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Supporting Information

Inhibition of carbonic anhydrases by a substrate analog: benzyl carbamate directly coordinates the catalytic zinc ion mimicking bicarbonate binding

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Experimental methods

CA inhibition

Benzyl carbamate was purchased from Sigma-Aldrich. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[1] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water and dilutions up to 0.1 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min – 6 h at room temperature (15 min) or 4 °C (6 h) prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier,[2-4] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[2-4]

Crystallization, X-ray data collection, and refinement

cDNA encoding hCA II was cloned in pETM13 expression vector which allows to obtain a final product with a C-terminal Histidine tag. However, due to the presence of 5 histidine residues at the N-terminus of the protein sequence, it has been not necessary an additional $(His)_6$-tag. Indeed, in the reverse primer designed for PCR cloning a stop codon was added in order to avoid the translation of the C-terminal $(His)_6$-tag. The protein was thus expressed in *E. coli* and purified by IMAC using a His-Trap column taking advantage of the presence of the 5 His residues. A second step of purification was performed by affinity chromatography using a resin activated with pAMBS, a molecule able to selectively bind to the zinc ion of CA active site. A final yield of around 80 mg/L of growth medium was obtained.

hCA II/**1** complex was obtained by soaking hCA II preformed crystals, obtained using the hanging drop vapor diffusion technique, for 30 minutes in the crystallization solution (1.3 M sodium citrate, 60 mM Tris-HCl, pH 8.0), supplemented with 10% glycerol and saturated with the inhibitor. Diffraction data were collected to 1.46 Å resolution, in-house at -180 °C, using a Rigaku MicroMax-007 HF generator producing Cu Kα radiation and equipped with a Saturn 944 CCD detector.

Diffraction data were indexed, integrated and scaled using the HKL2000 software package.[5] A total of 189200 reflections were measured and reduced to 41125 unique reflections. Crystal parameters and relevant X-ray data collection statistics can be found in Table S1. Initial phases were calculated using hCA II crystallized in the $P2_1$ space group (PDB code 6EQU)[6] as starting model after deletion of non-protein atoms. An initial round of rigid body refinement followed by simulated annealing and individual B factor refinement was performed using the program CNS.[7, 8] Model visualization and rebuilding was performed using the graphics program O.[9] After an initial refinement, limited to the enzyme structure, a model for the inhibitor was easily built and introduced into the atomic coordinates set for further refinement; restraints on inhibitor bond angles and distances were taken from the Cambridge Structural Database[10] and standard restraints were used on protein bond angles and distances throughout refinement. Water molecules were built into peaks >3σ in |Fo| - |Fc| maps that demonstrated appropriate hydrogen-bonding geometry. Crystallographic refinement was carried out against 97.8% of the measured data. The remaining 2.2% of the observed data, which was randomly selected, was used for R-free calculations to monitor the progress of refinement. Several alternate cycles of refinement and manual model building were performed to reduce the R-work and R-free to the final values of 0.169 and 0.184, respectively. The correctness of protein stereochemistry was finally checked using PROCHECK.[11] Relevant refinement statistics can be found in Table S1. The refined model contained 2072 protein atoms, 263 waters, and 1 inhibitor molecule. Coordinates and structure factors have been deposited with the Protein Data Bank (accession code 6H29).

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Table S1. Data collection and refinement statistics for hCA II/**1** complex. Values in parentheses refer to the highest resolution shell (1.49-1.46 Å).

Crystal parameters	
Space group	$P2_1$
a (Å)	42.3
b (Å)	41.6
c (Å)	71.8
β (°)	104.4
Data collection statistics	
Resolution (Å)	28.8-1.46
Temperature (K)	100
Total reflections	189200
Unique reflections	41125
Completeness (%)	97.3 (74.7)
$\langle I \rangle / \langle \sigma(I) \rangle$	25.6 (4.1)
Redundancy	4.6 (2.1)
R_{merge}[a]	0.046 (0.194)
R_{meas}[a]	0.051 (0.245)
R_{pim}[a]	0.020 (0.147)
Refinement statistics	
Resolution (Å)	28.8-1.46
R_{work}[b] (%)	16.9
R_{free}[b] (%)	18.4
r.m.s.d. from ideal geometry:	
Bond lengths (Å)	0.01
Bond angles (°)	1.5
Number of protein atoms	2072
Number of inhibitor atoms	11
Number of water molecules	263
Average B factor (Å^2)	
All atoms	12.2
Protein atoms	11.0
Inhibitor atoms	17.4
Water molecules	21.5

[a] $R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$; $R_{meas} = \Sigma_{hkl}\{n(hkl)/[n(hkl)-1]\}^{1/2}\Sigma_i|I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$; $R_{pim} = \Sigma_{hkl}\{1/[n(hkl)-1]\}^{1/2}\Sigma_i|I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl)\rangle$ is the mean value for its unique reflection; summations are over all "n" reflections.

[b] $R_{work} = \Sigma_h||F_o(h)|-|F_c(h)||/\Sigma_h|F_o(h)|$, where F_o and F_c are the observed and calculated structure-factor amplitudes, respectively. R_{free} was calculated with 2.2% of the data excluded from the refinement.