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Neutron Structure of Human Carbonic Anhydrase II: A Hydrogen-Bonded Water Network "Switch" Is Observed between pH 7.8 and 10.0

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Supporting Information

ABSTRACT: The neutron structure of wild-type human carbonic anhydrase II at pH 7.8 has been determined to 2.0 Å resolution. Detailed analysis and comparison to the previously determined structure at pH 10.0 show important differences in the protonation of key catalytic residues in the active site as well as a rearrangement of the H-bonded water network. For the first time, a completed H-bonded network stretching from the Zn-bound solvent to the proton shuttling residue, His64, has been directly observed.

Human carbonic anhydrase II (HCA II) is a 29 kDa monomeric Zn-metalloenzyme that catalyzes the reversible hydration of CO_2 into HCO_3^- and a proton and is found prominently in red blood cells and secretory tissues. HCA II is one of the fastest known enzymes with a k_{cat} for CO_2 hydration of 10^6 s^{-1}, and its activity is close to being diffusion-limited.[1] HCA II serves as a relatively simple model enzyme for the study of long-range proton transfer mechanisms in more complex systems, such as ATP synthase, bacteriorhodopsin, and cytochrome *c* oxidase. There is also increasing industrial interest in using engineered carbonic anhydrase for extraction of carbon dioxide from flue gas in coal-fired power plants.[2] Analyses of HCA II neutron structures provide unique data and insights into proton transfer mechanisms and could have broad applicability to many other systems.

All CAs identified to date use a metal hydroxide mechanism to mediate the two-step ping-pong catalytic reaction.[3] The first step of catalysis by HCA II is a nucleophilic attack on incoming CO_2 by a Zn-bound OH^- to produce HCO_3^-. The binding of HCO_3^- at the metal is weak, and accordingly, the product is easily displaced by a water molecule. The second step activates the metal-bound water to OH^- through a series of proton transfers.[1,4] This is thought to occur through a network of well-ordered H-bonded waters.[4,5] This network stretches from the metal to the proton shuttling residue, His64, and is H-bonded to several hydrophilic residues that line the active site (Figure S1 of the Supporting Information).[6,7] In structural studies, residue His64 has been observed to occupy two distinct positions, termed the inward and outward conformations, pointing toward and away from the Zn active site, respectively.[8,9] Exactly how protons move in the active site of proteins is not clear, but it has been proposed that a Grotthus proton hopping mechanism or the formation of a series of Zundel ($H_5O_2^+$) or Eigen cations (H_9O4^+) may be a factor.[10] It has been postulated that the inward conformation of His64 might be poised to accept the excess proton from the water network, while the outward conformation is ready for proton shuttling to the bulk solvent.[8,11] This observed flexibility of His64 is most likely related to its protonation state.[7,12] The enzyme displays very strong pH dependence for both k_{cat} and k_{cat}/K_M that are defined by a single ionization corresponding to a pK_a of ∼7.[1]

The details of CO_2 binding and subsequent hydration became clearer once the structure of CO_2 at the active site revealed a side-on binding mode.[13] Currently, neutron crystallography studies on HCA II have focused on the structural details of the active site hydrophilic residues and water molecules involved in proton transfer. As such, the analysis of the neutron structure at high pH (∼10) has been previously reported.[14]

The approach taken in this study was to change the pH of the crystal as a way to modulate the changes in protonation that occur during catalysis. Discussed here is the pH 7.8 crystal structure that is compared to the previously determined pH 10.0 form. The advantage of neutron crystallography is that H and its isotope D diffract neutrons very strongly and to approximately the same extent as the heavier atoms found in proteins such as N, C, or O (scattering lengths, −3.74 fm for H, 6.67 fm for D, 9.4 fm for N, 6.6 fm for C, and 5.8 fm for O). Accordingly, H and D atoms appear as negative and positive peaks, respectively, in nuclear scattering density maps. In this study, the crystal was subjected to H−D vapor exchange, to replace labile H with D, thus promoting strong positive scattering from D atoms. The benefit of using vapor exchange instead of soaking is that damage to the large crystals required

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for neutron studies is less likely. Using neutron diffraction on samples that have been subjected to H−D exchange, it is possible, even at medium (2.0−2.2 Å) resolution, to directly observe the protonation states of amino acid residues (e.g., neutral vs charged histidine residues) and to distinguish among D_3O^+, D_2O, and OD^-.[15,16] The room-temperature neutron structures were complemented with X-ray data from a similar crystal prepared and subjected to H−D exchange in the same manner. Using the joint neutron and X-ray methodology for model refinement leads to more accurate structures in that X-rays provide highly accurate information for the heavier atoms in proteins while neutrons are used to refine the positions of H and D atoms, effectively increasing the data-to-parameter ratio for refinement. In this way, the data are highly complementary and allow a detailed understanding of hydration and H-bonding in proteins.[17]

Careful analyses of omit and difference nuclear density maps revealed several interesting features in the pH 7.8 structure that are different from those at high pH. The most interesting one, from a proton transfer point of view, is that the water network has rearranged in response to the reduced pH and forms a completed H-bonded network, not observed before, between the Zn-bound solvent (ZW) and W2 (Figure 1).

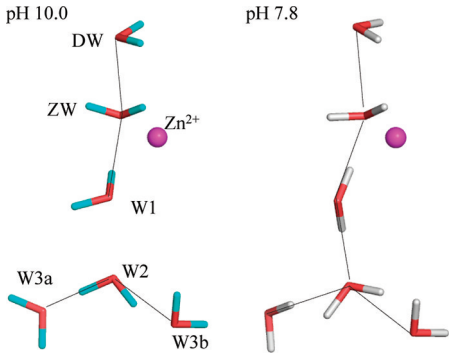

Figure 1. Comparison stick diagram of the water networks in the active site of HCA II determined by neutron crystallography at pH 10 (left) and 7.8 (right). Zn is included for reference, and waters are labeled. Observed hydrogen bonds are shown as black lines, and active site amino acid residues have been omitted for the sake of clarity.

At high pH, W1 was acting as an H-bond donor to the Zn-bound water (ZW) and Thr200 (left panel in Figure 1 and Figure S1 of the Supporting Information). In this configuration, there is a break in the H-bonded water network between W1 and W2. This can, in part, explain the lower observed rate constant for the transfer of a proton between His64 and the Zn-bound solvent molecule. At pH 7.8 in the current structure, the positions of the water termed "deep water" (DW) because of its location in the active site and ZW are unchanged. However, W1 has flipped to engage in an H-bond to W2 and now acts as an H-bond acceptor from Thr200 (right panel in Figure 1). Accordingly, W2 and W3a have changed their orientation in a compensatory manner to remain H-bonded to each other and W1. This rearrangement positions one of the W2 D atoms to act as an H-bond donor to His64 (right panel of Figure 1). This represents an unbranched proton transfer pathway between ZW and His64, something that is thought to be essential for efficient proton transfer.[18] Previous computational studies have implied that proton transfer occurs through W3a, but this observation supports the notion that proton transfer most likely occurs through W2.[12] It also provides a clue about the specific participation of an explicit water molecule in proton transfer. The cluster of H-bonds among W3b, Asn62, and Asn67 is completely unchanged from that of the high-pH structure. Table 1 shows the O···O distances and O−D···O H-bond

Table 1. O···O Distances and Angles between H-Bond Donors and Acceptors for the Water Networks Determined at pH 10 and 7.8

pH 10.0	O···O (Å) [O−D···O (deg)]	pH 7.8	O···O (Å) [O−D···O (deg)]
W1−ZW	2.5 (170)	W1−ZW	2.7 (170)
W1−W2	2.8 (not applicable)	W1−W2	2.7 (170)
W2−W3a	2.7 (150)	W3a−W2	2.9 (150)
W2−W3b	2.8 (160)	W2−W3b	2.7 (170)

donor−acceptor angles between waters in the pH 10 and 7.8 structures. Within the structural coordinate errors at 2.0 Å resolution, the O distances are identical while the D atoms rearrange.

The fact that the solvent O positions are unchanged while only the D atoms are reorganizing is consistent with the significant role of the hydrophilic residues of the active site, such as Asn62 and Asn67, in stabilizing the positions of water molecules in the structure. The change in H-bonding of the water structure is most likely due to the significant changes in the charged states of two key residues, Tyr7 and His64, demonstrating the effect of changing electrostatics in the active site cavity. An unanticipated result observed in the high-pH structure was that Tyr7 was deprotonated, implying that this residue has a pK_a lower than that for free Tyr in solution (Figure 2).[14]

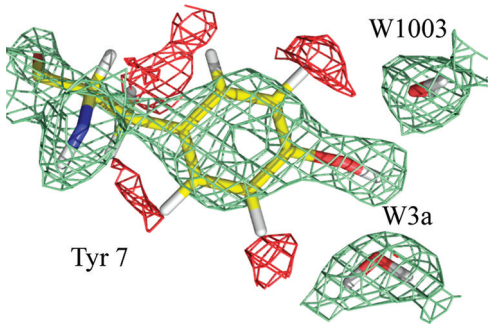

Figure 2. Tyr7 is protonated in the pH 7.8 structure and can act as a bifurcated H-bond donor to W3a and W1003. Positive $2F_o - F_c$ nuclear maps are colored green (contoured at 1.5σ), with the negative $2F_o - F_c$ nuclear map colored red (contoured at 2.0σ), indicating the unexchanged and/or nonlabile H density.

As anticipated, Tyr7 is indeed protonated at pH 7.8 (Figure 2), but it was thought that it would still act as an H-bond acceptor from W3a and that the phenolic D atom would point toward the buried water W1003.[13] Instead, at pH 7.8, the Tyr7 OD group makes a bifurcated H-bond to buried water W1003 and W3a with O−D···O angles of ~100° each (Figure 2). The O···O distances between the phenolic O of Tyr7 in W1003 and W3a are 2.6 Å each. This significant electrostatic change from the negatively charged deprotonated oxygen to a neutral protonated OD group may be causing W3a to flip and establish an H-bond with W2 (Figures 1−3). This, along with the changes observed in His64, is causing a cascade of flipping and

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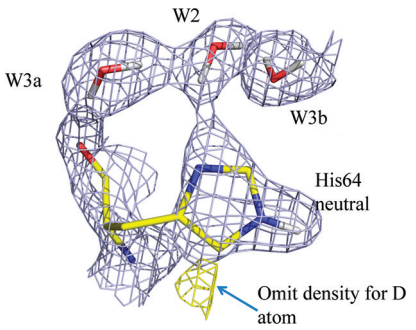

Figure 3. Predominant conformation of the His64 side chain that is neutral and in the inward position. The $2F_o - F_c$ nuclear map (contoured at 1.5σ) is colored blue, with the positive omit $F_o - F_c$ nuclear density map (contoured at 3.0σ) colored yellow, indicating the D atom density for the flipped neutral histidine conformation. Waters are labeled, and H atoms have been omitted from the His imidazole ring for the sake of clarity.

re-establishing of H-bonds along the water network. This raises interesting implications for Tyr affecting proton transfer at high pH *in vitro*, such as the possibility thatTyr7 may act as a proton trap at high pH.

As expected, at high pH, His64 was observed to be neutral and in a single inward conformation. At pH 7.8, His64 occupies three superimposed forms. The predominant one is neutral (~60% occupancy); another one is flipped and neutral [~30% occupancy (Figure S3 of the Supporting Information)], and finally, we observed a minor flipped charged species [estimated ~10% occupancy (not shown)]. The predominant form directs the Nδ1 electron lone pair toward W2's D atom, indicating formation of an H-bond between W2 and His64 [O···N distance of 3.2 Å and O−D···N angle of 170° (Figure 3)]. The orientations of the 30% neutral and minor protonated form, with their rings flipped ~180°, are similar to that observed for the neutral imidazole ring at high pH. The observation of the minor protonated species is consistent with the pK_a of His64 being ~7 considering the pH of the crystal (pH 7.8). Because of limitations of the resolution of the neutron data, we included only 60 and 40% for the two major conformations in the deposited structure.

These observations reveal important contributions to the understanding of the molecular details of proton transfer in HCA II. A change in the pH of the crystals has permitted the observation of what might happen during catalysis. This has allowed the observation, for the first time, of a completed H-bonded network connecting ZW to His64. In addition, His64 was observed in an intermediate state where a minor form is protonated, possibly poised to rotate to the outward conformation to deliver the excess proton to the bulk solvent.

To further probe the role of Tyr7 during catalysis, we are preparing ^{13}C-labeled Tyr HCA II for pH titrations using NMR to look at the pK_a of all seven Tyr residues and the active pursuit of a low pH-crystal form to shed more structural light on proton transfer.

■ ASSOCIATED CONTENT

Supporting Information

Details of crystallization, data collection, and structure refinement; crystallographic data table for both room-temperature neutron and X-ray diffraction data; additional figures of waters, His64, and the active site. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Structural coordinates and experimental data have been deposited in the Protein Data Bank as entry 3TMJ.

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■ ABBREVIATIONS

HCA II, human carbonic anhydrase II; H-bond, hydrogen bond; PCS, Protein Crystallography Station.

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