Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

HYDROXAMATE REPRESENTS A VERSATILE ZINC BINDING GROUP FOR THE DEVELOPMENT ON NEW CARBONIC ANHYDRASE INHIBITORS

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EXPERIMENTAL SECTION

Chemistry and CA Inhibition

Phenyl hydroxamate **4** has been prepared by the routine procedure, by reacting benzoyl chloride with hydroxylamine in the presence of triethylamine.[1,2]

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity.[3] 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 (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature 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, as reported earlier,[4,5] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[6-8]

Human purified MMPs (MMP-2, and MMP-8) were purchased from Calbiochem (Inalco, Milano, Italy). They were activated in the assay buffer by adding bovine trypsin (from Sigma, 50 μL, 0.6 mg/mL) to the proenzyme, followed by incubation at 37 °C for 10 min. The trypsin was then inactivated with aprotinin (50 μL, 1.2 mg/mL). Initial rates for the hydrolysis of the thioester substrate AcProLeuGly-S-LeuLeuGlyOEt, coupled to the reaction with 5,5′-dithiobis(2-

nitrobenzoic acid), were used for assessing the catalytic activity and inhibition of the two MMPs mentioned above, as reported earlier.[1] The change of absorbance ($\Delta epsilon$ = 19 800 M^{-1} cm^{-1}) at 405 nm was monitored continuously at room temperature, using a Cary 3 spectrophotometer interfaced with a PC. A typical 100-μL reaction contained 50 mM MES, pH 6.0, 10 mM $CaCl_2$, 100 μM substrate, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 5 nM MMP. For the K_I determinations, DMSO solutions of the inhibitor were included in the assay, resulting in a final concentration of 2% DMSO in the reaction mixture. In these conditions, K_I values varied from 5-10% in replicate experiments. K_I's were then determined by using Easson-Stedman plots and a linear regression program, as reported earlier.[1]

Crystallization, X-ray data Collection and refinement

Crystals of hCA II in complex with N-(Hydroxy)-benzamide **4** have been obtained by soaking techniques. In particular, enzyme crystals were grown at room temperature by the vapor diffusion hanging drop method. Equal volumes of protein (10 mg/ml in 0.1 M TRIS-HCl pH 8.5) and of a solution containing 2.4 M ammonium sulphate, 0.3 M sodium chloride, 0.1 M Tris-HCl, pH 8.6 and 5 mM 4-(hydroxymercurybenzoate) were mixed and equilibrated against a 500 microliters reservoir containing the same precipitant solution. A few hCA II native crystals were then transferred in a 2 µl drop of freshly prepared precipitant solution containing also the inhibitor at the concentration of 20 mM. These crystals were kept in the soaking solution for about one hour, and then were transferred to the cryoprotectant (15% glycerol) and flash-frozen in N_2. A complete dataset was collected at 1.85 Å resolution by copper rotating anode generator developed by Rigaku and equipped with Rigaku Saturn CCD detector, at 100K. Data were processed using the HKL crystallographic data resolution package.[9] The crystal symmetry corresponds to the $P2_1$ space group with unit cell dimensions of a=42.16 Å, b=41.50Å, c=71.99Å and β=104.39°. Data collection statistics are reported in Table S1.

The atomic coordinates of native hCA II (PDB accession code 1CA2)[10] less solvent molecules were used as starting model for refinement with CNS.[11] An initial round of rigid body refinement followed by simulated annealing and isotropic thermal factor (B-factor) refinement was performed.[11] The analysis of electron density maps in correspondence of the active site region after this single round of refinement showed the presence of an inhibitor molecule, which was easily built into the model. The geometry of metal-ligand interaction of hCA II-**4** adduct was treated applying restraints in refinement. In particular, the information on bond lengths were derived from accurately determined structures of small-molecule crystals in the Cambridge Structural Database.[12,13] Many cycles of manual rebuilding and positional and temperature factor refinement were necessary to reduce the crystallographic Rfactor and Rfree values (in the 20.00–1.85 Å resolution range) to 0.163 and 0.203, respectively. Statistics for refinement are summarized in Table S1. Coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (Accession code 4FL7).

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Table S1 Crystal parameters, data collection and refinement statistics

Cell parameter	
Space group	$P2_1$
a (Å)	42.16
b (Å)	41.50
c (Å)	71.99
β (°)	104.39
Data collection statistics	
Resolution (Å)	20.00-1.85
Temperature (K)	100
Total reflections	60270
Unique reflections	19721
Completeness (%)	94.7 (78.4)
R-merge*	0.046 (0.067)
Mean I/sigma(I)	19.70 (12.05)
Refinement statistics	
Resolution (Å)	20.00-1.85
R-factor** (%)	16.3
R-free** (%)	20.3
rmsd from ideal geometry:	
Bond lengths (Å)	0.009
Bond angles (°)	1.5
Number of protein atoms	2069
Number of inhibitor atoms	10
Number of water molecules	301
Average B factor ($Å^2$)	12.6

* $R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, 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 reflections.

**Rfactor = $\Sigma_h||Fo(h)|-|Fc(h)||/\Sigma_h|Fo(h)|$, where Fo and Fc are the observed and calculated structure-factor amplitudes, respectively. R_{free} was calculated with 5% of the data excluded from the refinement. Values in parenthesis are referred to the highest resolution shell (1.92-1.85 Å).