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Structure and Catalysis by Carbonic Anhydrase II: Role of Active-Site Tryptophan 5

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Abstract

The tryptophan residue Trp5, highly conserved in the α class of carbonic anhydrases including human carbonic anhydrase II (HCA II), is positioned at the entrance of the active site cavity and forms a π -stacking interaction with the imidazole ring of the proton shuttle His64 in its outward orientation. We have observed that replacement of Trp5 in HCA II caused significant structural changes, as determined by X-ray diffraction, in the conformation of eleven residues at the N-terminus and in the orientation of the proton shuttle residue His64. Most significantly, two variants W5H and W5E HCA II had His64 predominantly outward in orientation, while W5F and wild type showed the superposition of both outward and inward orientations in crystal structures. Although Trp5 influences the orientation of the proton shuttle His64, this orientation had no significant effect on the rate constant for proton transfer near $1 \mu\text{s}^{-1}$, determined by exchange of ^{18}O between CO_2 and water measured by mass spectrometry. The apparent values of the pK_a of the zinc-bound water and the proton shuttle residue suggest that different active-site conformations influence the two stages of catalysis, the proton transfer stage and the interconversion of CO_2 and bicarbonate.

Keywords

carbon dioxide; carbonic anhydrase; enzyme mechanism; proton transfer; bicarbonate

INTRODUCTION

Carbonic anhydrase catalyzes the dehydration of bicarbonate in a two-stage mechanism [1–4]. The initial stage is the reaction of bicarbonate with zinc-bound water releasing CO_2 and resulting in zinc-bound hydroxide (Eq. 1). In the second stage, proton transfer from bulk solution to the zinc-bound hydroxide regenerates the zinc-bound water. In the α class of carbonic anhydrases including human carbonic anhydrase II (HCA II)¹, this stage proceeds

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Coordinates and structure factors have been deposited in the Protein Data Bank as entries 3RG3, 3RG4 and 3RGE.

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predominantly via the intermediate transfer of the proton to the shuttle residue His64 (Eq. 2) [5, 6].

Here B represents buffer in bulk solution. The intramolecular proton transfer of Eq. 2 is the rate-limiting step in the maximal turnover of the catalytic dehydration of bicarbonate near 10^6 s^{-1} . The imidazole side chain of His64 is about 7 Å from the zinc, and proton transfer is believed to proceed via intervening, hydrogen-bonded water molecules [1, 2].

In human carbonic anhydrase II (HCA II)¹, two orientations of the proton shuttle His64 have been identified from difference electron density maps in crystal structures [7, 8]. At pH near 8.5, the inward orientation predominates with the side chain of His64 pointing toward the zinc in the active site (χ_1 and χ_2 are near 47° and 99° [8]). Whereas, the outward orientation predominates at pH 6.0 with His64 pointing out of the active site cavity towards bulk solution (χ_1 and χ_2 are near -55° and 111° ; Figure 1). The imidazole side chain of His64 extends into the active-site cavity with only one significant interaction; in the outward orientation, the imidazole side chain appears to form a π -stacking interaction with the indole ring of Trp5 (Figure 1) which perhaps stabilizes His64 in the outward conformation. The side chain of Trp5 also extends into the active-site cavity but is located closer to the surface of the enzyme than His64. Martensson et al. [9] replaced each tryptophan by phenylalanine in HCA II to identify individual contributors to fluorescence emission and quenching processes. They demonstrated that Trp5 fluorescence was efficiently quenched by His64, as shown by the variants H64A and W5F, both of which fluoresced at increased levels over wild type at 333 nm. This demonstrated the interaction between Trp5 and His64.

Molecular dynamics calculations show that the energy barrier for side-chain rotation of His64 between inward and outward is near 6 kcal/mol [10]. The energy barrier for the catalyzed reaction is near 10 kcal/mol. Hence, the conformational rotation between the inward and outward orientations of the proton shuttle is orders of magnitude faster than the catalyzed reaction and an average or thermally equilibrated ratio of inward to outward orientations occurs during catalysis. Multistate valence bond calculations show that the energy barrier for proton transfer in catalyzed hydration is smaller by about 1.4 kcal/mol for the inward compared with outward orientations [11]. A number of experiments suggest that the rate constant for proton transfer in variants of HCA II is independent of whether His64 is inward or outward in crystal structures [12]. In addition, combined quantum mechanical-molecular mechanical simulations suggest that this intramolecular proton transfer is governed by electrostatics and is not sensitive to distance or number of water molecules within the active-site cavity [13].

In this work we investigate the role of Trp5 in the control and stabilization of conformations of HCA II. Replacement of this residue had effects on the orientation of eleven residues at the N-terminus and on the side chain of the proton shuttle His64. In the variant W5F HCA II both the inward and outward orientations of His64 were observed, as in wild type. The variants W5H and W5E HCA II show His64 predominantly in the outward conformation.

¹Abbreviations used: HCA II, human carbonic anhydrase II; W5F, the variant in which which Trp5 is replaced with Phe

Despite these differences in conformation, the maximal values of rate constants for catalytic interconversion of bicarbonate and CO₂ and for proton transfer were relatively unchanged for the variants. However, the pH maximum for proton transfer was altered from pH 7 in wild type to near 6 for the variants, out of the physiological range. Both the apparent pK_a of His64 and of the zinc-bound water were decreased in the variants to values near 6 when obtained in pH profiles of the second stage of catalysis (Eq. 2). This is discussed in terms of different active-site conformations for the two stages of catalysis in the variants.

METHODS

Expression and purification

Cloning and expression of the variants of HCA II with replacements of Trp5 followed the procedures previously used in this laboratory [14]. Mutations in the cDNA of HCA II were made by site-directed mutagenesis using Stratagene Quick Change II kit and primers from Invitrogen. The mutations were confirmed by sequencing the entire coding region of the enzyme. The HCA II and variant genes were expressed in *Escherichia coli* (BL21(DE3)pLysS). Purifications were carried out using affinity chromatography based on the tight binding of HCA II to sulfonamides [15]. Briefly, cells were lysed with hen egg white lysozyme, and the lysate was passed through an agarose resin coupled with the HCA II inhibitor p-(aminomethyl)-benzene-sulfonamide. The bound HCA II was eluted with 0.4 M sodium azide, 100 mM Tris, pH 7.0, and the azide was removed by extensive dialysis against 10 mM Tris, pH 8.0. Protein concentration was carried out by centrifugal ultrafiltration and purity was assessed by SDS-PAGE. The final enzyme concentration was determined by titration of active sites with ethoxzolamide measured by the catalyzed exchange of ¹⁸O between CO₂ and bicarbonate, analyzing the data using the approach of Henderson [16].

Crystallization and diffraction data collection

Crystals of the variants containing replacements at residue 5 were obtained via hanging drop vapor diffusion with a reservoir of 1.3 M sodium citrate and 100 mM Tris pH 8.0. For W5H and W5F HCA II, the suspended protein drop was a 1:1 mixture of protein: reservoir solution. In the case of the W5E mutant, the protein drop was a 2:2:1 mixture of protein: reservoir: 1% Tween-detergent. Diffraction data were measured at 298 K on an in-house Rigaku R-AxisIV⁺⁺ image plate detector using an RU-H3R rotating anode generator operating at 50 kV, 100 mA. X-rays were focused via Osmic optics, followed by a helium-purged beam path. For each W5 mutant, oscillation images were measured with an exposure time of 6m at a crystal to detector distance of 75 mm. *HKL2000* [17] was used to integrate, merge, and scale the data in the monoclinic space group *P*2₁, to resolutions of 1.5Å (W5F), 1.9Å (W5E), and 2.1Å (W5H). A summary of data statistics is provided in Table 1.

Structure solution and refinement of the molecular model

Initial phases for the three W5 mutants were calculated using the coordinates of the isomorphous wild-type HCA II structure (PDB code 2ILI; [8]). *PHENIX.REFINE* [18] was used in cycles of restrained refinement of the molecular model, alternating with manual model building using COOT [19]. Electron density maps for all three mutants were weak for the disordered N-terminal residues 1–4, and in addition, residues 5–11 for the W5E mutant. The converged, refined models each consist of one HCA II chain, one catalytic zinc atom, and first shell water molecules. R_{cryst} and R_{free} values were 13.3 and 17.0% (W5E), 13.6 and 16.6% (W5F), and 14.1 and 18.8% (W5H), respectively. *PROCHECK* [20] was used to assess the quality of the final model. Final model statistics are included in Table 1.

Catalysis

The catalysis was measured by the exchange of ^{18}O between CO_2 species and water using membrane inlet mass spectrometry [21]. The method is based on the measurement of the ^{18}O content of CO_2 that passes across a membrane inlet into a mass spectrometer (Extrel EXM-200) allowing a continuous measurement of isotopic enrichment of CO_2 . This ^{18}O exchange is catalyzed by carbonic anhydrase. The initial step of the catalysis by HCA II is the dehydration of labeled bicarbonate with a probability of leaving ^{18}O -labeled hydroxide at the zinc (Eq. 3). The second step involves the protonation of the zinc-bound hydroxide subsequently releasing a H_2^{18}O which is very greatly diluted by solvent H_2^{16}O (Eq. 4).

The ^{18}O -exchange method allows a rate for two stages of the catalysis to be determined [21]. The rate of the first stage, exchange between CO_2 and HCO_3^- at chemical equilibrium, is determined from Eq. 5 and termed R_1 [21]. The maximal rate constant of the conversion between CO_2 and HCO_3^- is $k_{\text{cat}}^{\text{ex}}$ and $K_{\text{eff}}^{\text{CO}_2}$ is an apparent binding constant of the substrate to enzyme [22].

$$R_1/[E] = k_{\text{cat}}^{\text{ex}} [\text{CO}_2]/(K_{\text{eff}}^{\text{CO}_2} + [\text{CO}_2]) \quad (5)$$

The rate of the second stage, the transfer of a proton and release of ^{18}O -labeled water (Eq. 4), is termed $R_{\text{H}_2\text{O}}$. $R_{\text{H}_2\text{O}}$ is interpreted in terms of a rate constant k_B for proton transfer to the zinc-bound hydroxide (Eq. 6). The ionization constants of the proton donor and the zinc-bound water molecule are $(K_a)_{\text{donor}}$ and $(K_a)_{\text{ZnH}_2\text{O}}$.

$$R_{\text{H}_2\text{O}}/[E] = k_B / ([1 + (K_a)_{\text{donor}}/[H^+]][1 + [H^+]/(K_a)_{\text{ZnH}_2\text{O}}]) \quad (6)$$

The uncatalyzed and carbonic anhydrase catalyzed ^{18}O exchange at chemical equilibrium were measured in the absence of buffer at a total substrate concentration (all CO_2 species) of 25 mM using membrane-inlet mass spectrometry [21]. Reactions were carried out at 25 °C, and total ionic strength of solution was maintained at 0.2 M by the addition of Na_2SO_4 . The determination of the kinetic constants and ionization constants of Eqs. 5 and 6 were carried out by nonlinear least-squares methods (Enzfitter, Biosoft).

The catalysis of the hydrolysis of 4-nitrophenylacetate by HCA II and variants was measured by the method of Verpoorte et al. [23]. The hydrolysis was followed at 348 nm, the isosbestic point of nitrophenol and the conjugate nitrophenylate ion using the molar absorptivity $5.0 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. A Beckman Coulter DU 800 spectrophotometer was used to measure both the uncatalyzed and carbonic anhydrase catalyzed initial velocities. A range of buffers from pH 5.0 to 9.0 was used at 100 mM (MES, ACES, HEPES, TAPS, CHES). The values of k_{cat}/K_m are reported; the value of K_m is too large to measure k_{cat} .

RESULTS

Structure

Based upon the electron densities of the three mutant structures, the N-terminal residues 4–11 appear to be less well-ordered when compared to wild-type HCA II. Depending on the mutation involved, the electron density becomes qualitatively weaker than wild-type HCA II in the order W5F > W5H > W5E, in which W5E was the weakest. The relative destabilization can be somewhat quantified by comparing the ratio of the average main-chain isotropic temperature factors of the N-terminus (to residue 11) with that of the rest of the protein (residues 12 to 261). For W5F those values are 27.0/16.0 (1.7 fold), for W5H they are 39.9/15.5 (2.6 fold), and for W5E they are 71.7/15.4 (4.6 fold) ($\text{\AA}^2/\text{\AA}^2$), while for the high-resolution wild-type HCA II structure (PDB code 2ILI, [8]) these values are 22.4/11.1 (2.0 fold).

In wild-type HCA II, the side chain of His64 in the outward conformation forms a π -stack with the indole ring of Trp5 with a distance of 3.4 $\text{\AA}$ between the planes of the aromatic rings (Figure 1). In the conservative W5F mutation, the phenyl ring of Phe5 superimposes closely on the side chain indole ring of Trp5 of wild type (Figure 2). Thus π -stacking is also present in W5F HCA II in which the side chain of His64 is observed in inward and outward orientations in the crystal structure (Figure 3).

In the crystal structures of W5H and W5E HCA II, the side chain of His64 is predominantly outward (Figure 3). The imidazole side chain of His5 in W5H does not superimpose with the indole ring of Trp5 in wild type; instead, the His5 side chain makes a hydrogen bond to the OD2 of Glu19, which also makes a salt-bridge to Arg182 from a symmetry-related molecule. In the W5E variant, the Glu5 residue is disordered in the crystal and was therefore not modeled or interpreted.

As a result of the lost stacking interaction, the side chains of His64 in the two mutants W5E and W5H adopt slightly different orientations, which are shown in Figure 3. Neither His64 of the W5E or W5H variant structures has an alternate conformation: they are both in the outward position. The side chain torsion angles of His64 in W5E are -80° and 178° for χ_1 and χ_2 ; and for W5H, they are -80° and 137° . In the structure of the W5F mutation, both inward and outward conformations of His64 are present, similar to the wild-type structure (Figure 3).

Although, the mutations W5H and W5E result in less-well ordered N-terminal residues and adopt different conformations of the proton shuttle residue His64, the water structure in the active site remains remarkably well conserved. Superposition of the coordinates of the ordered water molecules in the variants examined here reveals that no significant changes occur in the positions of W1, W2, W3A or W3B, as can be seen in Figures 2, 3.

Catalysis

Catalysis was examined for each of the variants of Table 2 by measuring rates of exchange of ^{18}O between CO_2 and water using membrane inlet mass spectrometry. The capacity of these variants to catalyze the hydrolysis of 4-nitrophenyl acetate was also determined. Values of $R_1/[E]$ were obtained for each mutant over the pH range from 5.0 to 9.0 and Eq. 5 was used to determine the catalytic constants $k_{\text{cat}}^{\text{ex}}/K_{\text{eff}}^{\text{CO}_2}$ for hydration of CO_2 (Figure 4). Fits to a single ionization appear satisfactory, a characteristic of carbonic anhydrases reflecting the pK_a of the zinc-bound water. The data at $\text{pH} < 6$ showed decreased $k_{\text{cat}}^{\text{ex}}/K_{\text{eff}}^{\text{CO}_2}$ values especially for W5E HCA II which may be due to the influence of a second ionizable group (Figure 4). The maximal pH independent values of $k_{\text{cat}}^{\text{ex}}/K_{\text{eff}}^{\text{CO}_2}$ were similar to wild type (Table 2) and the resulting pK_a ZnH_2O for the zinc-bound water,

determined from Figure 4, were all close to 7.0, similar to that for wild-type HCA II (Table 3). The values determined from the esterase activity of these enzymes also showed pK_a ZnH_2O near 7.0 (Table 3) with maximal values of k_{cat}/K_m somewhat decreased compared with wild type (Table 2). The similarity of these values between the variants and wild type reflects the significant distance (more than 10 Å) between residue 5 and the catalytic zinc.

The ^{18}O exchange method allows the mutants to be examined in solutions without added buffer yielding a quantitative measure of rates of intermolecular proton transfer. The rate constant $R_{H_2O}/[E]$ measures the second stage of catalysis (Eq. 4) wherein rate-limiting intermolecular proton transfer releases $H_2^{18}O$ from the active site. The pH profiles of $R_{H_2O}/[E]$ for each mutant were predominantly bell shaped (Figure 5), reflecting the proton transfer from the imidazolium side chain of His64 to the zinc-bound hydroxide. Each of the variants at position 5 between pH 8 and 9 showed a region of smaller pH dependence in $R_{H_2O}/[E]$ compared with what was observed in the wild type (Figure 5). This behavior has been observed in other variants of HCA II [24] and has not been adequately explained.

In order to interpret the bell-shaped data correctly, proper assignment of the pK_a values for the proton donor (His64) and acceptor (ZnH_2O) groups is necessary. In previous reports using this method [14] this was done by using values of pK_a ZnH_2O from first stage of the catalysis ($k_{cat}^{ex}/K_{eff}^{CO_2}$ in Figure 4) and from the ester hydrolysis to assign the pK_a values in the pH profiles of $R_{H_2O}/[E]$. Then the bell-shaped curves were fit according to Eq. 6 to yield a rate constant for proton transfer k_B and estimates of two values of pK_a (pK_a ZnH_2O and pK_a His64). The values of the two pK_a 's from $R_{H_2O}/[E]$ were similar in magnitude, near 6 (columns 2 and 3 of Table 3), so the assignment was not crucial and gave values of the rate constant for proton transfer k_B near that of wild type, about $1 \mu s^{-1}$ (Table 2). However, the values of pK_a ZnH_2O determined from R_{H_2O} (Eq. 6) were about a unit lower than the values of pK_a ZnH_2O found from $k_{cat}^{ex}/K_{eff}^{CO_2}$ and from the esterase activity (Table 3).

There was no evidence from the data that His5 participates in the proton transfers of the second stage of catalysis. The rate constant k_B for W5H HCA II was near that of wild type (Table 2). This may in part reflect the outward orientation of His5 with respect to the active site (Figures 2, 3).

DISCUSSION

The highly conserved Trp5 in the α class of carbonic anhydrases is located near the opening of the active-site cavity and extends into the cavity. That it is prevalent in many forms of carbonic anhydrases in the α class and appears at an approximately similar location in a carbonic anhydrase of the γ class [25] suggests a possible catalytic or structural function. We have observed that replacement of Trp5 in HCA II caused significant structural changes in the enzyme. The replacements introduce instability in the N-terminal eleven residues. The electron density becomes qualitatively weaker than wild-type HCA II in the order W5F > W5H > W5E, in which W5E is the weakest. It appears that Trp5 and Phe 5 provide a means of stabilizing these eleven residues, perhaps acting as an anchor to shift the N-terminus into the position observed in the wild-type structure. This is supported by the environment of Trp5 the indole ring of which is located in a hydrophobic environment (Trp16, Phe20, Pro201).

The crystal structures of wild type and W5F HCA II showed the superposition of both outward and inward orientations of His64 while the two variants W5H and W5E had His64 predominantly outward in orientation (Figures 2, 3). The conservative replacement W5F, likely because of the π stacking with the imidazole of His64, is able to retain the balance of

inward and outward orientations of His64. However, in W5H and W5E HCA II the conformational balance no longer holds and the side chain of His 64 exists predominately in the outward conformation.

Although Trp5 influences the orientation of the proton shuttle His64, this orientation had no significant effect on k_B the rate constant for proton transfer with a maximal value near $1 \mu s^{-1}$ in the variants examined here (Table 2). This is consistent with quantum mechanical/molecular mechanical simulations showing that polar and charged residues along the proton transfer pathway play a more significant role in the barrier to proton transfer than the number of intervening water molecules [13]. Experimental evidence suggested that the inward and outward orientations of His64 as observed in the crystal structures are not associated with significant changes in the magnitude of the rate constant for proton transfer during catalysis, as discussed in a recent review [12]. Moreover, the large distance of residue 5 from the zinc ($> 10 \text{ \AA}$) is consistent with little effect of mutations at residue 5 on the interconversion of CO_2 and bicarbonate as measured by $k_{cat}^{ex}/K_{eff}^{CO_2}$ (Table 2). There is a significant association of an ordered water structure in the active-site cavity with rapid proton transfer in catalysis [12, 14, 26], and the water structures in the variants examined here are very similar to that of wild type (Figures 2, 3).

However, there is a change in the pH maximum of the proton transfer process as shown in Figure 5. The pH at which the maxima occur is near 6 for the variants studied here and not the more physiological value of 7 for wild type (Figure 5). The shift to pH 6 results from the shifts in pK_a of the zinc-bound water and His64 obtained from the bell-shaped curves of $R_{H_2O}/[E]$, as in Figure 5. The apparent values of $pK_{a \text{ His64}}$ near 6.0 observed here are lower by one pK unit compared with wild type (Table 3). We had previously observed such lowered values of $pK_{a \text{ His64}}$ in variants of HCA II with His64 in the inward orientation, as in N62L HCA II [12, 27], a result that was also found in the multistate empirical valence bond computations of Maupin et al. [11]. Variants of HCA II in which His64 is oriented outward, as in N67L, had $pK_{a \text{ ZnH}_2O}$ somewhat higher, 7.5 to 8.4 [27]. The variants W5H and W5E are the first observation of a substantially lowered value of $pK_{a \text{ His64}}$ with His64 oriented predominantly outward. Perhaps in these two mutants the outward orientation of His64 allows interactions (including crystal packing interactions) to stabilize the uncharged imidazole side chain of His64, although we cannot identify these from the crystal structure.

Also significant are the values of $pK_{a \text{ ZnH}_2O}$ near 6 obtained from $R_{H_2O}/[E]$ for the mutants (Table 3, column 3) that are lower by about one pK unit than the values obtained from $k_{cat}^{ex}/K_{eff}^{CO_2}$ and the esterase data (Table 3). They indicate that the apparent $pK_{a \text{ ZnH}_2O}$ near 6 reflecting the proton transfer stage of catalysis (Table 3, column 3) is lower than the apparent $pK_{a \text{ ZnH}_2O}$ near 7 in the catalysis of the interconversion of CO_2 and bicarbonate (Table 3, column 4). Hence, these data suggest different conformations in the active site of the mutants for the two stages of catalysis, Eqs. 1 and 2. This could also indicate that although His64 is outward in the crystal structure and in catalysis of stage 1 (Eq. 1), in the transition state of the proton transfer (Eq. 2) it is inward.

Acknowledgments

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Trp5 stabilizes the amino-terminal end of human carbonic anhydrase II
Trp5 affects the orientation of the proton shuttle residue His64
Replacement of Trp5 altered the pH maximum for activity to a non-physiological value

Figure 1. N-terminus of HCA II. The wild-type structure depicting Trp 5 through Tyr 7 and the proton shuttle residue His 64. Coordinates taken from PDB ID: 2ILI [8]. Figure made using PyMOL (DeLano Scientific).

Figure 2.

Stereo figure of the active site of HCA II and variants. Wild-type structure of residues in the active-site cavity of HCA II (yellow) superimposed on the structures of W5H (cyan); W5E (green); and W5F (magenta). The wild type structure is from PDB ID: 2ILI [8]. The active-site zinc is depicted as a black sphere, and solvent molecules are depicted as spheres in the color of the respective variants. The side chain of Glu5 in W5E HCA II is not shown because it is indistinct and apparently disordered in the electron density map. Figure made using PyMOL (DeLano Scientific).

Figure 3.

Conformations of the proton shuttle residue His 64. Wild-type structure of HCA II (yellow) superimposed on the structures of W5H (cyan); W5E (green); and W5F (magenta). The active-site zinc is depicted as a black sphere, and solvent molecules are depicted as spheres in the color of the respective variants. Red lines represent hydrogen bonds. Amino acids are as labeled. Figure made using PyMOL (DeLano Scientific).

Figure 4.

The pH profiles for $k_{\text{cat}}^{\text{ex}}/K_{\text{eff}}^{\text{CO}_2}$ ($\text{M}^{-1}\text{s}^{-1}$) for the hydration of CO_2 catalyzed by (◆) wild-type HCA II; (○) W5E HCA II; (△) W5H HCA II; (■) W5R HCA II; and (□) W5F HCA II. Data were obtained by ^{18}O exchange between CO_2 and water using solutions at 25°C containing 25 mM of all species of CO_2 and sufficient Na_2SO_4 to maintain 0.2 M ionic strength. The solid lines are a fit to a single ionization.

Figure 5.

The pH profiles for $R_{H_2O}/[E]$ (s^{-1}) the proton-transfer dependent rate of release of ^{18}O -labeled water catalyzed by (◆) wild-type HCA II; (○) W5E HCA II; (△) W5H HCA II; (■) W5R HCA II; and (□) W5F HCA II. Conditions were as described in Figure 3. The solid lines are a fit of Eq. 6 to the bell-shaped components of these data.

Table 1

Data processing and refinement statistics for the structures of the W5 mutants.

Parameter	W5E	W5F	W5H
PDB code	3RG3	3RG4	3RGE
Space group	$P 2_1$	$P 2_1$	$P 2_1$
Cell dimensions a, b, c (Å)	42.7, 41.5, 72.7	42.7, 41.50, 72.7	42.7, 41.5, 72.6
Cell angles α , β , γ (°)	90.0, 104.4, 90.0	90.0, 104.4, 90.0	90.0, 104.3, 90.0
Resolution (Å)	20–1.9 (1.97–1.9) ^a	20–1.5 (1.58–1.5)	50–2.1 (2.18–2.1)
R_{sym} ^b (%)	9.5 (29.8)	8.6 (25.3)	10.0 (12.1)
$I/(\sigma)I$	9.7 (3.9)	17.7 (3.2)	13.0 (4.7)
Completeness (%)	99.5 (99.6)	89.9 (84.8)	87.4 (90.9)
Average Redundancy	2.6 (2.6)	2.1 (2.0)	2.6 (2.4)
R_{cryst} ^c / R_{free} ^d (%)	13.3/17.0	13.6/16.6	14.1/18.8
No. atoms – Protein	2070	2112	2048
Water	149	202	131
B-factors (Å ²)			
Protein (main/side)	16.7/23.2	16.3/22.9	16.2/23.3
Water	32.6	34.6	31.2
RMSD (bond (Å)/angle(°))	0.011/1.3	0.010/1.3	0.003/0.8
Ramachandran plot (%)			
Allowed	88.4	89.4	88.4
Additionally allowed	11.2	11.2	11.2
Generously allowed	0.5	0.5	0.5

^aValues in parentheses are for the highest resolution shell.^b $R_{\text{sym}} = (\Sigma |I - \langle I \rangle| / \Sigma \langle I \rangle) \times 100$.^c $R_{\text{cryst}} = (\Sigma ||F_o| - |F_c|| / \Sigma |F_o|) \times 100$.^d R_{free} is calculated the same as R_{cryst} , except with 5% of the data omitted from refinement.

Table 2

Maximal Values of Rate Constants for the Hydration of CO₂ and Hydrolysis of 4-Nitrophenylacetate Catalyzed by HCA II and Variants

Enzyme	$k_{\text{cat}}^{\text{exch}}/K_{\text{eff}}^{\text{CO}_2}$ CO ₂ hydration ($\mu\text{M}^{-1}\text{s}^{-1}$)	$k_{\text{cat}}/K_{\text{m}}$ esterase ($\text{M}^{-1}\text{s}^{-1}$)	k_{B} proton transfer (μs^{-1})
Wild Type ^a	120	2800	0.8
W5H	120 ± 10	1900 ± 200	0.42 ± 0.05
W5R	150 ± 20	1800 ± 100	0.9 ± 0.3
W5F	90 ± 10	1700 ± 100	1.2 ± 0.3
W5E	80 ± 10	1500 ± 100	0.8 ± 0.1

^aFrom [14]

Table 3Values of Apparent pK_a obtained by Various Kinetic Measurements of Catalysis by HCA II and Mutants

Enzyme	$pK_{a \text{ His64}}^a$ (Eq. 6)	$pK_{a \text{ ZnH}_2\text{O}}^a$ (Eq. 6)	$pK_{a \text{ ZnH}_2\text{O}}^b$ ($k_{\text{cat}}^{\text{exch}}/K_{\text{eff}}^{\text{CO}_2}$)	$pK_{a \text{ ZnH}_2\text{O}}$ (esterase)
Wild type ^c	7.2	6.8	6.9	6.9
W5H	6.3	5.9	7.3	6.7
W5R	6.4	6.0	7.0	7.0
W5F	6.4	6.4	7.0	6.6
W5E	6.4	6.5	7.0	7.1

^aThe pK_a was derived from the fits of Eq. 6. Standard errors were mostly ± 0.1 and no greater than ± 0.2 .

^bThe pK_a was determined from a fit of $k_{\text{cat}}^{\text{exch}}/K_{\text{eff}}^{\text{CO}_2}$ determined from Eq. 5. Minor deviations from the fit to a single ionization that could be interpreted as a second ionization are not included. Standard errors were mostly ± 0.1 and no greater than ± 0.2 .

^cFrom [14].