Two-Site Binding of Phenol in the Active Site of Human Carbonic Anhydrase II: Structural Implications for Substrate Association

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In the human erythrocyte, carbonic anhydrase II (CAII) catalyzes the reversible hydration of carbon dioxide into bicarbonate and a proton.[1] The forward direction of catalysis comprises two consecutive reactions: (1) nucleophilic attack of zinc-bound hydroxide at substrate CO_2 to form product bicarbonate anion, and (2) transfer of a proton from zinc-bound water to bulk solvent in order to regenerate zinc-bound hydroxide. The 1.54-Å-resolution crystal structure of CAII reveals that the catalytically-obligatory zinc ion resides at the base of a 15-Å-deep cleft, liganded by His-94, His-96, His-119, and hydroxide ion.[2] The hydroxyl side chain of Thr-199 accepts a hydrogen bond from zinc-bound hydroxide and donates a hydrogen bond to Glu-106, and this hydrogen bond network plays an important role in catalysis.[3] A hydrophobic pocket adjacent to the zinc-bound hydroxide, defined by enzyme residues Trp-209, Val-121, Leu-198, and Val-143, is believed to be the precatalytic association site for substrate carbon dioxide,[4] and this proposal is indirectly supported by numerous theoretical[3a,5] and genetic–structural studies[6] of CAII. The so-called "deep" water molecule resides at the mouth of this pocket and donates a hydrogen bond to zinc-bound hydroxide; this water molecule is probably displaced by substrate CO_2.[2]

There is a dearth of structural data on complexes of CAII with competitive inhibitors of the forward, hydration reaction, despite the fact that several structures of CAII complexes with competitive inhibitors of the reverse, dehydration reaction have been determined.[2,7] Phenol is the only known competitive inhibitor of CAII for the hydration reaction (K_i = 10 mM at pH 8.7);[8] here, we report the crystal structure of the complex between CAII and phenol at a resolution of 2.0 Å. Surprisingly, two molecules of phenol are observed to bind to the enzyme, and their binding modes provide important structural inferences on enzyme–substrate association.

Crystals of CAII (Sigma) were prepared as described[9] and then transferred to a stabilizing buffer solution of 4 M potassium phosphate, pH 10.[10] Increasing concentrations of ultrapure phenol (Sigma) were then added to achieve a final concentration of 100 mM phenol.[11,12] X-ray data to 2.0 Å were collected and processed as previously described.[10] Raw data frames were analyzed using BUDDHA,[13] and replicate and symmetry-related intensities were merged using PROTEIN[14] (R_{merge} = 0.10). The atomic coordinates of human blood CAII[2] retrieved from the Brookhaven Protein Data Bank,[15] less active site solvent molecules, served as the starting model for refinement. Refinement utilized the simulated annealing protocol of Brünger[16] installed on a Silicon Graphics IRIS workstation, and active site solvent molecules and inhibitor molecules were added to the model when the crystallographic R factor dropped below 0.19. Refinement converged smoothly to a final crystallographic R factor of 0.156 with root mean square deviations from ideal values of bond lengths and angles of 0.010 Å and 2.8°, respectively. Atomic coordinates have been deposited in the Brookhaven Protein Data Bank.[15]

Overall, the three-dimensional structure of the complex between phenol and CAII is similar to that of the native blood enzyme;[2] the root mean square difference in Cα coordinates between the two protein structures is 0.20 Å. Additionally, there are no significant changes in the zinc coordination polyhedron between the two structures, and the geometry and coordination distances of His-94, His-96, His-119, and zinc-bound solvent are identical to those of the native enzyme within experimental error. Unexpectedly, two molecules of phenol are observed in electron density maps of the enzyme–inhibitor complex. The first molecule binds to a hydrophobic patch about 15 Å away from zinc near the symmetry-related molecule at $-x, y + {}^1/_2, -z$ (data not shown). This phenol makes van der Waals contacts with residues Leu-57 and Ile-91 and a hydrogen bond with Asp-72 (3.1 Å). This phenol also makes a van der Waals contact with Pro-237 and a weak hydrogen bond with the backbone carbonyl of Gly-235 (3.4 Å) in the symmetry-related molecule.[17] We note that this location does not correspond to the molecular trajectory or secondary CO_2 binding sites predicted in molecular dynamics calculations.[3a,5]

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(10) Direct transfer of preformed CAII crystals into a 4 M solution of potassium phosphate had no effect on the diffraction quality of the crystals. Moreover, the stabilizing buffer does not affect the structure of the zinc-coordination polyhedron or the overall structure of CAII.

(11) Attempts to soak phenol into crystals grown and stored in ammonium sulfate buffer were unsuccessful. X-ray analysis of these crystals revealed a spherical, 8σ peak corresponding to the nonprotein zinc ligand and equal populations of both the "in" and "out" conformers of residue His-64.[9]

(12) The occupancy of the inhibitor in the CAII–phenol complex demonstrated a dependence on the pH of the buffer solutions. Best results were achieved at pH 10.

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(17) Interactions of phenol with Asp-72 and the backbone carbonyl of symmetry-related Gly-235 imply hydrogen bond acceptor interactions for both, despite the fact that phenol has only one proton. One possibility is that phenol interacts with both residues via a bifurcated hydrogen bond. Alternatively, Asp-72 may be protonated due to its hydrophobic environment, thus donating a hydrogen bond to phenol.

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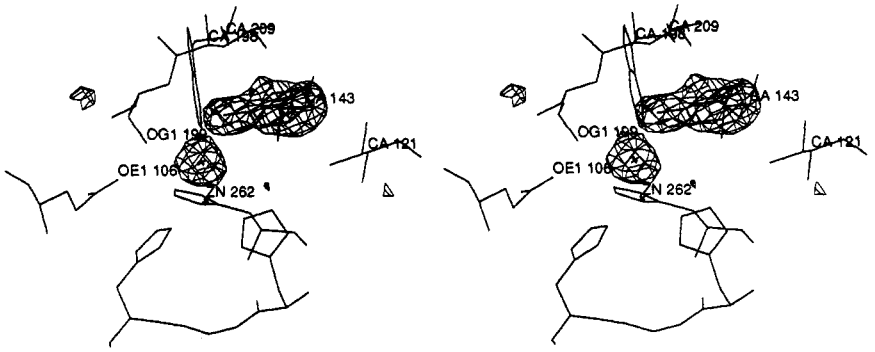

Figure 1. Difference electron density map of the active site in the CAII–(phenol)$_2$ complex generated with Fourier coefficients $|F_o| - |F_c|$ and phases calculated from the final model less the atomic coordinates of zinc-bound hydroxide and both phenol molecules. The map is contoured at 2.8σ, and refined atomic coordinates are superimposed; Glu-106, Val-121, Val-143, Leu-198, Thr-199, Trp-199, and zinc are indicated, and zinc-bound solvent appears as a star.

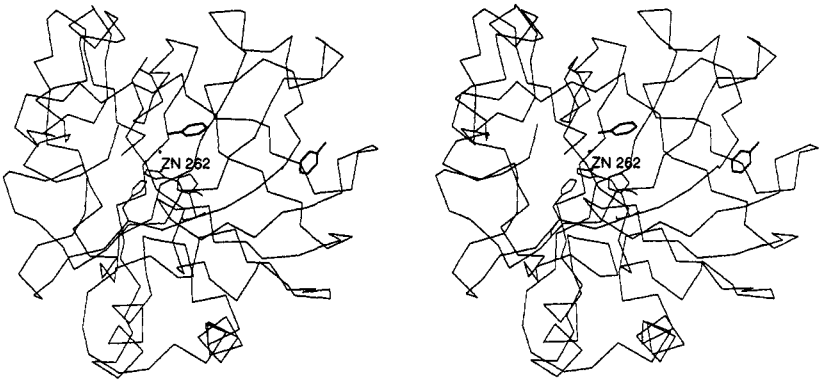

Figure 2. Stereoview of the CAII–(phenol)$_2$ complex. Protein Cα atoms, selected side chains (His-94, His-96, and His-119), zinc, zinc-bound solvent, and the two phenol molecules are shown. Zinc and zinc-bound solvent appear as stars.

The second molecule of phenol binds in the hydrophobic pocket of the CAII active site and displaces the "deep" water molecule (Figure 1), making van der Waals contacts with pocket residues Val-121, Val-143, Leu-198, and Trp-209. Importantly, this phenol molecule does not coordinate to zinc but instead hydrogen bonds to zinc-bound solvent (2.6 Å). A second, poorly-oriented hydrogen bond[18] is formed between the phenolic hydroxyl and the backbone amide of Thr-199 (3.2 Å). We emphasize that this phenol molecule does not coordinate to zinc. This result is the first direct confirmation of spectroscopic studies of Co(II)-substituted CAII and its phenol complex,[8] and of molecular modeling studies based on ^{13}C NMR data,[19] which conclude that the coordination of zinc is not altered by phenol binding. A stereoview of the CAII–(phenol)$_2$ complex is provided in Figure 2.

Given that the pK_a values of phenol and zinc-bound solvent are about 10 and 7, respectively,[20] it is not immediately clear what the ionization state of the active site phenol molecule is in the CAII–(phenol)$_2$ complex at pH 10. Although earlier spectroscopic data on the Co(II)-substituted CAII–phenol complex suggest that neutral phenol binds to the enzyme,[8] recent ^{13}C-NMR studies appear to indicate that phenol is ionized when bound to CAII (these studies did not indicate the binding of two phenol molecules to the enzyme).[19] Given the different pK_a values of zinc-bound solvent[20] and phenol and the presumed directionality of hydrogen bond patterns in the enzyme active site[2,3] it is difficult to rationalize the latter results unless the pK_a of zinc-bound solvent is elevated upon phenol binding. Intriguingly, since the occupancy of phenol is best at pH 10, the binding of ionized phenol may be indicated.

Spectroscopic studies of various metallo-substituted carbonic anhydrase isozymes suggest that enzyme–substrate association does not involve an inner-sphere interaction between a CO_2 oxygen and zinc.[6d,21] Hence, the hydrophobic pocket is a logical precatalytic association site for substrate CO_2, and recent genetic–structural studies support this conclusion.[6] Moreover, the three-dimensional structure of the complex between CAII and the competitive inhibitor phenol provides the first direct inference for the role of this pocket in CO_2 association: since phenol binds to this pocket and since phenol is a competitive inhibitor of CO_2 hydration, it follows that CO_2 similarly binds to this pocket prior to catalytic turnover.[22]

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