Novel Binding Mode of Hydroxamate Inhibitors to Human Carbonic Anhydrase II

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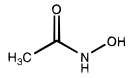
Peptidylhydroxamic acids are potent inhibitors of zinc enzymes, such as thermolysin and the pharmaceutically-important matrix metalloproteinases.[1] The X-ray crystallographic structure determinations of enzyme−inhibitor complexes reveal that the coordination of the active site zinc ion by the hydroxamate group is the primary determinant of enzyme−inhibitor affinity: the C═O and OH groups simultaneously coordinate to zinc to form an energetically-favorable 5-membered chelate complex.[2] Since this interaction contributes to nanomolar affinity between appropriately designed peptidylhydroxamic acids and target proteinases,[1−3] we wondered whether hydroxamic acids could be exploited as inhibitors of other pharmaceutically-important zinc enzymes. In particular, will hydroxamic acids effectively inhibit human carbonic anhydrase II (CAII)?

CAII is a metalloenzyme of 260 amino acids containing one catalytically-obligatory zinc ion at the base of a 15 Å deep conical active site cavity.[4] Zinc is liganded by His-94, His-96, His-119, and hydroxide ion in a tetrahedral fashion.[5] 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.[6] A hydrophobic pocket is adjacent to zinc and is the precatalytic binding site for substrate CO_2.[7] In the native enzyme, a water molecule resides at the mouth of this pocket and has been designated the "deep water".[5] The molecular details of mechanism and inhibitor binding to this metalloenzyme are important for understanding the structural basis of catalysis as well as the structure-assisted design of potent therapeutic agents for glaucoma.[8] To date, the only functional group incorporated into rationally-designed CAII inhibitors for zinc coordination is the sulfonamide moiety.[8] Therefore, the exploration of different functional groups capable of zinc coordination in the CAII active site may lead to the design of new and potent inhibitors.

The three-dimensional structures of CAII complexed with acetohydroxamic acid (**1**, IC_{50} = 47 μM at pH = 7.2)[9] and trifluoroacetohydroxamic acid (**2**, IC_{50} = 3.8 μM at pH = 7.2)[9] have each been determined by X-ray crystallographic methods to 2.1 Å resolution.



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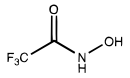

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Surprisingly, these hydroxamic acids do not bind to zinc via a 5-membered chelate with the C═O and OH groups; instead, they bind through the coordination of an ionized nitrogen directly to zinc. In the CAII·**2** complex, nitrogen−zinc coordination is augmented by a C−F → Zn^{2+} interaction to yield a novel 5-membered chelate. Interestingly, certain features of hydroxamate binding are reminiscent of those observed in enzyme−product complexes.[10]

Hydroxamate **1** was purchased from Sigma, and hydroxamate **2** was synthesized as described.[11] Crystals of recombinant wild-type CAII were prepared as described,[12] and complexes with hydroxamates **1** and **2** were prepared by gradually transferring crystals to a buffer solution containing 2.6 M ammonium sulfate, 50 mM Tris-HCl (pH = 8.0 at room temperature) and either 820 mM **1** or 4 mM **2**. Crystals were soaked in these solutions for 4 days. For each crystalline CAII−hydroxamate complex, X-ray diffraction data to 2.1 Å resolution were collected and processed as previously described[12] (CAII·**1** complex 93.1% complete, R_{merge} = 0.045; CAII·**2** complex 93.5% complete, R_{merge} = 0.051). The atomic coordinates of human blood CAII[5] less active site solvent molecules served as the starting model for refinement with X-PLOR.[13] Initial difference electron density maps of each enzyme−inhibitor complex revealed coordination of the hydroxamate to zinc through an ionized nitrogen. Neither inhibitor nor active site solvent molecules

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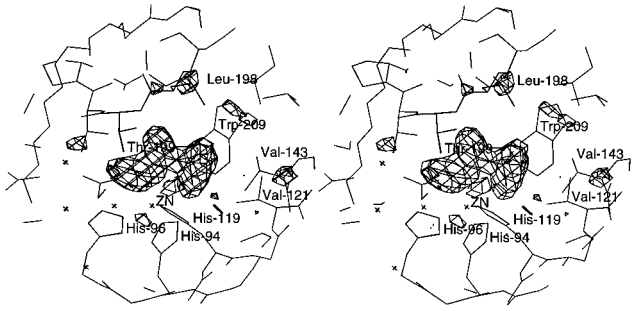

Figure 1. Difference electron density map of the CAII·**2** complex, generated with Fourier coefficients $|F_o| - |F_c|$ and phases calculated from the final model less the atomic coordinates of the inhibitor. The map is contoured at 2.8σ. Refined atomic coordinates are superimposed, and selected active site residues are indicated (solvent molecules appear as unlabeled stars).

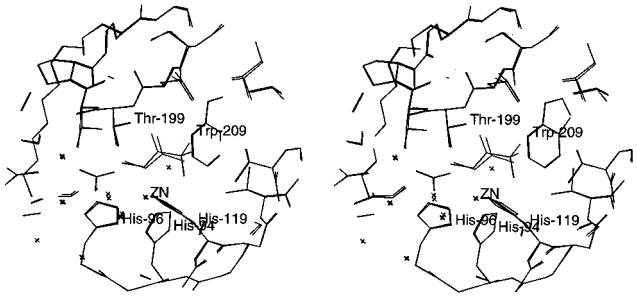

Figure 2. Least-squares superposition of the CAII·**1** and CAII·**2** complexes; selected active site residues are indicated.

were included in each model until the crystallographic *R* factor dropped to 0.20. Refinement of the CAII·**1** complex converged to a crystallographic *R* factor of 0.160 (R_{free} = 0.220), and refinement of the CAII·**2** complex converged to a crystallographic *R* factor of 0.157 (R_{free} = 0.227). Root mean square deviations from ideal values of bond lengths and angles were 0.008 Å and 1.6° for each complex. Atomic coordinates have been deposited in the Brookhaven Protein Data Bank.

Both **1** and **2** bind to CAII through a novel coordination interaction with the active site zinc ion. To illustrate, an electron density map of the CAII·**2** complex is found in Figure 1, and a superposition of the CAII·**1** and CAII·**2** complexes is found in Figure 2. The ionized nitrogen of each inhibitor displaces hydroxide ion and coordinates to zinc. Both inhibitors also displace the deep water molecule, and both inhibitors contact Thr-199: each hydroxamate carbonyl group accepts a hydrogen bond from the Thr-199 backbone NH group and each hydroxamate hydroxyl and zinc-bound nitrogen atoms are within hydrogen-bonding distance to the Thr-199 side chain hydroxyl group. The coordination of hydroxamate nitrogen to zinc and the hydrogen bond between the hydroxamate carbonyl and the Thr-199 NH group mimics the observed binding of bicarbonate in stable enzyme−product complexes[10] (Figure 3). The hydrogen bond between the Thr-199 NH group and the hydroxamate carbonyl apparently contributes to the unexpected hydroxamate−zinc coordination mode: this hydrogen bond could not be made if the hydroxamate coordinated to zinc through its carbonyl group.

The trifluoro moiety of **2** forms van der Waals interactions in the CAII active site, which presumably contribute to its enhanced affinity relative to its nonfluorinated analogue. All three fluorine atoms of **2** contact residues in the hydrophobic pocket: Val-121, Val-140, Val-143, Leu-198, and Trp-209.

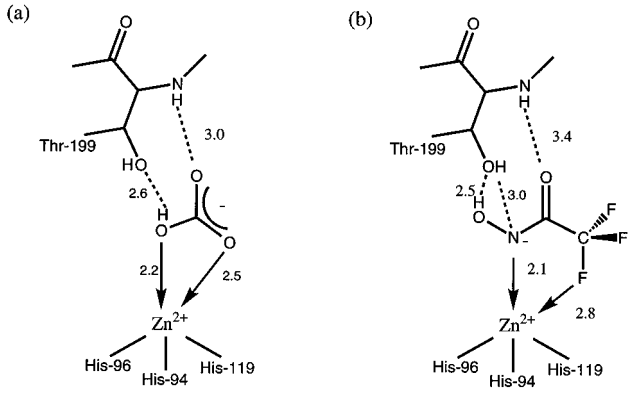

Figure 3. Scheme of bicarbonate[10a] (a) and trifluorohydroxamate **2** (b) binding to CAII; interatomic separations are indicated in angstroms.

Similar van der Waals interactions are observed in the binding of trifluoromethanesulfonamide to CAII.[14] In addition, the CAII·**2** complex reveals a weakly polar C−F → Zn^{2+} interaction (F → Zn^{2+} separation = 2.8 Å). The fluorine and ionized nitrogen of **2** form a 5-membered chelate with zinc; coordination geometry is approximately trigonal bipyramidal, with the nitrogen atoms of the hydroxamate and His-119 serving as apical ligands and His-94, His-96, and the inhibitor fluorine atom serving as equatorial ligands (Figures 1 and 3).

Several C−F → M^{n+} interactions are characterized for small molecule complexes containing the alkali metal ions Na^+, K^+, and Rb^+.[15] The bidentate chelate complex between **2** and CAII meets three geometrical criteria defined by Murray-Rust and colleagues which characterize effective C−F → M^{n+} interactions.[15] First, the length of the F → M^{n+} separation in the CAII·**2** complex is 2.8 Å, which is within the tabulated range of 2.4−3.2 Å. Second, the C−F → M^{n+} angle is 112.5°, which is consistent with an interaction between an sp^3-hybridized lone electron pair on fluorine and the metal ion. Third, the F → M^{n+} contact completes a metal chelate complex, a feature commonly observed in other examples of C−F → M^{n+} interactions.

The discovery of avid hydroxamate binding to CAII through an unexpected binding mode mimicking aspects of bicarbonate binding provides a starting point for the design of high-affinity inhibitors. Importantly, hydroxamate inhibitors of CAII exploit a binding interaction fundamentally different from that of any other structurally-characterized hydroxamate−metalloenzyme complex. The ionized nitrogen of the hydroxamic acid inhibitor binds directly to zinc, which is in contrast with the binding of hydroxamate inhibitors to other zinc enzymes, such as thermolysin, collagenase, and matrilysin.[2] Given that hydroxamic acid **2** binds to CAII with micromolar affinity, the subsequent redesign of the inhibitor with modifications targeting the Pro-202 and Phe-131 hydrophobic patches in the CAII active site[16] may yield nanomolar affinity inhibitors with desired properties of affinity, charge, and solubility for possible therapeutic applications.

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