

Activation of carbonic anhydrase II by active-site incorporation of histidine analogs

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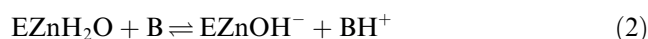
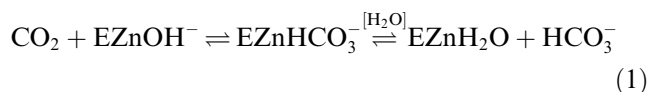
Abstract

The hydration of CO₂ catalyzed by human carbonic anhydrase II (HCA II) is accompanied by proton transfer from the zinc-bound water of the enzyme to solution. We have replaced the proton shuttling residue His 64 with Ala and placed cysteine residues within the active-site cavity by mutating sites Trp 5, Asn 62, Ile 91, and Phe 131. These mutants were modified at the single inserted cysteine with imidazole analogs to introduce new potential shuttle groups. Catalysis by these modified mutants was determined by stopped-flow and ¹⁸O-exchange methods. Specificity in proton transfer was demonstrated; only modifications of the Cys 131-containing mutant showed enhancement in the proton transfer step of catalysis compared with unmodified Cys 131-containing mutant. Modifications at other sites resulted in up to 3-fold enhancement in rates of CO₂ hydration, with apparent second-order rate constants near 350 μM⁻¹ s⁻¹. These are among the largest values of *k*_{cat}/*K*_m observed for a carbonic anhydrase.

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The carbonic anhydrases (CAs)¹ in the α class contain the mammalian isozymes. These are zinc metalloenzymes with a molecular mass near 30 kDa that catalyze the hydration of CO₂ to bicarbonate in two distinct and separate stages [1]. The conversion of carbon dioxide into bicarbonate is catalyzed by a zinc-bound hydroxide, with the dissociation of bicarbonate leaving a water molecule coordinated at the zinc (Eq. (1)).



The second stage of catalysis involves the transfer of a proton to solution to regenerate the zinc-bound hydroxide (Eq. (2)). This stage includes a proton transfer from the zinc-bound water molecule to a proton acceptor residue, which can be part of the enzyme itself or an exogenous proton acceptor in solution, designated B in Eq. (2). This proton transfer has been shown to be rate-limiting under maximal velocity conditions [1].

Human carbonic anhydrase isozyme II (HCA II) is among the most efficient of the 14 known isozymes from the α class [1]. This isozyme, and many others in the α class, contains His 64 as the proton shuttle residue B of equation (2) [2–4]. The mutation of His 64 to alanine in isozyme II (H64A) results in an enzyme with a turnover number, *k*_{cat}, decreased more than 10-fold compared

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¹ Abbreviations used: CA, Carbonic anhydrase; HCA II, human carbonic anhydrase II; H64A HCA II, the mutant of human carbonic anhydrase II with His 64 replaced with Ala; 2-CMI, 2-chloromethylimidazole; 4-CMI, 4-chloromethylimidazole; and 4-CEI, 4-chloroethylimidazole; Mops, 3-(*N*-morpholino) propanesulfonic acid; Taps, *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid.

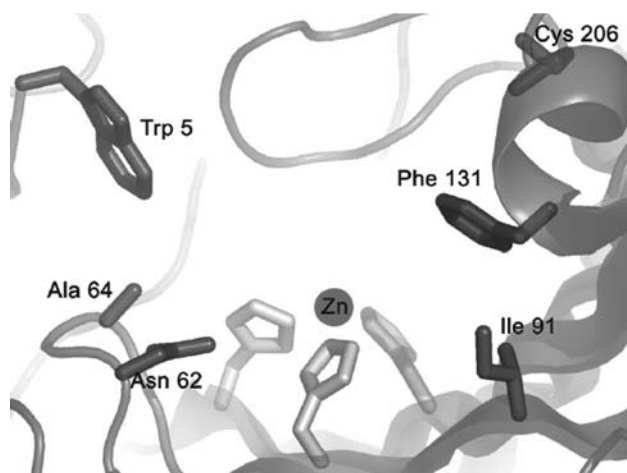


Fig. 1. Active-site of H64A HCA II showing the zinc ion coordinated to His 94, 96, and 119 (not labeled). Cys 206 is shown in two different conformations as determined by X-ray crystallography [18]. Shown in dark gray are the sites targeted for cysteine modification: Trp 5, Asn 62, Ile 91, and Phe 131.

with wild type HCA II [3]. The catalytic activity of H64A HCA II can be rescued by addition of exogenous proton donors such as imidazole and pyridine [3,5]. Although the kinetic properties of this chemical rescue have been studied, a binding site for these activators from which an efficient proton transfer can be achieved has not been identified.

In this study, we have investigated four locations in the active-site cavity of HCA II for their capacity to sustain proton transfer in catalysis. Trp 5, Asn 62, Ile 91, and Phe 131 (Fig. 1) have each been replaced with Cys, which was subsequently alkylated with imidazole-containing reagents. Stopped-flow spectrophotometry and the exchange of ^{18}O between CO_2 and water measured by mass spectrometry were used to determine catalytic rates. We have observed an enhancement in the rate constant of proton transfer only for mutants modified at Phe 131; a 2-fold enhancement of about 10 ms^{-1} over that of the unmodified mutant. Interestingly, several of the unmodified mutants show as much as a 3-fold enhancement of the apparent second-order rate constant for hydration of CO_2 when compared to wild type and H64A HCA II. These enhancements to values near $350\ \mu\text{M}^{-1}\text{ s}^{-1}$ represent examples of CA with this steady-state constant approaching the diffusion-controlled limit.

Materials and methods

Enzyme preparation

Mutations of carbonic anhydrase were made by site-directed mutagenesis using expression vectors containing

the HCA II coding region [6,7]. Residues Trp 5, Asn 62, Ile 91, and Phe 131 were separately mutated to cysteine using a plasmid encoding the sequence of H64A-C206S HCA II. In the H64A mutation, the proton shuttling residue His 64 has been replaced with an alanine to abolish the efficient proton transfer pathway provided by this group. The C206S mutation replaces the only naturally occurring cysteine in HCA II and was prepared to ensure absolute specificity since the alkylating agents chosen for modification are reactive towards cysteine residues [8–11]. The sequences of the triple mutants, W5C-H64A-C206S, N62C-H64A-C206S, I91C-H64A-C206S, and F131C-H64A-C206S, were confirmed by sequencing the DNA of the entire coding region for CA in the expression vector. Expression of the mutated vectors was done by transforming into *Escherichia coli* BL21(DE3)pLysS, which does not express any indigenous CA under our conditions [7]. Purification of H64A HCA II was performed by using affinity chromatography on a gel containing *p*-(aminomethyl)benzenesulfonamide coupled to agarose beads [12], followed by dialysis against a 15 mM Tris solution at pH 8.0. Enzyme concentration was determined from the molar absorptivity at 280 nm of wild type isozymes II ($5.5 \times 10^4\ \text{M}^{-1}\text{ cm}^{-1}$). Isozyme II binds sulfonamides tightly; therefore, the enzyme concentration was confirmed by titration of active-sites with ethoxzolamide measured by the ^{18}O exchange between CO_2 and water (see below). Electrophoresis on a 10% polyacrylamide gel stained with Coomassie blue was used to confirm that the purity of the enzyme sample was greater than 95%.

Chemical modification

Imidazole analogs were introduced by reacting each of the mutants containing single cysteines at positions 5, 62, 91 or 131 with the alkylating reagents: 2-chloromethylimidazole (2-CMI; $\text{p}K_a$ 7.9), 4-chloromethylimidazole (4-CMI; $\text{p}K_a$ 7.6), and 4-chloroethylimidazole (4-CEI; $\text{p}K_a$ 6.9) (Fig. 2). The alkylating agents were

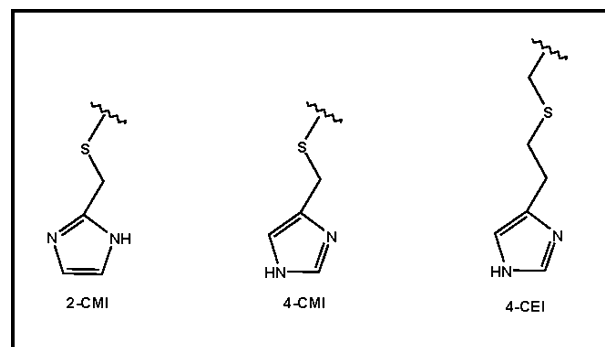


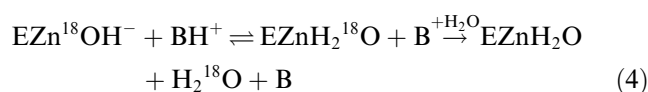
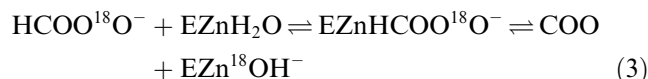
Fig. 2. Representation of the chemical structure of modified residues. Mutated cysteines were modified with imidazole analogs: 2-CMI, 4-CMI, and 4-CEI.

synthesized as described previously [13]. Chemical modifications were performed in 250 mM degassed borate buffer, pH 8.8, using enzyme concentrations of 0.5–1.0 mg/mL and amino acid analog reagent concentrations of 10–40 mM. A typical reaction was incubated under nitrogen with stirring for 8 h at 4 °C and then quenched by extensive dialysis against 20 mM Tris–HCl, pH 8.0, to remove excess reagent. The stoichiometry of cysteine modification was determined spectrophotometrically at 412 nm by monitoring the presence of the free cysteine content of the enzyme with 2,2'-dithiobis-(5-nitrobenzoate) (DTNB) [14,15]. Modified protein samples were concentrated by centrifugal ultrafiltration (Centricon, Amicon).

Oxygen-18 exchange

The uncatalyzed and carbonic anhydrase catalyzed exchange of ^{18}O between CO_2 and water at chemical equilibrium was measured in the absence of buffer at a total substrate concentration of 25 mM, using membrane-inlet mass spectrometry [16,17]. The temperature was 25 °C and the total ionic strength of solution was maintained at 0.2 M by the addition of Na_2SO_4 .

This method can measure (1) the rate of depletion of ^{18}O from CO_2 caused by the appearance of the label in H_2^{18}O where it is greatly diluted by H_2^{16}O (Eq. (3)) and (2) a rate of proton transfer to the transitory ^{18}O label at the active site of carbonic anhydrase (Eq. (4)) [16].



The reaction solution is in contact with a membrane permeable to gases. CO_2 passing across the membrane enters a mass spectrometer (Extrel EXM-200) providing a continuous measure of isotopic content of CO_2 . Two rates at chemical equilibrium can be determined for this exchange [16]. R_1 is the rate of interconversion of CO_2 and HCO_3^- (Eq. (3)). $R_{\text{H}_2\text{O}}$ is the rate of release of H_2^{18}O from the enzyme (Eq. (4)).

The value of $R_{\text{H}_2\text{O}}$ can be interpreted in terms of the rate constant from an exogenous donor group to the zinc-bound hydroxide according to Eq. (5), in which k_B is the rate constant for proton transfer to the zinc-bound hydroxide, $(K_a)_{\text{donor}}$ is the ionization constant of the donor group, and $(K_a)_{\text{ZnH}_2\text{O}}$ is the ionization constant of the non-interacting zinc-bound water molecule. The determination of kinetic constants k_B from equation (5) was carried out by nonlinear least-squares methods (Enzfitter, Elsevier-Biosoft).

$$R_{\text{H}_2\text{O}}/[\text{E}] = k_B / \{ (1 + (K_a)_{\text{donor}}/[\text{H}^+]) \times (1 + [\text{H}^+]/(K_a)_{\text{ZnH}_2\text{O}}) \} \quad (5)$$

Stopped-flow kinetics

Initial rates of CO_2 hydration were measured by following the change in absorbance of a pH indicator on an Applied Photophysics (SX.18MV) stopped-flow spectrophotometer as described earlier [3]. The pK_a values and wavelengths for the pH indicator-buffer pairs used to create pH profiles were as follows: Mes ($pK_a = 6.1$) and chlorophenol red ($pK_a = 6.3$), $\lambda = 574$ nm; Mops ($pK_a = 7.2$), and *p*-nitro phenol ($pK_a = 7.1$), $\lambda = 401$ nm; Hepes ($pK_a = 7.5$) and phenol red ($pK_a = 7.5$), $\lambda = 557$ nm; Taps ($pK_a = 8.4$) and *m*-cresol purple ($pK_a = 8.3$), $\lambda = 578$ nm; and Ches ($pK_a = 9.3$), and thymol blue ($pK_a = 8.9$), $\lambda = 596$ nm. Final buffer concentrations were 25 mM and total ionic strength was kept at 0.2 M by addition of Na_2SO_4 . CO_2 solutions were prepared by bubbling CO_2 into water at 25 °C with final concentrations after mixing ranging from 0.7 to 17 mM. The mean initial rates at each pH were determined from 5 to 8 reaction traces comprising the initial 10% of the reaction. The uncatalyzed rates were determined in a similar manner and subtracted from the total observed rates. Determination of the kinetic constants k_{cat} and k_{cat}/K_m was carried out by a nonlinear least-squares method (Enzfitter, Elsevier-Biosoft).

Results

Introduction of new proton shuttle groups

We have placed cysteine residues at strategic locations along the surface of the active-site cavity of HCA II (Fig. 1), and then chemically modified each of them with alkylating agents containing an imidazolium group as a potential proton shuttle group. His 64, the natural proton shuttle residue of HCA II, was replaced with Ala so that the proton transfer properties of the imidazole groups introduced at specific sites can be measured in the absence of the competing natural shuttle pathway. HCA II contains a single cysteine residue (Cys 206), which is located on the surface of the active-site cavity. The side-chain of Cys 206 is positioned with its sulfur 12 Å from the zinc and lies along the cavity wall but not extended into the cavity [18]. This cysteine residue was replaced with Ser to avoid chemical modifications at this alternate site during modifications of the introduced cysteine. Each of the cysteine mutant enzymes was modified by three alkylating agents as described under Materials and methods: 2-chloromethylimidazole (2-CMI), 4-chloromethylimidazole (4-CMI), and

4-chloroethylimidazole (4-CEI) (Fig. 2). In each case, the extent of chemical modification of the single cysteine residue was in excess of 90% as determined by DTNB titration. Non-reducing SDS–PAGE showed no dimer formation before or after chemical modification of carbonic anhydrase.

Hydration of CO₂

The apparent second-order rate constant k_{cat}/K_m describing the catalytic conversion of CO₂ into bicarbonate (Eq. (1)) was determined by stopped-flow spectrophotometry. The pH profile for k_{cat}/K_m was determined for several of the modified and unmodified mutants of Table 1. For these mutants, the pH profile could be described by a single ionization with a $\text{p}K_a$ around 7.3 ± 0.2 and a maximum at high pH, with a result shown in Fig. 3. For other variants of carbonic anhydrase, measurements only at pH 9.0 were taken as the maximal value of k_{cat}/K_m . Although the values of the $\text{p}K_a$ did not change significantly as a result of mutation and chemical modification, the maximal values of k_{cat}/K_m varied over 10-fold as shown in Table 1.

The maximal values of k_{cat}/K_m for the unmodified mutants H64A and H64A-C206S were close in magnitude to that of wild type HCA II, which is $120 \pm 20 \mu\text{M}^{-1} \text{s}^{-1}$ (Table 1). This value is similar to the values of k_{cat}/K_m determined for CA II by stopped-flow methods [2,19]. However, replacement of residues at positions 5, 62, and 91 with Cys caused significant increases in k_{cat}/K_m , as large as 3-fold for I91C-H64A-C206S (Table 1). In each of these cases, chemical modification by 4-CMI at the inserted Cys residue caused

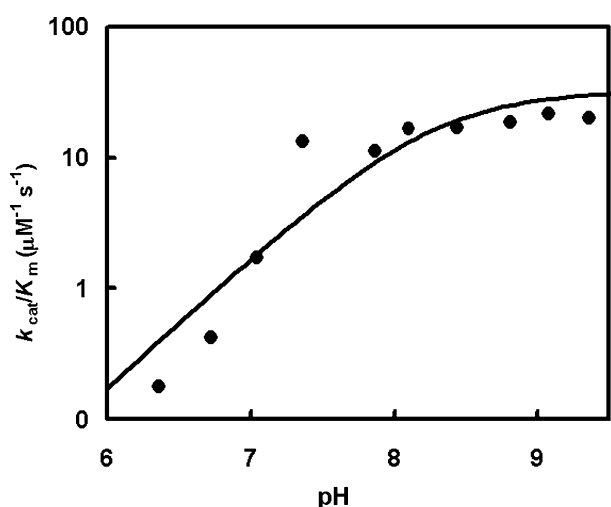


Fig. 3. Dependence of k_{cat}/K_m for CO₂ hydration on pH for catalysis by unmodified F131C-H64A-C206S HCA II. The solid line is a least-squares fit to a single ionization with $\text{p}K_a$ of 7.3 ± 0.2 and a maximal $k_{\text{cat}}/K_m = 22 \pm 2 \mu\text{M}^{-1} \text{s}^{-1}$ at high pH. The temperature was 25 °C and the total ionic strength of solution was maintained at 0.2 M by the addition of Na₂SO₄.

Table 1

The apparent second-order rate constant k_{cat}/K_m for CO₂ hydration catalyzed by mutants of HCA II and their chemically modified forms^a

Enzyme	Modifying reagent	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$) ^b
Wild type	Unmodified	120
H64A	Unmodified	78
H64A-C206S	Unmodified	73
W5C-H64A-C206S	Unmodified	330
	4-CMI	160
N62C-H64A-C206S	Unmodified	260
	4-CMI	56
I91C-H64A-C206S	Unmodified	350
	4-CMI	130
F131C-H64A-C206S	Unmodified	22
	4-CMI	24

^a The temperature was 25 °C and the total ionic strength of solution was kept at 0.2 M by the addition of Na₂SO₄.

^b Standard errors were less than 20%.

significant loss of activity in k_{cat}/K_m (Table 1). In contrast, k_{cat}/K_m catalyzed by F131C-H64A-C206S was less than wild type by about 5-fold and was unchanged by chemical modification with 4-CMI (Table 1).

Proton transfer

The rate constant $R_{\text{H}_2\text{O}}/[E]$ measures the proton transfer dependent release of ¹⁸O-labeled water from the enzyme, as in Eq. (4). The pH profiles for modified and unmodified mutants are rather complex and cannot be described by a single or even easily by two ionizations. Of the four cysteine-containing mutants in the active-site cavity, only the F131C-containing mutant showed enhancement of the proton transfer step $R_{\text{H}_2\text{O}}$ upon modification, and here the enhancement was observed for all three imidazole-containing alkylating agents (Fig. 4). The release of ¹⁸O-labeled water from the active site is enhanced by proton donation to the zinc-bound ¹⁸O-labeled hydroxide (Eq. (4)); accordingly the enhancements were observed at pH less than 7, as anticipated for the imidazolium group acting as a proton donor. This pH-dependent enhancement is clearest for modification of the F131C-containing mutant with 4-CMI; a difference plot showing the pH profile for enhancement caused by modification with 4-CMI is fit by Eq. (5) (inset, Fig. 4) representing a proton donor and proton acceptor both with $\text{p}K_a$ values near 6.3.

The W5C- and I91C-containing mutants modified by alkylating agents showed no significant difference in $R_{\text{H}_2\text{O}}/[E]$ when compared with the respective unmodified mutants (data not shown). In contrast, when compared with the unmodified N62C-containing mutant, the profile of the chemically modified counterparts showed up to 90% inhibition of $R_{\text{H}_2\text{O}}/[E]$ in a pH range from 5 to 9 (Fig. 5). This inhibition is not pH dependent, with the same degree of inhibition observed across the pH range for each modified enzyme.

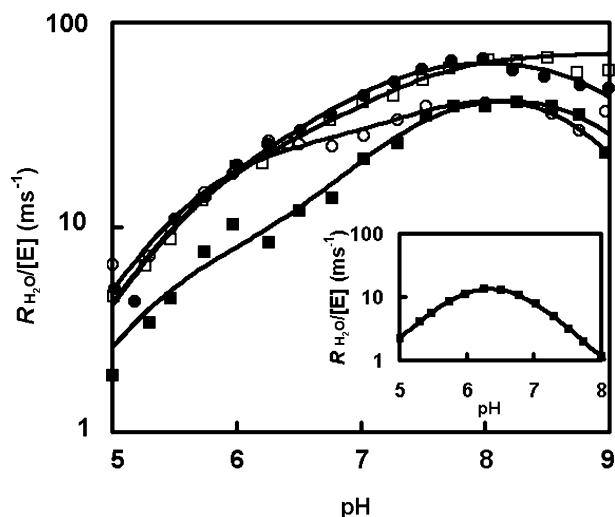


Fig. 4. The pH dependence of $R_{H_2O}/[E]$ catalyzed by unmodified F131C-H64A-C206S HCA II (■) and chemically modified by 2-CMI (●), 4-CMI (○), and 4-CEI (□). The temperature was 25 °C and the total concentration of CO_2 and HCO_3^- was 25 mM. The total ionic strength of solution was maintained at 0.2 M by the addition of Na_2SO_4 . Inset: Difference plot for $R_{H_2O}/[E]$ between unmodified and 4-CMI modified F131C-H64A-C206S showing a maximum near pH 6.3. The solid line is a least-square fit of Eq. (5) yielding ionizations of pK_a near 6.3 for both the zinc-bound water and introduced proton donor group. For comparison, the maximal value of $R_{H_2O}/[E]$ for wild type HCA II is about 500 ms^{-1} and occurs at pH 7.

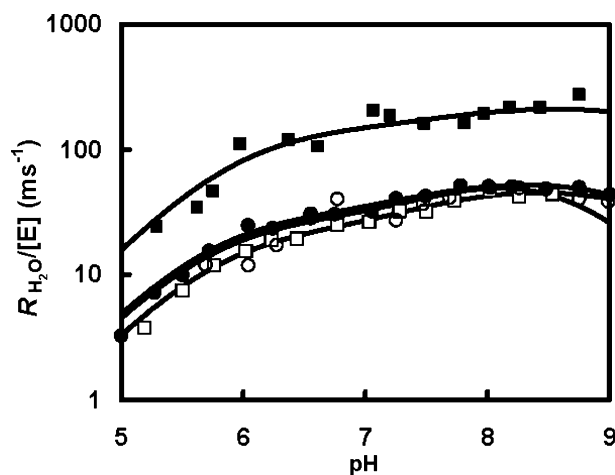


Fig. 5. The pH dependence of $R_{H_2O}/[E]$ catalyzed by unmodified N62C-H64A-C206S HCA II (■) and chemically modified by 2-CMI (●), 4-CMI (○), and 4-CEI (□). The temperature was 25 °C and the total concentration of CO_2 and HCO_3^- was 25 mM. The total ionic strength of solution was maintained at 0.2 M by the addition of Na_2SO_4 .

Discussion

The well-characterized structure and catalytic mechanism of carbonic anhydrase provide an opportunity to examine the redesign of proton shuttle pathways in the context of an enzyme active site. The natural proton

shuttle residue in this enzyme, His 64, was replaced with Ala to provide a background to detect new proton transfer pathways. This replacement experiment has been reported by Earnhardt et al. [8] for murine CA V, an isozyme of CA that has Tyr 64 instead of His 64. Murine CA V has four cysteine residues that were not replaced by Earnhardt et al. [8], and chemical modification with 4-CMI at cysteine residues placed at positions 91 and 131 showed activation of the proton transfer dependent rate constant for ^{18}O exchange, $R_{H_2O}/[E]$, only for modification at position 131.

This work expands on that initial study by using the very efficient isozyme HCA II, replacing the single cysteine of the wild type, and studying four positions for activation, residues 5, 62, 91, and 131. These sites were chosen because their side chains extend into the active-site cavity with distances from the $C\alpha$ to the zinc ranging from 12.5 to 14.5 Å. Trp 5 and Ile 91 were chosen as sites for the introduction of proton shuttle groups because crystallographic evidence indicates binding sites for the exogenous proton donor 4-methylimidazolium near Trp 5 and Ile 91 [20,21]. A crystal structure of H64A HCA II shows that the phenyl ring of Phe 131 forms a π -stacking interaction with the exogenous proton donor pyridine [Duda et al., unpublished]. A fourth site of interest for our study was Asn 62; in a study done by Liang et al. [22], it was found that mutation of Asn 62 to a histidine in a double mutant of HCA II (H64A-N62H) results in catalytic activity for CO_2 hydration larger by about 5% when compared with the H64A single mutant.

The hydration of CO_2 , Eq. (1), is separate and distinct from the proton transfer steps of Eq. (2); k_{cat}/K_m is a measure of the rate of this catalyzed hydration. In our study, we observed about 3-fold enhancements of k_{cat}/K_m for the unmodified W5C-, N62C-, and I91C-containing mutants compared with wild type HCA II (Table 1).² These enhanced values are unusual for mutants of HCA II and are among the largest rate constants that have been observed for variants of CA II. We note that in each case of enhancement in these mutants, the value of k_{cat}/K_m is greatest for the unmodified mutants and significantly less for the modified mutants (Table 1). The most likely explanation is that the kinetics of this stage in catalysis are mainly diffusion controlled and the mutations which replace bulky side chains with much smaller ones have opened the active site for enhanced diffusion of substrate and product. The absence of such an effect for the mutant containing a

² The values of $R_1/[E]$, determined by ^{18}O exchange, also measure the rate of interconversion of CO_2 and bicarbonate [16]. These values were in qualitative agreement with the values of k_{cat}/K_m in Table 1, but were not in quantitative agreement, generally showing larger enhancements for the unmodified and modified mutants compared with wild type. The most likely explanation for this difference between the results from ^{18}O exchange and stopped-flow lies in the different conditions of these experiments. Work is in progress on this topic.

replacement at residue 131 may reflect its location in the active-site cavity (Fig. 1); this site may not lie along a prominent substrate access path yet, its replacement decreases activity. In fact, the much lower values of k_{cat}/K_m , near $0.1 \mu\text{M}^{-1} \text{s}^{-1}$, for HCA III have been attributed in part to the sterically constrained nature of this active site [23]. Other, but much less likely, explanations are that cysteine at sites 5, 62, and 91 situated 9–12 Å from the zinc might facilitate the nucleophilic attack by zinc-bound hydroxide, or may enhance the departure of the product bicarbonate through nonspecific effects in the active-site cavity.

Of the four sites, each tested with three different alkylating agents, we determined that only the introduction of shuttle groups at position 131 was capable of enhancing proton transfer (Fig. 4). A pH profile of the difference in $R_{\text{H}_2\text{O}}/[\text{E}]$ between 4-CMI-modified and unmodified F131C-containing mutant demonstrates a maximum rate enhancement at pH 6.3 (inset Fig. 4). This pH profile is fit by Eq. (5) describing proton transfer from a chemically introduced imidazolium group of $\text{p}K_a$ near 6.3 to the zinc-bound hydroxide with a $\text{p}K_a$ also near 6.3. This $\text{p}K_a$ is consistent with the imidazole group of 4-CMI and with the expected $\text{p}K_a$ near 7 for the zinc-bound water. Modification at this position by 2-CMI and 4-CEI also showed enhanced values of $R_{\text{H}_2\text{O}}/[\text{E}]$ in this region, and in the higher pH region. The enhancements at higher pH are not explained by the introduction of imidazolium groups with a $\text{p}K_a$ near 7. The pH profile of unmodified F131C-H64A-C206S HCA II has no apparent proton donor although rate constants for proton transfer exceed 10ms^{-1} at $\text{pH} > 7$. This is possibly due to proton transfer involving residues more distant from the zinc than His 64, as described by Qian et al. [24].

It is interesting that residue 131 is the only modified site among the four sites tested from which efficient proton transfer occurs. Moreover, this is also the site found in CA V from which proton transfer is observed. Jude et al. [25] solved the crystal structure for murine CA V modified with methylimidazole at Cys 131, a modified mutant with proton transfer activity enhanced about 3-fold, which is similar to the extent of enhancement found for the modified-Cys 131 mutant of HCA II (Fig. 4). The modified Cys 131 side chain in CA V is seen in the crystal structure to extend toward the zinc and also show considerable disorder, due to conformational mobility. A chain of three hydrogen-bonded water molecules connects the imidazole ring with the zinc-bound solvent. In HCA II, the side chains of residues 5, 62, and 91 all point into the active-site cavity at distances that are comparable to position 131, yet chemical modification at these sites showed no enhancement of proton transfer. Features of the active-site cavity may orient these modified side chains away from the metal, or could possibly engage the imidazole nitrogens in

electrostatic or hydrogen bonds in a manner that decreases their ability to participate in proton transfers. The modified N62C HCA II mutant showed about a 10-fold inhibition of $R_{\text{H}_2\text{O}}/[\text{E}]$ compared with unmodified mutant (Fig. 5).

From the crystal structure of the modified Cys 131 in CA V, Jude et al. [25] concluded that activation of this mutant is consistent with a proton transfer through a hydrogen-bonded solvent bridge between the proton donor and the zinc-bound solvent molecule. It is likely that the reason why modified Cys 131 in HCA II and V is a site of proton transfer is because the imidazole analogs at this position possess the same essential properties; proximity to the zinc and sufficient conformational flexibility to hydrogen bond with the water molecules of the proton wire extending to the zinc-bound hydroxide. Our results emphasize the similarity of the proton transfer process in CA II and CA V. These isozymes have very similar backbone conformations, but different residues in the active-site cavity. Yet, both isozymes show equivalent enhancement of $R_{\text{H}_2\text{O}}/[\text{E}]$ by chemical modification of Cys 131 with 4-CMI [8]. We also point out another example of the specificity involved in this proton transfer; N62H HCA II showed enhanced catalysis consistent with enhanced proton transfer from His 62 [22], yet chemical modification of Cys 62 to produce histidine analogs at this position results in inhibition (Fig. 5). The opposite effect is observed at position 131 in CA V. Mutation of the tyrosine at this position to histidine yields no enhancement of proton transfer, yet the introduction of longer histidine analogs by chemical modification results in up to a 3-fold enhancement [8]. Clearly efficient proton transfer requires the optimization of the distance and orientation between the donor and acceptor, a tuning of the respective $\text{p}K_a$ values, and the conformational flexibility for the introduced shuttle group to both accept and deliver the proton.

Acknowledgments

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References

- [1] S. Lindskog, *Pharmacol. Ther.* 74 (1997) 1–20.
- [2] H. Steiner, B.H. Jonsson, S. Lindskog, *Eur. J. Biochem.* 59 (1975) 253–259.
- [3] C.K. Tu, D.N. Silverman, C. Forsman, B.H. Jonsson, S. Lindskog, *Biochemistry* 28 (1989) 7913–7918.
- [4] D.N. Silverman, S. Lindskog, *Acc. Chem. Res.* 21 (1988) 30–36.

- [5] H. An, C.K. Tu, D. Duda, I. Montañez-Clemente, K. Math, P.J. Laipis, R. McKenna, D.N. Silverman, *Biochemistry* 41 (2002) 3235–3242.
- [6] S.M. Tanhauser, D.A. Jewell, C.K. Tu, D.N. Silverman, P.J. Laipis, *Gene* 117 (1992) 113–117.
- [7] F.W. Studier, A.H. Rosenberg, J.J. Dunn, J.W. Dubendorff, *Methods Enzymol.* 185 (1990) 60–89.
- [8] J.N. Earnhardt, S.K. Wright, M. Qian, C.K. Tu, P.J. Laipis, R.E. Viola, D.N. Silverman, *Arch. Biochem. Biophys.* 361 (1999) 264–270.
- [9] T. Yoshimura, Y. Matsushima, K. Tanizawa, M.H. Sung, T. Yamauchi, M. Wakayama, N. Esaki, K. Soda, *J. Biochem. (Tokyo)* 108 (1990) 699–700.
- [10] G. DeSantis, P. Berglund, M.R. Stabile, M. Gold, J.B. Jones, *Biochemistry* 37 (1998) 5968–5973.
- [11] H.B. Smith, F.W. Larimer, F.C. Hartman, *Biochem. Biophys. Res. Commun.* 152 (1988) 579–584.
- [12] R.G. Khalifah, D.J. Strader, S.H. Bryant, S.M. Gibson, *Biochemistry* 16 (1977) 2241–2247.
- [13] J.F. Schindler, R.E. Viola, *J. Protein Chem.* 15 (1996) 737–742.
- [14] G.L. Ellman, *Arch. Biochem. Biophys.* 82 (1959) 70–77.
- [15] S.K. Wright, R.E. Viola, *Anal. Biochem.* 265 (1998) 8–14.
- [16] D.N. Silverman, *Methods Enzymol.* 87 (1982) 732–752.
- [17] D.N. Silverman, C.K. Tu, X. Chen, S.M. Tanhauser, A.J. Kresge, P.J. Laipis, *Biochemistry* 32 (1993) 10757–10762.
- [18] A.E. Eriksson, T.A. Jones, A. Liljas, *Proteins* 4 (1988) 274–282.
- [19] R.G. Khalifah, *J. Biol. Chem.* 246 (1971) 2561–2573.
- [20] D. Duda, C.K. Tu, M. Qian, P.J. Laipis, M. Agbandje-McKenna, D.N. Silverman, R. McKenna, *Biochemistry* 40 (2001) 1741–1748.
- [21] D. Duda, L. Govindasamy, M. Agbandje-McKenna, C. Tu, D.N. Silverman, R. McKenna, *Acta Crystallogr. D* 59 (Pt 1) (2003) 93–104.
- [22] Z. Liang, B.H. Jonsson, S. Lindskog, *Biochim. Biophys. Acta* 1203 (1993) 142–146.
- [23] A.E. Eriksson, A. Liljas, *Proteins* 16 (1993) 29–42.
- [24] M.Z. Qian, N.J. Earnhardt, N.R. Wadhwa, C.K. Tu, P.J. Laipis, D.N. Silverman, *Biochim. Biophys. Acta* 1434 (1999) 1–5.
- [25] K.M. Jude, S.K. Wright, C. Tu, D.N. Silverman, R.E. Viola, D.W. Christianson, *Biochemistry* 41 (2002) 2485–2491.