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Carbonic anhydrase inhibitors: Crystallographic and solution binding studies for the interaction of a boron-containing aromatic sulfamide with mammalian isoforms I–XV

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ABSTRACT

We investigated the inhibition of carbonic anhydrase (CA, EC 4.2.1.1) isoforms I–XV with 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylsulfamide and other simple or sugar sulfamides, a class of less investigated CA inhibitors (CAIs). The crystal structure of the adduct of hCA II with the boron-substituted sulfamide shows the organic scaffold of this compound bound in the hydrophilic half of the active site where it makes a large number of van der Waals contacts with Ile91, Gln92, Val121, Phe131, Leu198, and Thr200. The data here reported provide further insights into sulfamide binding mechanism confirming that this zinc-binding group could be usefully exploited for obtaining new potent and selective CAIs.

Sulfamide $H_2NSO_2NH_2$, a simple molecule incorporating the sulfonamide functionality, is widely used by medicinal chemists for the design of derivatives with pharmacological applications. It may give rise to many types of derivatives, by substituting one to four hydrogen atoms present in it. All these derivatives show specific biological activities[1,2]; indeed, primary sulfamides ($RNHSO_2NH_2$) are inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1),[1–4] whereas sulfamide derivatives with different substitution patterns significantly inhibit a large number of proteases belonging to the aspartic protease (HIV-1 protease, γ-secretase), serine protease (elastase, chymase, tryptase and thrombin among others) and metalloprotease (carboxypeptidase A (CPA) and matrix metalloproteinases) families.[1–6] In all these compounds, many of which show low nanomolar affinity for the target enzymes, the free or substituted sulfamide moiety plays an important role for the binding of the inhibitor to the enzyme active site. For example, in some metalloenzymes (CAs, CPA, steroid sulfatase) the sulfamide moiety directly coordinates to a metal ion found within the active site,[1–4] while in the case of HIV protease, cyclic sulfamides act as inhibitors interacting with the catalytically critical aspartic acid residues of the active site by means of an oxygen atom belonging to the HN–SO_2–NH motif,[7] which substitutes a catalytically essential water molecule.[5–7] In other cases, the sulfamide moiety is important for inducing desired physico-chemical properties to the drug-like compounds incorporating it, such as, for example, enhanced water solubility, or better bioavailability, due to the intrinsic properties of this highly polarized moiety when attached to an organic scaffold.[1,2,5]

CAs catalyze a very simple but essential reaction, carbon dioxide hydration to bicarbonate and protons.[8,9] This reaction also occurs without a catalyst but is very slow. Thus, due to the fact that CO_2, bicarbonate and protons are essential molecules/ions in many important physiologic processes in all life kingdoms (*Bacteria*, *Archaea*, and *Eukarya*) throughout the phylogenetic tree, high rates of their interconversion are necessary for physiological requirements. These enzymes are excellent examples of convergent evolution, as five distinct genetic families (α-, β-, γ-, δ-, and ε-CAs) were discovered so far.[8,9] Mammals possess 16 different α-CA isoforms, which are involved in many crucial physiological or pathological processes connected with respiration and transport of CO_2/bicarbonate, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, etc.[8,9] Some of them are cytosolic (CA I, CA II, CA

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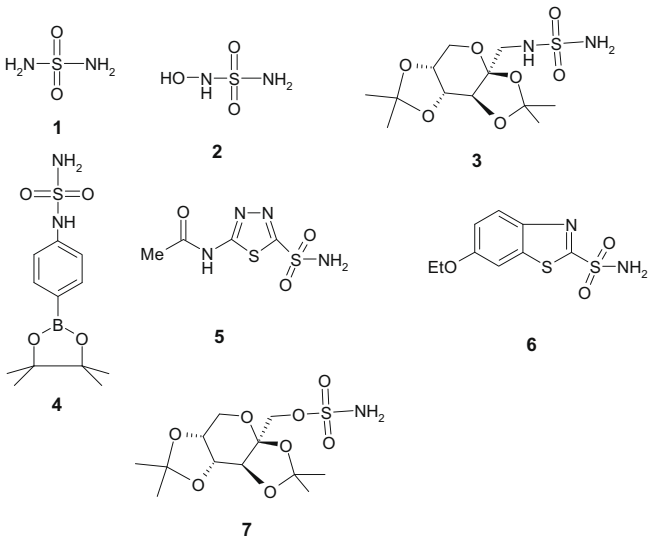
III, CA VII, and CA XIII), others are membrane-bound (CA IV, CA IX, CA XII, CA XIV, and CA XV), CA VA and CA VB are mitochondrial, and CA VI is secreted in saliva and milk. Three acatalytic isoforms are also known, denominated sometimes CA related proteins (CARP), that is, CA VIII, CA X and CA XI, which seem to be cytosolic proteins too.[8,9]

Primary sulfonamides (RSO_2NH_2)[8,9] and their bioisosteres, such as the sulfamates ($ROSO_2NH_2$)[10] and sulfamides ($RNHSO_2NH_2$),[1–4] are the most investigated class of CA inhibitors (CAIs). Many such compounds (mainly of the sulfonamide and sulfamate type)[8] are clinically used as diuretics and antiglaucoma agents, but it has recently emerged that they have potential as anti-convulsant, anti-obesity, anti-cancer, anti-pain, and anti-infective drugs.[8–12] However critical barriers to the design of CAIs as therapeutic agents are encountered, due to the high number of isoforms in humans, their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors of the sulfonamide/sulfamate type.[8–10] Thus, many classes of CAIs are constantly being reported. The knowledge of the inhibition profile of such compounds against all mammalian isoforms, as well as their detailed binding to the enzyme (which can be obtained from crystallographic data), allow a better understanding of the features associated with isoform-selective CAIs.[13] However, only three sulfamide-containing derivatives have been characterized so far by means of X-ray crystallography for their interaction with CA: the simple sulfamide **1**,[4a] *N*-hydroxy-sulfamide **2**,[4c] and the sulfamide derivative of the antiepileptic drug topiramate, compound **3**.[4b] Here we report the detailed CA inhibitory activity against all mammalian isoforms as well as the X-ray crystal structure in complex with isoform II of a boron-containing aromatic sulfamide, 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylsulfamide **4**. This molecule has been originally reported as a potential anti-cancer compound for boron neutron capture therapy by this group.[3e]



The inhibitory activity of **4** has been reported previously only against the cytosolic isozymes hCA I and II, and the transmembrane tumor-associated one hCA IX.[3e] Here we extend those data and investigate the complete inhibition profile of this sulfamide against all 13 catalytically active isoforms, comparing it to those of derivatives **1–3** (Table 1). Data of Table 1 also include the inhibition with the classical sulfonamide inhibitors in clinical use acetazolamide **5**, ethoxzolamide **6** and topiramate **7**.[8,13b] Thus, the boron-containing inhibitor **4** showed K_I values in the range of 7.1–92 nM against isoforms hCA I, II, VA, VB, VII, IX, XII, XIV, and mCA XV, being thus an effective CAI. The isoforms which were inhibited in the low nanomolar range were the transmembrane ones hCA XII (cancer-associated) and hCA XIV. Two other isoforms hCA VI and mCA XIII were on the other hand modestly inhibited by this sulfamide (K_Is of 749–994 nM) whereas hCA III and hCA IV were only weakly inhibited (K_Is of 8.62–8.77 μM). Thus, as seen from data of Table 1, the boron-containing sulfamide **4** possesses an inhibition profile distinct of those of the clinically used sulfonamides **5, 6** (which have low nanomolar activity against all isoforms except hCA III, and are thus promiscuous inhibitors), of that of sulfamate **7**, or of other sulfamides investigated earlier.[1–4] Indeed, the simple, unsubstituted sulfamide **1** is a very weak, generally millimolar CAI against all isoforms, as it lacks an organic scaffold and its inhibitory power is due only to the coordination to the metal ion and some hydrogen bonds with Thr199 and Thr200.[4a] *N*-Hydroxy-sulfamide **2**, with a supplementary OH moiety compared to **1**, is already a micromolar inhibitor of the four isoforms against which it has been tested (hCA I, II, IX, and XII),[4c] whereas the sugar sulfamide **3** has a very interesting inhibition profile.[4b] In fact, unexpectedly this compound behaved as a weak hCA II inhibitor (K_I of 2.13 μM) although the cognate sulfamate, the antiepileptic drug topiramate, possessing exactly the same scaffold as **3**, is a potent, low nanomolar CAI (K_I of 10 nM).[3c] This very different behavior has been explained when the X-ray crystal structure of the two compounds have been compared.[3c,4b] Furthermore, compound **3** showed interesting CA inhibitory activity against other isoforms, such as, for example, hCA VA, hCA VB, hCA VII, mCA XIII, or hCA XIV (K_Is in the range of 21–35 nM). It is also a weak, micromolar inhibitor of hCA I, hCA IV, hCA IX, and hCA XII (Table 1). All these data show that the nature of the organic scaffold present in a sulfamide CAI is very much influencing the inhibition profile against various CA isoforms of such a compound.

In order to assess the molecular basis responsible for the inhibitory properties of **4** toward CAs, we solved the crystal structure of the hCA II–**4** complex, which was crystallized by a soaking experiment as previously reported for other sulfonamide CA inhibitors.[13a,15] The three-dimensional structure was analyzed by difference Fourier techniques, the crystals being isomorphous to those obtained for the native enzyme[16] and refined using the CNS program.[17] The statistics for data collection and refinement are summarized in Table 2. The structure of the hCA II–**4** complex is very similar to that of the non-inhibited enzyme, with a rmsd of 0.27 Å calculated by superposing the Cα atoms in the adduct and non-inhibited enzyme. Examination of the electron density at various stages of the refinement afforded the observation of one molecule of inhibitor **4** bound in the hCA II active site (Figs. 1 and 2). The inhibitor fills the active site channel, as shown in Figure 1.

The sulfamide **4** is coordinated at the Zn(II) ion from the enzyme active site, by means of one deprotonated nitrogen atom belonging to the sulfamide moiety (N1···Zn(II) = 2.01 Å), similarly to other sulfamides (compounds **1–3**) or sulfonamides/sulfamates whose crystal structures in adduct with CAs have been reported.[4,13] As for other CA–sulfonamide/sulfamate/sulfamide adducts investigated earlier,[4,13] this nitrogen atom of the inhibitor also interacts with Thr199 (Thr199OG1···N1 = 2.68 Å) whereas one of its oxygen atoms forms a second hydrogen bond with the backbone NH of Thr199 (Thr199N···O2 = 2.86 Å). The second oxygen of the sulfamide moiety is at 3.06 Å from the Zn(II) ion. An additional H-bond interaction is observed between the Thr200OG atom and the second nitrogen atom of the sulfamide moiety (Thr199OG1···N2 = 3.21 Å) (Fig. 2). No other polar interactions have been evidenced between the organic scaffold of **4** and amino acid residues in the hCA II active site (Fig. 2). For example, the 1,3,2-dioxaborolan ring does not make hydrogen bonds with any amino acid side chain, even if it incorporates heteroatoms able to participate in such interactions.

By superposing the hCA II–**4** adduct structure reported here with those of the adducts of hCA II with sulfamides **1–3** reported

Table 1
Inhibition data with the sulfamides **1**–**4** and clinically used sulfonamides **5**, **6**, (as standards) against mammalian isozymes CA I–XV

Isozyme[a]	K_I[b] (nM)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
hCA I[c]	0.31×10^6	4050	3450	92	250	25	250
hCA II[c]	1.13×106	566	2135	48	12	8	10
hCA III	1.09×10^6	nt	4×10^6	8770	2×10^5	1×10^6	7.8×10^5
hCA IV[c]	880	nt	941	8620	74	93	4900
hCA VA[c]	0.84×10^6	nt	32	87	63	25	63
hCA VB[c]	0.84×10^5	nt	21	75	54	19	30
hCA VI	7.0×10^4	nt	nt	749	11	43	45
hCA VII[c]	6800	nt	35	84	2.5	0.8	0.9
hCA IX[d]	9.6×10^3	865	4580	81	25	34	58
hCA XII[d]	0.83×10^6	1340	1875	8.9	5.7	22	3.8
mCA XIII[c]	0.14×10^6	nt	30	994	17	50	47
hCA XIV[c]	0.75×10^6	nt	25	7.1	41	25	1460
mCA XV[c]	3.74×10^4	nt	nt	89	72	58	78

[a] h, human; m, murine isozyme; nt, not tested (no data available).
[b] Errors in the range of 5–10 % of the reported value (from three different assays).
[c] Human (h) or murine (m) full length, cloned isozymes, by the CO_2 hydration method.[14]
[d] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.[14]

Table 2
Crystal parameters, data collection and refinement statistics for the hCA II–**4** adduct

Cell parameter	
Space group	$P2_1$
Unit cell parameters (Å, °)	
a	42.08
b	41.33
c	71.88
β	104.45
Data collection statistics (20.00–1.80)	
Temperature (K)	100
Total reflections	64,133
Unique reflections	20,270
Completeness (%)	91.8 (89.7)
R-sym[a]	0.067 (0.350)
Mean *I*/sigma(*I*)	15.4 (3.16)
Refinement statistics (20.00–1.80)	
R-factor[b] (%)	17.4
R-free[b] (%)	20.5
rmsd from ideal geometry	
Bond lengths (Å)	0.007
Bond angles (°)	1.5
Number of protein atoms	2104
Number of inhibitor atoms	20
Number of water molecules	288
Average *B*-factor (Å^2)	20.15

[a] $R_{sym} = \Sigma|I_i - \langle I\rangle|/\Sigma I_i$; over all reflections.
[b] $R_{factor} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$; R_{free} calculated with 5% of data withheld from refinement. Values in parenthesis are referred to the highest resolution shell (1.86–1.80 Å).

Figure 1. Ribbon diagram of the hCA II–**4** complex. The inhibitor, metal coordinating residues H94, H96, H119 and the zinc ion are represented in stick.

earlier (Fig 3),[4] one may observe that the sulfamide zinc anchoring moiety is well superposable for all compounds **1–4** except for the *N*-hydroxy-sulfamide **2**,[4c] which presents a supplementary OH moiety bound to the second nitrogen of the sulfamide functionality. In the hCA II–**2** complex this additional OH group is hydrogen bonded to the Thr200OG1 atom (Thr200OG1···O3 = 3.06 Å), leading to a small tilting of the molecule when bound to the Zn(II) ion so that neither the SO_2 oxygens, nor the second NH moiety are perfectly superposable with the corresponding groups in the remaining hCA II–sulfamide (**1, 3**, and **4**) adducts (Fig. 3).

The organic scaffold of sulfamide **4** is orientated towards the hydrophilic half of the hCA II active site, with the phenylene moiety being in van der Waals contacts (<4.5 Å) with the side chains of Gln92, Val121, Phe131, Leu198, and Thr200 and the 1,3,2-dioxaborolan ring making hydrophobic interactions with Gln92, Ile91, and Phe131 (Fig. 2). The binding of compound **4** within the hCA II active site is further stabilized by a strong stacking interaction with the aromatic ring of Phe131 (Fig. 2), a residue playing a key role in inhibitor binding, by orientating the inhibitor scaffold within the enzyme active site cavity, towards the hydrophilic or hydrophobic halves, as reported earlier by this group for adducts of various sulfonamides with hCA II.[13,18,19]

In order to understand the diverse inhibition of two physiologically relevant isoforms, that is, hCA II (cytosolic, dominant, ubiquitous one) and IX (transmembrane, present only in hypoxic tumors) with compound **4**, the hCA II–**4** complex has been superimposed with hCA IX structure, recently resolved by this group (Fig. 4).[12,20,21]

As clearly observed in Figure 4, the organization of the active sites in the two enzymes is rather similar. However, at least two amino acid residues that in hCA II are involved in inhibitor binding are not conserved in hCA IX and could be considered responsible of the lower inhibitory activity of **4** against the tumor associated hCA IX (K_I of 81 nM) compared to hCA II (K_I of 48 nM). These residues

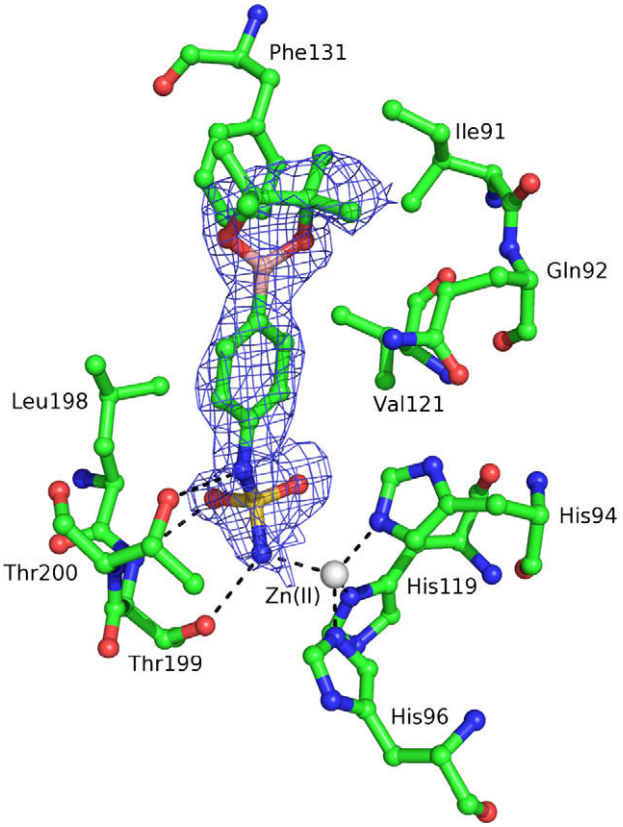


Figure 2. Active site region of the hCA II–**4** complex. The simulated annealing omit $|2F_o - F_c|$ electron density map relative to the inhibitor molecule is shown. Zn(II) coordination and hydrogen bonds are also shown as dotted lines.

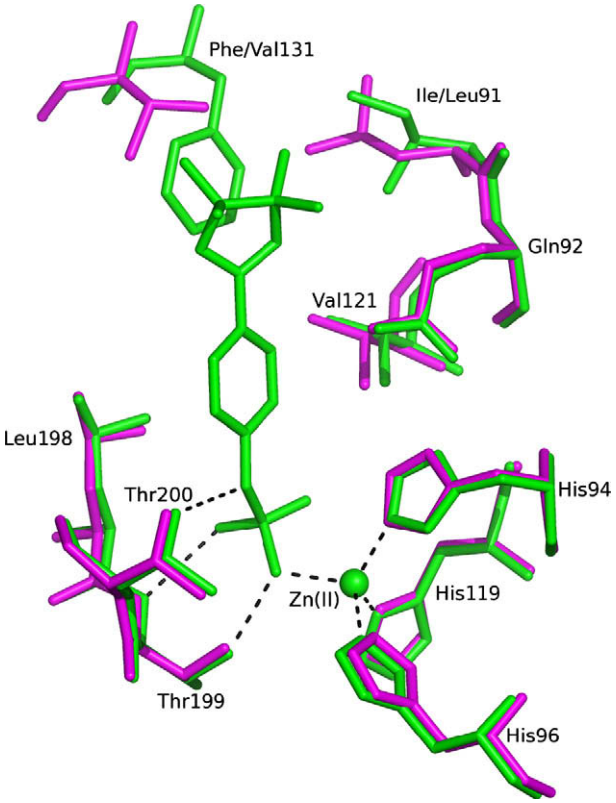


Figure 4. Structural superposition of the active sites of hCA II–**4** complex (green) and hCA IX (magenta).[12]

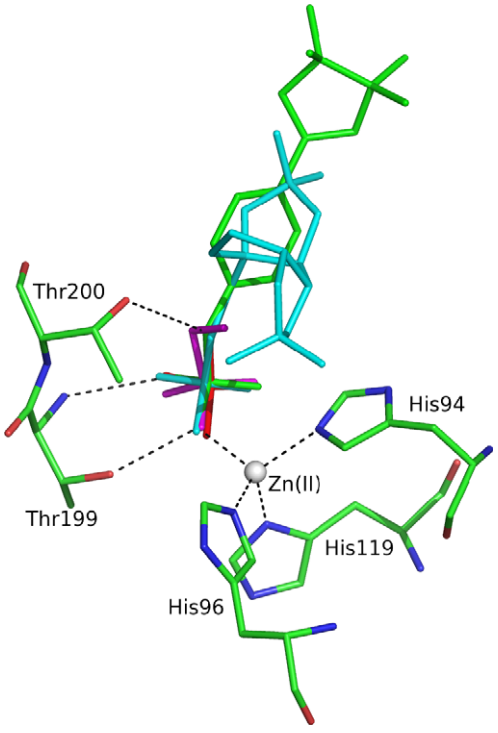


Figure 3. Superposition of hCA II-inhibitor adducts: **1** is reported in red,[4a] **2** in magenta,[4c] **3** in cyan,[4b] and **4** in green. The Zn(II) ion and its three protein ligands are also shown.

are those in position 91 and 131. Indeed, hCA II has Ile91 and Phe131, whereas hCA IX has Leu91 and Val131. We speculate that the Phe131/Val131 'substitution' in the hCA IX active site plays a major role in the lower affinity of **4** against the transmembrane isoform. Indeed, as described above, in the hCA II–**4** adduct Phe131 plays an important role in inhibitor recognition and its substitution in hCA IX with a much less bulky residue could significantly reduce the number of hydrophobic interactions.

In conclusion, we investigated the inhibition of all mammalian CA isoforms (CA I–XV) with 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylsulfamide, and other sulfamides, a class of less investigated CAIs. The X-ray crystal structure of the adduct of hCA II with the boron-substituted sulfamide showed the organic scaffold of the compound bound in the hydrophilic half of the active site where it participates in a large number of van der Waals contacts with Ile91, Gln92, Val121, Phe131, Leu198, and Thr200. The data here reported provide further insights into sulfamide binding mechanism confirming that this zinc-binding group could be usefully exploited for obtaining new potent and selective CAIs. Thus, a sulfamide incorporating an organic scaffold with several moieties (tails) appended to it, may exploit the favorable interactions with amino acid residues from the hydrophilic part of the CA active site observed in the present adduct, and probably make other interactions with the hydrophobic half of it. Such interactions were not observed in the current structure due to the fact that our compound has only one rather compact 'tail', the 4,4,5,5-tetramethyl-1,3,2-dioxaborolan one. It may be thus envisaged that introducing bulkier groups that the methyl ones on the dioxaborolan ring may lead to an enhancement of activity and diverse interactions with the various isoforms, since such moieties should bind at the entrance of the enzyme active site cavity where the amino acid residues of the different isoforms are very much variable.

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14. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An applied photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. 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 for the α-CAs) 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 (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 μM were done thereafter with distilled-deionized water. 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,[12,13b] and represent the mean from at least three different determinations. Human CA isozymes were prepared in recombinant form as reported earlier by our groups.[10–12] Sulfamides **2**–**4** were reported earlier by our groups,[3e,4b] whereas **1** and sulfonamides **5**, **6** are commercially available (Sigma–Aldrich)..
15. Crystals of the hCA II–**4** adduct were obtained by the soaking technique.[13a] hCA II 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 2.6 M ammonium sulfate, 0.3 M sodium chloride, 0.1 M Tris–HCl, pH 8.5, and 5 mM 4-(hydroxymercurybenzoate) were mixed and equilibrated against a 500 μl reservoir containing the same precipitant solution. A few hCA II native crystals of $0.3 \times 0.2 \times 0.1$ mm size were then transferred in a 3 l drop of freshly prepared precipitant solution containing also inhibitor **4**, at the concentration of 20 mM. These crystals were kept in the soaking solution for about twenty minutes, until they started to be damaged, and then were immersed briefly in a cryoprotectant (15% glycerol) and flash-frozen in liquid nitrogen. X-ray diffraction data were collected at Synchrotron source Elettra in Trieste, using a Mar CCD detector, at 100 K. Data were measured to a 1.80 Å resolution and processed using the HKL package.[22] A total of 64,133 reflections were measured (unit cell parameters: $a = 42.08$ Å, $b = 41.33$ Å, $c = 71.88$ Å, and $\beta = 104.45°$) and reduced to 20,270 unique reflections (completeness = 91.8%, R-sym = 6.7 % in the 20.00–1.80 Å resolution range). The structure of the complex was analyzed by difference Fourier techniques, using the PDB file 1CA2[16] as starting model for refinement. Non-protein atoms were removed from the model prior to structure factor and phase calculations. An initial round of rigid body refinement followed by simulated annealing and isotropic thermal factor (B-factor) refinement was performed using the CNS 1.1 program.[17] The inspection of electron density maps in correspondence of the active site region after this single round of refinement (R-free = 0.307; R-factor = 0.276) indicated the presence of an inhibitor molecule, which was gradually built into the model. Introduction of solvent molecules and several cycles of manual rebuilding and energy minimization and B-factor refinement gave a final model with $R_{free} = 0.205$ and $R_{factor} = 0.174$ in the 20.00–1.80 Å resolution range. The statistics for refinement are summarized in Table 2. Coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (Accession code 3MNU).
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