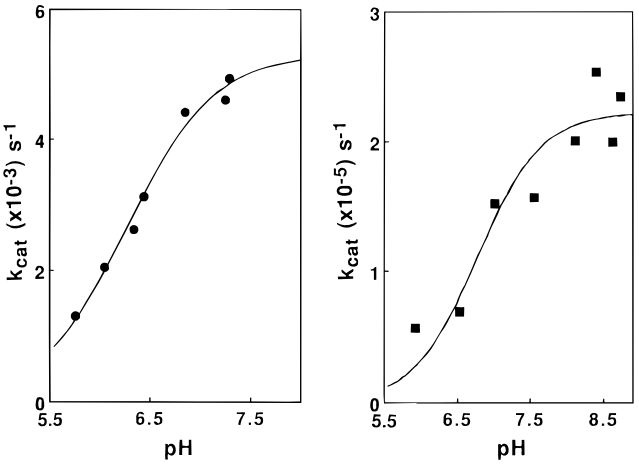

FIGURE 4: (A, left) The difference $k_{cat(Y64H)} - k_{cat(wild\ type)}$ as a function of pH, taken from the data of Figure 3. The solid line describes a single ionization of p$K_a = 6.3 \pm 0.1$ with a maximum at $(5.3 \pm 0.2) \times 10^3$ s^{-1}. (B, right) The difference $k_{cat(Y64H/F65A)} - k_{cat(wild\ type)}$ as a function of pH, taken from the data of Figure 3. The solid line describes a single ionization of p$K_a = 6.8 \pm 0.2$ with a maximum at $(2.2 \pm 0.1) \times 10^5$ s^{-1}.

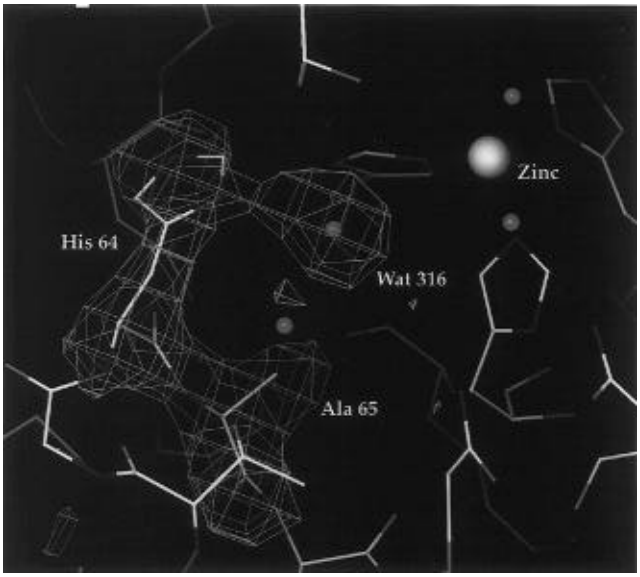

FIGURE 5: Difference electron density map of Y64H/F65A murine carbonic anhydrase V calculated with Fourier coefficients $|F_o| - |F_c|$ and phases derived from the final model less H64 and A65 and contoured at 2.5σ. His 64, Ala 65, water 316, and zinc are indicated.

al., 1991). At the resolution of 2.8 Å, active site solvent structure is not sufficiently well determined to pinpoint a specific solvent-mediated proton transfer trajectory between zinc-bound solvent and His 64, but it is clear that such a trajectory would not be perturbed by the Ala 65 side chain.

DISCUSSION

In probing the proton transfer mechanism of mouse carbonic anhydrase V, useful comparisons can be made to CA II, where the proton transfer scheme is well studied (Silverman & Lindskog, 1988; Tu et al., 1989). A histidine at position 64 in the active site cavity of CA II serves as a proton shuttle; as described in eq 3, the role of His 64 is to shuttle protons between buffers in solution and the zinc-bound water. The maximal value of k_{cat} for CA II is near 10^6 s^{-1} and shows an apparent pK_a near 7, consistent with

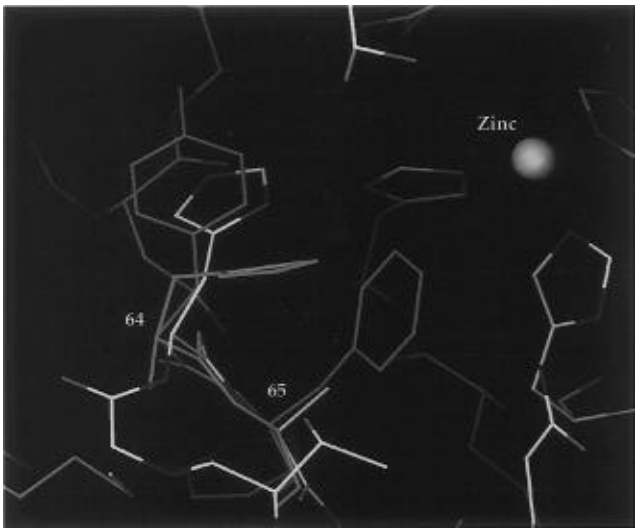

FIGURE 6: Least squares superposition of Y64H/F65A murine carbonic anhydrase V (multicolored), wild-type carbonic anhydrase V (red), and carbonic anhydrase II (green). Residues 64 and 65 are indicated. Note that the conformation of His 64 in the mutant Y64H/F65A CA V resembles that of Tyr 64 in CA V more than that of His 64 in CA II.

the histidine 64 side chain as a proton shuttle. When His 64 was replaced by alanine in CA II, a side chain with no proton transfer capability, the maximal turnover k_{cat} was reduced 20-fold and had no apparent pH dependence (Tu et al., 1989).

In wild-type CA V position 64 is occupied by tyrosine, and k_{cat} has a pH profile showing a pK_a near 9 with a maximum of 3×10^5 s^{-1} (Figure 3). This higher apparent pK_a suggests a proton transfer group that is more basic, such as tyrosine or lysine. However, Heck et al. (1994) demonstrated that replacing Tyr 64 with His in mouse CA V did not show significant activation of catalysis, suggesting that position 64 was not a prominent proton shuttle site in either wild-type or Y64H CA V. Thus, the source of the intramolecular proton transfer in wild-type CA V remains unclear. We measured catalysis using mutants of CA V in which the two residues possibly involved in proton transfer, Tyr 64 and Tyr 131, were replaced. The side chain of Tyr 64 is forced away from the zinc in large part by the neighboring Phe 65; the side chain of Tyr 131 is pointed toward the zinc with its hydroxyl oxygen 9.1 Å from the metal (Figure 1; Boriack-Sjodin et al., 1995). Neither the replacement Tyr 64 → Ala nor the replacement Tyr 131 → Ala caused a decrease in k_{cat} sufficient to suggest that these residues are major proton shuttle groups (Table 1). The double mutant containing both replacements, Y64A/Y131A CA V, had k_{cat} deceased to 25% that of the wild type, which may indicate some joint contribution to proton transfer by these two residues. This decrease does not match the 20-fold decrease in CA II upon replacement of the proton shuttle His 64. However, the maximal value of k_{cat} for Y64A/Y131A CA V is larger by at least 2−4-fold (depending on buffers) than k_{cat} for H64A CA II, which may indicate that this double mutant of CA V has other proton transfer residues (Lys 91 and Lys 132) not present in CA II, as discussed further below.

Another approach to determining whether the tyrosines at positions 64 and 131 in CA V could function in proton transfer was to replace the tyrosines with histidines. The

k_{cat} would be enhanced and its pH dependence shifted toward the expected lower pK_a of histidine if the replacements were functional. This effect was not observed for the mutant with the replacement Tyr 64 → His (Table 1), with the exception of a minor deviation observed at low pH (Figure 3) which is discussed below. The replacement Tyr 131 → His caused a significant decrease in k_{cat} (Table 1). Consequently, these experiments indicate that neither Tyr 64 nor Tyr 131 are sole or prominent sources of intramolecular proton transfer in wild-type CA V.

The crystal structure of the wild-type murine CA V (Boriack-Sjodin et al., 1995) shows why position 64 may not be effective in shuttling protons to the active site; the mobility of the side chain of residue 64 appears to be hindered by the bulky side chain of Phe 65 (Figure 1). When this residue was replaced by alanine in the mutant F65A CA V, there was no appreciable change in the steady-state constants compared with the wild-type enzyme (Table 1). However, in the double mutant containing both the replacements Tyr 64 → His and Phe 65 → Ala, there was considerable enhancement of k_{cat} for hydration of CO_2 (Figure 3, Table 1). Furthermore, the pH profile for k_{cat} can be considered as the superposition of the contribution from the wild type and an additional contribution to k_{cat} with an apparent pK_a of 6.8 and a maximal value of k_{cat} of 2.2×10^5 s^{-1}. This is shown by the plot of the difference in k_{cat} for Y64H/F65A CA V and wild type (Figure 4B). This strongly suggests that His 64 in this double mutant is acting as a proton shuttle in the manner of His 64 in HCA II. In fact, such a plot for the single mutant Y64H CA V (Figure 4A) shows a similar activation although lesser in magnitude with a maximal value of 5.3×10^3 s^{-1} and a pK_a of 6.3, suggesting that His 64 in the single mutant Y64H CA V may have a minor contribution to catalysis by proton transfer. There was no significant effect of His 64 on the interconversion of CO_2 and HCO_3-; that is, the replacement Tyr 64 → His had no apparent effect on k_{cat}/K_m under the conditions of these measurements (Figure 2). This further supports the interpretation that His 64 is a proton shuttle residue rather than alternative interpretations that, for example, an electrostatic effect of His 64 has a significant influence on active site properties. Moreover, this demonstrates the success of this structure-based design experiment.

No physical barrier for the conformational change of His 64 is apparent from the crystal structure of Y64H/F65A mCA V (Figure 5), so His 64 may be conformationally mobile in its role as a proton shuttle group in this mutant just as His 64 is in wild-type CA II (Krebs et al., 1991; Nair & Christianson, 1991). Although the resolution of the structure of Y64H/F65A mCA V is not sufficiently high to determine a specific hydrogen-bonded solvent network between zinc-bound water and H64, this structure shows that a solvent-mediated proton transfer pathway would be facilitated by the F65A amino acid substitution. The conformation of His 64 in Y64H/F65A mCA V is directed away from zinc-bound solvent, but it is to an alternative "out" conformation differing by ~130° about χ_1 from the out conformer occasionally observed in CA II (Krebs et al., 1991; Nair & Christianson, 1991). A comparison of the orientations of His 64 in CA V and CA II is shown in Figure 6. However, regardless of the predominant conformation of His 64 in CA II, it functions efficiently as a proton shuttle group (Krebs et al., 1991); the same conclusion is relevant to His 64 in Y64H/F65A mCA V.

There remains the question of what residues are important for proton transfer in wild-type CA V. That a shuttle residue exists is evident from the considerable value of k_{cat} in the double mutant Y64A/Y131A CA V (Table 1) indicating appreciable proton transfer. Because k_{cat} for wild-type CA V can be adequately described by a single ionization does not necessarily imply that a single proton transfer pathway predominates. The decrease in k_{cat} seen for mutations at position 131 (Table 1) may be caused by proximity effects on a nearby proton transfer group, possibly Lys 91 or Lys 132. These sites have not yet been tested. The side chain Nζ atoms of Lys 91 and Lys 132 are 7.2 and 10.1 Å, respectively, from the hydroxyl oxygen of Tyr 131. Since the current experiments were performed on a form of CA V lacking 21 residues from the amino terminus, it is clear that neither His 7 nor the amino terminus itself participates in the proton transfers observed here. It is possible that proton transfer can be facilitated by multiple pathways in CA V and that the elimination of one pathway leaves considerable catalytic facility from other pathways.

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