Unexpected pH-Dependent Conformation of His-64, the Proton Shuttle of Carbonic Anhydrase II

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Abstract: The pH-dependent structural variations of human carbonic anhydrase II (CAII) have been studied by X-ray crystallographic methods at 2.3 Å resolution. The overall structure of CAII at pH 6.5 or 5.7 is quite similar to that determined at pH 8.5 (Eriksson, A. E.; Jones, T. A.; Liljas, A. *Proteins: Struct. Funct. Gen.* **1988**, *4*, 274-282). However, an important structural change is observed for His-64, the catalytic proton shuttle, at pH 5.7: its side chain rotates away from the active site by 64° about χ_1. This alternate conformation of His-64 is interpretable with low occupancy in enzyme structures at higher pH values, although in some cases the corresponding electron density may be interpretable as a partially ordered solvent molecule. Given the unexpected mobility revealed for His-64, it is intriguing that significant conformational changes may accompany the function of the proton shuttle in catalysis.

The zinc metalloenzyme human carbonic anhydrase II (CAII) catalyzes the hydration of carbon dioxide to yield bicarbonate ion plus a proton. The chemistry of catalysis occurs via two distinct processes: (1) the nucleophilic attack of zinc-bound hydroxide at substrate CO_2, which generates the product bicarbonate ion; and (2) the regeneration of zinc-bound hydroxide from zinc-bound water following bicarbonate release.[1] Given that $k_{cat}/K_M = 1.5 \times 10^8$ M^{-1} s^{-1} for CO_2 hydration, CAII is unique among known carbonic anhydrase isozymes in that the kinetics of hydrase activity are comparable to those of a diffusion control reaction. However, the measured turnover rate of 10^6 s^{-1} requires buffer to accept the product proton—in the absence of buffer, the upper limit for proton transfer from an enzyme-bound group with $pK_a = 7$ to bulk solvent is 10^3 s^{-1}.[2]

The structure of pH 8.5 CAII from human blood has been determined by X-ray crystallographic methods[3] and refined at 2.0 Å resolution.[4] The enzyme is roughly spherical and the active site lies within a conical cleft about 15 Å deep; the zinc ion lies at the bottom of this cleft and is tetrahedrally liganded by His-94, His-96, His-119, and hydroxide ion at physiological pH. Zinc-bound hydroxide donates a hydrogen bond to the hydroxyl group of Thr-199, which in turn donates a hydrogen bond to Glu-106. This hydrogen bond network orients nucleophilic zinc-bound hydroxide for optimal attack at the substrate.[5] A second hydrogen bond network engages zinc-bound hydroxide and the imidazole side chain of His-64 through two intervening solvent molecules,[4] and this network is likewise important for catalysis. It is believed that His-64 is a proton shuttle in catalysis, where it accepts the proton product (via the bridging solvent molecules) from zinc-bound water as zinc-bound hydroxide is regenerated; subsequently, the proton product is passed along to buffer.[6]

Table I. Data Collection and Refinement Statistics for CAII at low pH

	pH 6.5	pH 5.7
no. of crystals	1	1
no. of measured reflcns	12652	17568
no. of unique reflcns	6604	7192
max resolution (Å)	2.3	2.3
$R_m(F)$[a]	0.072	0.079
no. of water molecules in final cycle of refinement	68	112
no. of reflcns used in refinement (6.5-2.3 Å)	6062	6782
R factor[b]	0.164	0.168
rms deviation from ideal bond lengths (Å)	0.010	0.014
rms deviation from ideal bond angles (deg)	1.5	1.2
rms deviation from ideal planarity (Å)	0.008	0.008
rms deviation from ideal chirality (Å^3)	0.074	0.075

[a] R_{merge} for replicate reflections, $R = \sum|\|F_{hi}| - \langle|F_h|\rangle|/\sum\langle|F_h|\rangle$; $|F_{hi}|$ = scaled structure factor for reflection h in data set i. $\langle|F_h|\rangle$ = average structure factor for reflection h 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.

Interestingly, conformational mobility for the His-64 side chain about χ_2 (i.e., ring flipping) is proposed from the analysis of its surrounding solvent structure at pH 8.5; such mobility may accompany the role of His-64 as a proton shuttle.[4] In the current study, we demonstrate that His-64 is more conformationally mobile than previously ascertained: mobility about χ_1 is interpretable in electron density maps of CAII crystals equilibrated in buffer solutions at pH 5.7 and 6.5. These pH-dependent crystallographic studies, along with studies of recombinant wild-type and Thr-200→Ser CAII,[7] illuminate a conformational reaction coordinate for the χ_1 mobility of His-64 which may accompany catalysis.

Experimental Section

Lyophilized CAII was purchased from Sigma and used without further purification. Enzyme crystallizations were performed by the sitting-drop method. Typically, a 10-μL drop containing 0.3 mM enzyme, 50 mM Tris-HCl (pH 8.0 at room temperature), 150 mM NaCl, and 3 mM NaN_3 was added to a 10-μL drop containing 50 mM Tris-HCl (pH 8.0 at room temperature), 150 mM NaCl, and 3 mM NaN_3 with 1.75-2.5 M ammonium sulfate in the crystallization well. Both solutions were saturated with methyl mercury acetate and β-glucopyranoside in order to facilitate the growth of diffraction-quality parallelepipedons.[8] Crystals of typical dimensions 0.2 mm × 0.2 mm × 0.8 mm appeared within 2 weeks at 4 °C. CAII crystallized in space group $P2_1$ and exhibited typical unit cell parameters of $a = 42.7$ Å, $b = 41.7$ Å, $c = 73.0$ Å, and $\beta = 104.6°$.

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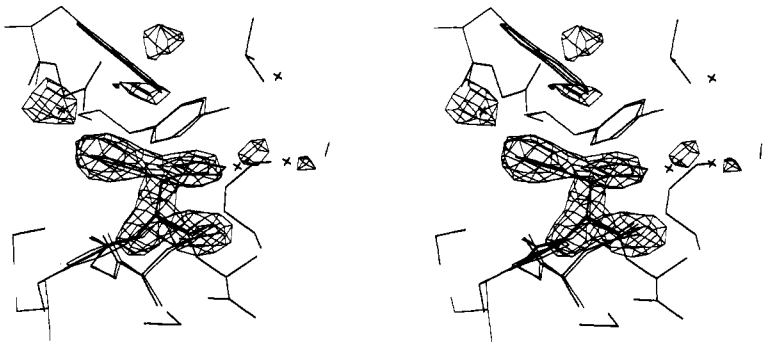

Figure 1. Difference electron density map of pH 6.5 CAII calculated with Fourier coefficients $|F_o| - |F_c|$ and phases derived from the final model less the atomic coordinates of His-64 and active site solvent molecules. The map is contoured at 2.7σ, and refined atomic coordinates of pH 6.5 CAII are superimposed (thick bonds); for comparison, the refined coordinates of pH 8.5 CAII are also superimposed (thin bonds).[4] Note the significant population of "in" and "out" conformers of His-64 (both with thick bonds).

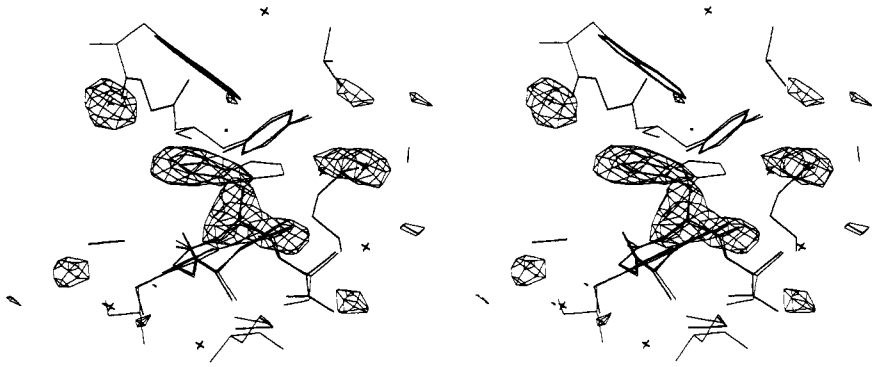

Figure 2. Difference electron density map of pH 5.7 CAII calculated with Fourier coefficients $|F_o| - |F_c|$ and phases derived from the final model less the atomic coordinates of His-64 and active site solvent molecules. The map is contoured at 2.7σ, and refined atomic coordinates of pH 5.7 CAII are superimposed (thick bonds). For comparison, the refined coordinates of pH 8.5 CAII are also superimposed (thin bonds);[4] His-64 predominantly occupies the "out" conformation at pH 5.7. Weak electron density, potentially interpretable as the "in" conformer of His-64, appears when this map is contoured at 1.7σ.

In order to obtain diffraction-quality crystals at low pH values, the pH of the solution in which the crystals were immersed had to be decreased gradually over a period of about 5 weeks. The direct transfer of crystals (in 50 mM Tris buffer, pH 8.0) to buffer solutions at pH values lower than 6.5 resulted in crystal cracking. However, allowing the pH to change by vapor diffusion[9] of the crystal buffer solution with a buffer solution at lower pH (50 mM *N*-(2-acetamido)iminodiacetic acid, pH 6.5; 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.7) maintained diffraction-quality crystals. The pH values of sitting drops containing pH-equilibrated crystals were confirmed to be 5.7 ± 0.2 and 6.5 ± 0.2 by testing with pH paper (ColorpHast, EM Science).

The pH-equilibrated crystals were mounted and sealed in 0.5-mm glass capillaries with a small portion of mother liquor. A Siemens X-100A multiwire area detector, mounted on a three-axis camera and equipped with Charles Supper double X-ray focusing mirrors, was used for X-ray data acquisition. A Rigaku RU-200 rotating anode X-ray generator operating at 45 kV/65 mA supplied Cu Kα radiation. All data were collected at room temperature by the oscillation method; the crystal-to-detector distance was set at 14 cm, and the detector swing angle was fixed between 20 and 27° in at least 4 runs per experiment. Data frames of 0.08° oscillation about ω were collected, with exposure times of 60 s/frame, for total angular rotation ranges about ω of at least 70° per run. Multiple data sets were collected, and diffraction intensities were measured to a limiting resolution of 2.3 Å for both pH 5.7 and 6.5 CAIIs. Raw data frames were analyzed using the BUDDHA package,[10] and replicate and symmetry-related structure factors were merged using PROTEIN;[11] relevant data reduction statistics are recorded in Table I.

In the analysis of CAII crystal structures at pH 5.7 and 6.5, structure factors obtained from the corrected intensity data were used to generate difference electron density maps using Fourier coefficients $(2|F_o| - |F_c|)$ and $(|F_o| - |F_c|)$ with phases calculated from the structure of refined human CAII at pH 8.5;[4] atomic coordinates were retrieved from the Brookhaven Protein Data Bank.[12] Fast Fourier transform routines were employed in all electron density map and structure factor calculations.[13] Inspection of the electron density maps revealed that only minor adjustments to the protein model were required. It was at this stage that the pH-dependent conformational change of His-64 was noticed, and this observation was confirmed by inspection of maps for which His-64 was omitted from the structure factor calculation.

Model building was performed with the graphics software FRODO[14] installed on an Evans and Sutherland PS390 interfaced with a VAXstation 3500. Atomic coordinates were refined against the observed data by the reciprocal space least-squares method using the stereochemically restrained least-squares algorithm of Hendrickson and Konnert.[15] Neither active site water molecules nor the atoms of His-64 were included in the initial stages of refinement. Residue conformations throughout the protein were examined during the course of refinement by using maps calculated with Fourier coefficients outlined above and phases derived from the in-progress atomic model. Only minimal adjustments of atomic positions were necessary, and active site water molecules and the coordinates of His-64 were added when the crystallographic *R* factor dropped below 0.19. In pH 6.5 CAII, coordinates for two partially-occupied conformers of His-64 were added. Refinements converged smoothly to final crystallographic *R* factors of 0.168 and 0.164 for pH 5.7 and 6.5 CAIIs. Pertinent refinement statistics are recorded in Table I. The coordinates of pH 5.7 and 6.5 CAIIs have been deposited in the Brookhaven Protein Data Bank.[12]

Results and Discussion

The three-dimensional structure of CAII at pH 5.7 or 6.5 is generally similar to that reported by Eriksson and colleagues at

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pH 8.5.[4] Least-squares superposition of Cα coordinates (using the software INSIGHT (Biosym, Inc.)) yields a root-mean-square (rms) difference of 0.2 Å between pH 5.7 and 8.5 CAIIs, and 0.2 Å between pH 6.5 and 8.5 CAIIs. Given the rms coordinate error of ca. 0.2 Å calculated for pH 5.7 and 6.5 CAIIs from relationships derived by Luzzati,[16] it appears that the overall CAII structure does not exhibit major pH-dependent variations within the pH range 5.7–8.5. However, an important structural difference is evident for His-64, the catalytic proton shuttle: the imidazole side chain of this residue appears to increasingly occupy an alternate conformation as the pH is decreased from 8.5 to 6.5 (Figure 1), and this alternate conformation predominates at pH 5.7 (Figure 2). The alternate conformer predominant at pH 5.7 is related to the conformer observed[4] at pH 8.5 by a 64° rotation about side chain torsion angle χ_1. We designate the alternate conformation of His-64 as the "out" conformation, since the imidazole side chain is directed away from the active site. When His-64 is in the "out" conformation, it apparently displaces a water molecule interpreted to be hydrogen bonded to an imidazole nitrogen at pH 8.5.[4] Since the imidazole side chain of His-64 is directed toward the active site at pH 8.5,[4] we designate that as the "in" conformation.

The "out" conformation of His-64 does not result in any hydrogen bond contacts between the imidazole side chain and enzyme or solvent atoms as judged from distance and stereochemical criteria.[17] Indeed, the "out" conformation places the imidazole group closer to a more hydrophobic region largely defined by Trp-5, Gly-6, Tyr-7, and Phe-231. A similar conformational change for His-64 is observed in the crystal structure of the Thr-200→Ser mutant of CAII at pH 8.0, where His-64 rotates about χ_1 by 105° (relative to its conformation in the recombinant wild-type enzyme) to a further "out" conformation; importantly, this CAII mutant exhibits normal CO_2 hydrase activity and proton transfer kinetics.[7] Therefore, different conformations for His-64 do not hinder efficient proton transfer in catalysis, as long as solvent structure satisfactorily accommodates different conformations. Likewise, the mobility of the proton shuttle among different conformations need not hinder catalysis, assuming that compensatory changes in solvent structure are sufficiently dynamic to support proton transfer. Given that imidazole buffer compensates for the deleted His-64 side chain in the proton transfer step of His-64→Ala CAII[6c] (imidazole in solution would correspond to an infinitely mobile histidine side chain), it is conceivable that efficient proton transfer in wild-type CAII tolerates the comparatively minor χ_1 and χ_2 mobility of His-64 in its function as a proton shuttle.

At higher pH values, electron density corresponding to the "out" conformer of His-64 may sometimes be interpretable as solvent.[4,7] Eriksson and colleagues[4] interpreted and refined this density as a water molecule in pH 8.5 CAII at 2.0 Å resolution, and the relatively high thermal *B* factor of 29 Å^2 indicates some disorder. In recombinant wild-type CAII at 2.1 Å resolution, the corresponding electron density is ambiguous and is interpretable as either a disordered solvent molecule or a low-occupancy "out" conformer of His-64.[7] This ambiguity is reminiscent of that encountered for His-119 in ribonuclease,[18] where theoretical and recent crystallographic studies suggest that a 2-conformer interpretation is correct.[19] We similarly favor a 2-conformer interpretation for His-64 in CAII, and we suggest that the pH-dependent crystallographic study of protein structures could reveal similar conformational ambiguities in other systems. Furthermore, such analysis may be applied toward the design of tight-binding protein ligands. For example, His-64 of CAII rotates to the "out" conformation in order to accommodate the binding of thienothiopyran-2-sulfonamides in the enzyme active site.[20]

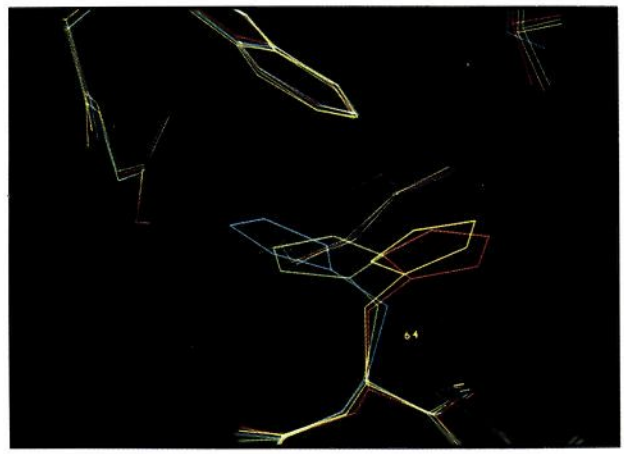

Figure 3. Superposition of residue His-64 from various crystallographic structure determinations of CAII, illustrating the reaction coordinate of conformational mobility; this reaction coordinate may characterize the role of His-64 as a proton shuttle (cyan, Thr-200→Ser CAII at pH 8.0;[7] green, native blood CAII at pH 5.7 (this study); yellow, recombinant wild-type CAII at pH 8.0;[7] red, native blood CAII at pH 8.5[4]). We define the "in" conformation as that observed for native blood (pH 8.5) and recombinant wild-type CAIIs and the "out" conformation as that observed for native blood (pH 5.7) and Thr-200→Ser CAIIs.

Table II. Zinc–Ligand Distances for CAII at pH 5.7, 6.5, and 8.5

	pH 5.7	pH 6.5	pH 8.5[4]
His-94 (Nε2)	2.3	2.5	2.0
His-96 (Nε2)	2.1	2.3	2.1
His-119 (Nδ1)	2.3	2.5	1.9
solvent	2.7	2.6	2.1

It is difficult to establish the molecular factors that are responsible for the predominance of "in" or "out" conformers of His-64. The imidazole group of His-64 must be positively charged at pH 5.7 since its pK_a is measured at 7.1.[21] However, it is difficult to conclude that the conformational change at low pH results from the protonation of the side chain, since the "out" conformer predominates at pH 8.0 in Thr-200→Ser CAII.[7] Interestingly, the conformational change of His-64 is only 64° at pH 5.7, whereas it is measured at 105° in Thr-200→Ser CAII; perhaps the conformational change at low pH is smaller since the greater the χ_1 rotation to the "out" conformation, the closer the positively charged imidazolium group would approach the nearby hydrophobic patch. The positively charged imidazolium group of His-64 could rotate away from the active site in order to minimize its electrostatic repulsion from the positively charged zinc coordination polyhedron (zinc-bound water has a pK_a of ca. 7,[22] so it would not be appreciably ionized at pH 5.7). Again, this explanation does not easily rationalize the trigger for the conformational change observed in Thr-200→Ser CAII. A general conclusion may be that the conformation of His-64 is sensitive to changes in solvent structure which, in turn, arise from changes in protein structure, counterion concentration, or pH.

If the mobility of His-64 arises from changes in solvent structure (here, the term "solvent" should be broadly interpreted to include

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water, hydronium ion, and counterions present in the crystallization medium), it is possible that the conformation of His-64 is responsive to the non-protein zinc ligand. Significant pH-dependent differences in the coordination of the non-protein ligand to zinc are observed, and zinc–ligand distances range from 2.1 to 2.7 Å (Table II; the metal coordination polyhedron remains tetrahedral at low pH, in contrast with that of human isozyme I[23]). However, these differences probably arise from a crystallographic ambiguity. Although the non-protein zinc ligand is modeled exclusively as a water molecule, it is conceivable that this ligand is an equilibrium mixture of different solvent species, including chloride and azide (present in the crystallization buffer at concentrations approaching their respective K_d values[24]). However, triatomic N_3^- will not fit into the egg-shaped peak of electron density corresponding to the non-protein zinc ligand. Since we find evidence for the "out" conformation of His-64 in crystals of CAII grown in the absence of azide and chloride,[25] we conclude that the equilibrium composition of the non-protein zinc ligand does not exclusively direct His-64 to the "in" or "out" conformations.

Elsewhere in the enzyme active site, solvent structure interpreted as water is generally similar among CAII structures determined at pH values 5.7, 6.5, and 8.5—refined solvent positions generally correlate to within 0.5–1.0 Å among the three structures. Strong electron density is not observed in pH 5.7 or 6.5 CAIIs for the so-called "deep" water molecule which is found at the mouth of the hydrophobic pocket in pH 8.5 CAII.[4] However, electron density corresponding to this water begins to appear when difference maps are contoured at the relatively low level of 1.7σ. Interestingly, this is a feature common to some, but not all, mutant CAIIs.[26] The "deep" water molecule hydrogen bonds to zinc-bound hydroxide and is presumably displaced by substrate.[1] Additionally, significant electron density corresponding to one of the two solvent molecules bridging zinc-bound hydroxide and His-64 at pH 8.5 is not apparent at low pH values; weak density corresponding to this water molecule becomes evident as difference electron density maps are contoured at the low level of 1.7σ.

Conclusions

Although it is difficult to pinpoint the molecular causes leading to the predominance of a particular conformation for His-64 of CAII, mobility for this catalytic proton shuttle is demonstrated in X-ray crystallographic studies (Figure 3). Despite the particular conformer of His-64—"in", "out", or somewhere else along the reaction coordinate of isomerization—it is likely that efficient proton transfer is accommodated.[7] The mobility of the proton shuttle need not compromise catalysis, as long as solvent structure endures dynamic and compensatory changes which maintain efficient proton transfer pathways.

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Registry No. His, 71-00-1; Zn, 7440-66-6; CAII, 9001-03-0.

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