A Short, Strong Hydrogen Bond in the Active Site of Human Carbonic Anhydrase II†,‡

Balendu Sankara Avvaru,§ Chae Un Kim,⊥ Katherine H. Sippel,§ Sol M. Gruner⊥,§ Mavis Agbandje-McKenna,§
David N. Silverman,*§∥ and Robert McKenna*§

§Department of Biochemistry and Molecular Biology,† Department of Pharmacology and Therapeutics, College of Medicine, University of Florida, Gainesville, Florida 32610,‡ Cornell High Energy Synchrotron Source (CHESS), and∥ Physics Department, Cornell University, Ithaca, New York 14853

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ABSTRACT: The crystal structure of human carbonic anhydrase II (HCA II) obtained at 0.9 Å resolution reveals that a water molecule, termed deep water, Dw, and bound in a hydrophobic pocket of the active site forms a short, strong hydrogen bond with the zinc-bound solvent molecule, a conclusion based on the observed oxygen–oxygen distance of 2.45 Å. This water structure has similarities with hydrated hydride found in crystals of certain inorganic complexes. The energy required to displace Dw contributes in significant part to the weak binding of CO₂ in the enzyme–substrate complex, a weak binding that enhances k_cat for the conversion of CO₂ into bicarbonate. In addition, this short, strong hydrogen bond is expected to contribute to the low pK_a of the zinc-bound water and to promote proton transfer in catalysis.

The hydration of CO₂ to produce bicarbonate and a proton is catalyzed by the carbonic anhydrases (CAs) and plays a significant role in a number of physiological processes, including respiration, fluid secretion, and pH control. There are 14 human gene products classified as CAs, including HCA II which is widespread in tissues and data collection, and refinement statistics is given in Table S1 (Supporting Information).

Of particular interest for this report is the structure of the apparently hydrogen bonded solvent water network that includes the zinc-bound solvent. This network emanates from the deep water (Dw) in the hydrophobic pocket formed in part by the side chains of Val121, Val143, Trp209, and Leu198 to the water molecules labeled W1, W2, W3a, W3b, and W4 shown in Figures 1, 2, and 3. In crystal structures, this chain extends to but is not in hydrogen bond contact with the proton shuttle residue His64. The zinc-bound solvent appears to form a hydrogen bond with the side chain of Thr199, and the deep water molecule (Dw) appears to participate in hydrogen bonds with the backbone amide of Thr199 and with the zinc-bound water molecule. The mechanism of the proton transfer utilizing pathways such as this has been the subject of considerable investigation (2, 7–12).

The current high-resolution structure provides a clearer view of the solvation at the active site (Figure 1). The hydrogen bonds in this water network have distances typical of solvent water, with O–O distances near 2.7–2.9 Å. A more detailed picture of distances and bond angles involving active site solvent is provided in Figures S1 and S2 (Supporting Information). However, there is a short hydroxyl group with an O–O distance estimated to be 2.45 ± 0.03 Å between Dw and the zinc-bound solvent (Figure 3). The crystallographic occupancy is near 100% for Dw, and both this water molecule and the zinc-bound solvent have B factors that are low (near 10 Å²) and close in value to the B factors of the...
Moreover, with a solution pK of the crystal structure is probably in large part zinc-bound hydroxide under the conditions of crystallization (pH 7.0). LBHB of the hydrated hydroxide anion HOHOH has similarities with the identification by crystallography of the environment and likely involves the zinc-bound hydroxide. Weak hydrogen bonds typical of water molecules in solution have a favorable enthalpy of formation near 5 kcal/mol; however, LBHBs can have such enthalpies near 15–25 kcal/mol. This is significant with respect to the catalysis by HCA II since the binding of CO2 to its catalytically productive binding site displaces the deep water molecule (Dw) (Figure 2) (16, 17) and thus requires the cleavage of the LBHB contributing to the very weak binding of CO2 at this site. A dissociation constant for CO2 at its catalytic site in HCA II has been estimated to be 100 mM measured by infrared spectroscopy (18, 19).

A tight binding of substrate at the reactive site is a disadvantage for catalysis by HCA II; it adversely affects its physiological function which requires it to enhance catalysis for a maximum velocity. When a thermodynamic well or pit that accumulates tightly bound substrate exists, the rate of catalysis is decreased. For an enzyme that requires rapid catalysis like carbonic anhydrase, it is advantageous for substrate binding to be weak and the active site to remain largely unbound at physiological levels of substrate CO2. The concentration of CO2 in plasma, for example, is near 1 mM; the value of Kcat for hydration is 10 mM, and the estimated dissociation constant of CO2 is 100 mM. It appears that HCA II evolved weak substrate binding by having it displace the Dw which participates in a LBHB.

There is likely another role for the LBHB as it may contribute to the low pK near 7 for the zinc-bound water molecule, the protolysis of which is enhanced using the energy of formation of the LBHB. In this respect, the role of the LBHB is analogous with the catalytic mechanism of liver alcohol dehydrogenase in which removal of a proton from the Zn-coordinated alcohol is promoted by formation of a LBHB with Ser48 in the reactant state. The alkoxide then undergoes hydride transfer to...
generate product. In each case, forming the LBHB provides the energy to pump the proton to His64 in HCA II and to His51 in horse liver alcohol dehydrogenase (21).

It is unclear whether these arguments will apply in the dehydration direction in which the maximal catalytic rates are slower than in hydration [maximal steady-state constants are $k_{\text{cat}}/K_m = 2 \times 10^7 \text{M}^{-1} \text{s}^{-1}$ and $k_{\text{cat}} = 0.6 \mu \text{s}^{-1}$ (22)]. Crystal structures of bicarbonate bound at the active site metal, the presumed catalytic site, have been obtained for the mutant of HCA II with Thr200 replaced with His (23), with Thr199 replaced with Ala (24), and for HCA II in which Zn(II) is replaced with Co(II) (25). Although the orientation of the bound bicarbonate is somewhat different in each of these examples, in all three cases the binding of bicarbonate displaces the deep water. The dissociation constant of bicarbonate at the active site of HCA II is estimated to be $\sim 100 \text{mM}$ by $^{13}\text{C}$ NMR measurements (26), with a similar $K_i$ value estimated by inhibition by bicarbonate of the esterase value of activation (13).

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**SUPPORTING INFORMATION AVAILABLE**

Materials and methods, a table of refinement statistics for the structure of human carbonic anhydrase II, a figure of bond lengths in the active site, and a figure of bond angles in the active site. This material is available free of charge via the Internet at http://pubs.acs.org.

**REFERENCES**