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Carbonic anhydrase inhibitors: Binding of an antiglaucoma glycosyl-sulfanilamide derivative to human isoform II and its consequences for the drug design of enzyme inhibitors incorporating sugar moieties☆

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Abstract—*N*-(4-Sulfamoylphenyl)-α-D-glucopyranosylamine, a promising topical antiglaucoma agent, is a potent inhibitor of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1). The high resolution X-ray crystal structure of its adduct with the target isoform involved in glaucoma, CA II, is reported here. The sugar sulfanilamide derivative binds to the enzyme in a totally new manner as compared to other CA–inhibitor adducts investigated earlier. The sulfonamide anchor was coordinated to the active site metal ion, and the phenylene ring of the inhibitor filled the channel leading to the active site cavity. The glycosyl moiety responsible for the high water solubility of the compound was oriented towards a hydrophilic region of the active site, where no other inhibitors were observed to be bound up to now. A network of seven hydrogen bonds with four water molecules and the amino acid residues Pro201, Pro202 and Gln92 further stabilize the enzyme–inhibitor adduct. Topiramate, another sugar-based CA inhibitor, binds in a completely different manner to CA II as compared to the sulfonamide investigated here. These findings are useful for the design of potent, sugar-derived enzyme inhibitors.

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) catalyzes a very simple but critically important physiological reaction: the interconversion between carbon dioxide, generated in huge amounts in all metabolic processes, and the bicarbonate ion.[1–6] Inhibitors of these zinc enzymes show a multitude of applications as diuretic, antiglaucoma, antiobesity or antitumour drugs, and are also used as diagnostic tools.[1–6] Various CA isoforms are responsible for specific physiological functions, and drugs with such a diversity of actions target different isozymes of the 15 presently known in humans.[2–6] In all of them, the inhibitor is bound as an anion to the catalytically critical Zn^{2+} ion, also participating in extensive hydrogen bond networks and van der Waals interactions with amino acid residues in both the hydrophobic and hydrophilic halves of the enzyme active site.[7–15] Among the three main classes of potent CA inhibitors (CAIs) described up to now, the sulfonamides, the sulfamates and the sulfamides, the first one is the most investigated one, since classical, clinically used drugs such as acetazolamide 1, methazolamide 2, ethoxzolamide 3, dichlorophenamide 4, dorzolamide 5 and brinzolamide 6 all belong to it.[1–6]

X-ray crystal stuctures are available for adducts of several isozymes (i.e., CA I, II, IV, V, XII and XIV)[7–14] mainly with sulfonamides, with some sulfamates and few sulfamides (including the simplest such derivatives,

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☆ Coordinates and structure factors have been deposited with the Protein Data Bank (Accession code 2HL4).
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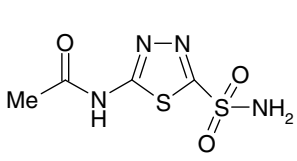
sulfamic acid and $H_2NSO_2NH_2$),[10] providing clear information on zinc coordination pattern in these enzymes as well as in the corresponding enzyme-inhibitor complexes. X-ray crystallography of CA–inhibitor adducts is in fact quite useful for drug design not only of this class of biologically active compounds, but also for investigating a much wider range of metalloenzyme inhibitors.[15] Thus, constantly, novel sulfonamide derivatives are synthesized and investigated as inhibitors of various enzymes in the search of compounds with selectivity for some of the many physiologically relevant isoforms, since the clinically used drugs **1**–**6** unselectively inhibit many isozymes and as a consequence show many undesired side effects.[1–6] For example, the fluorescent sulfonamide **7**, recently reported by our group, is in clinical development as an imaging agent, allowing the precise imaging of acute hypoxic tumours that are non-responsive to classical chemo- and radio-therapy.[7,16] On a totally different research area, we have also reported a series of sugar-sulfanilamide derivatives with potent topical antiglaucoma activity in an animal model of this disease.[17] The most promising compound of that series, the glucose derivative **8**, was shown to be a strong inhibitor of the key isozyme involved in glaucoma formation, that is, hCA II (h means human isoform), with an inhibition constant of 23 nM.[17] The compound was also shown to be highly water soluble and could be formulated as eye drops, which is not a straightforward property for sulfonamides, that are usually very poorly water soluble compounds. In order to understand at the molecular level the strong inhibitory activity of this compound against the target isoform involved in glaucomagenesis, and also for learning more regarding the possibility of using such data to obtain even more effective enzyme inhibitors with the desired physico-chemical properties, we here report the high resolution X-ray crystal structure (at 1.55 Å) of the adduct of hCA II with the sugar sulfonamide derivative **8**. A structural comparison with topiramate **9**, another interesting sugar derivative possessing good CA inhibitory activity and very interesting biological activity, being a clinically used antiepileptic,[15] is also reported. Topiramate possesses a



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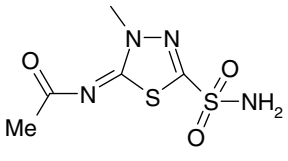

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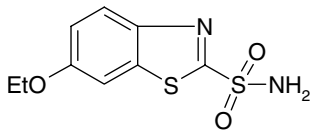

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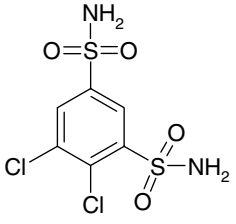

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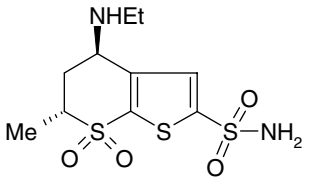

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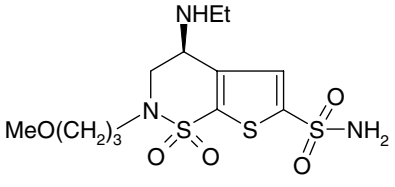

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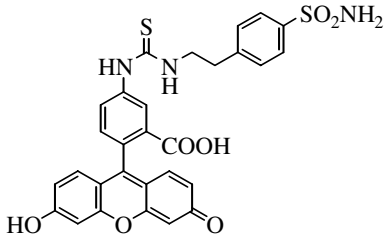

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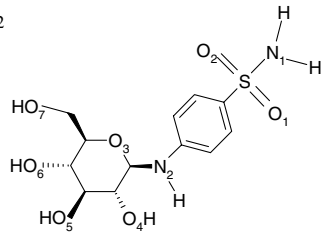

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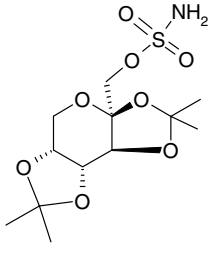

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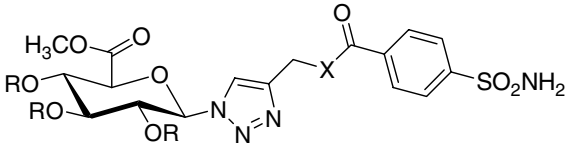
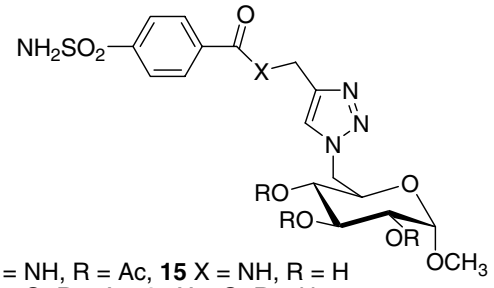
protected-fructose scaffold and a sulfamate zinc binding function. It has been proved earlier[9b] that this compound is a potent inhibitor of several CA isozymes, and its binding to hCA II has been investigated by means of high resolution X-ray crystallography.[9b]

Despite the synthesis of a vast multitude of CA inhibitors utilising the tail strategy [1–4] and the potential benefits of appending carbohydrate moieties to drug molecules, the combination of these two strategies to generate CAIs is essentially unexplored—there are just two literature examples[17,18] reporting this type of compounds, that is , our initial report of sulfanilamide-based inhibitors, to which various sugar moieties have been attached by reaction of monosaccharides with sulfanilamide in the presence of ammonium chloride,[17] and a newer example, in which a rather large number of sugar sulfonamide CAIs were obtained by means of click chemistry.[18] Indeed, using a 'click-tail' approach a novel class of glycoconjugate benzene sulfonamides of types **10**–**17** have been synthesized that contain diverse carbohydrate-triazole tails. Such compounds were shown to act as excellent CAIs against the isoforms CA I, II and IX, also possess desired physico-chemical properties, such as good water solubility.[18] Thus, this relatively untouched medicinal chemistry landscape has served as the primary inspiration for the X-ray crystallographic investigation reported in this study regarding the interaction of a sugar–sulfanilamide derivative with the main CA isozyme, hCA II, which might be important for a better understanding of the drug design of sugar-containing sulfonamide CAIs.



10 X = NH, R = Ac, **11** X = NH, R = H
12 X = O, R = Ac, **13** X = O, R = H



14 X = NH, R = Ac, **15** X = NH, R = H
16 X = O, R = Ac, **17** X = O, R = H

The hCA II–**8** complex was prepared and crystallized as previously reported for other sulfonamide/sulfamate CA inhibitors.[1–6] The three-dimensional structure was analyzed by difference Fourier techniques, the crystals being isomorphous to those obtained for the native enzyme,[19] and refined using the CNS program.[20] The statistics for data collection and refinement are summarized in Table 1.

Table 1. Data collection and refinement statistics for the hCA II–**8** adduct

Data collection statistics (20.00–1.55)	
Temperature (K)	100
Total reflections	1,22,533
Unique reflections	34,301
Completeness (%)	99.3 (96.7)
R-sym[a]	0.069 (0.264)
Mean $I/\sigma(I)$	15.1 (4.1)
Refinement statistics (20.00–1.55)	
R-factor[b] (%)	17.6
R-free[b] (%)	19.8
rmsd from ideal geometry	
Bond lengths (Å)	0.005
Bond angles (°)	1.4
Number of protein atoms	2096
Number of inhibitor atoms	22
Number of water molecules	321
Average *B*-factor (Å^2)	12.72

Values in parentheses refer to the highest resolution shell.
[a] $R_{sym} = \Sigma|I_i - < I >|/\Sigma I_i$; over all reflections.
[b] $R_{factor} = \Sigma||F_o|-|F_c||/\Sigma F_o$; R_{free} calculated with 5% of data withheld from refinement.

The three-dimensional structure of the enzyme was very similar to that of hCA II without any ligand bound,[19] as judged by an rms deviation for Cα atoms of 0.36 Å only. Examination of the initially calculated electron density maps in the active-site region showed clear evidence for the binding of the inhibitor within the active site cavity (Fig. 1).

The analysis of the active site environment showed that the tetrahedral geometry of the Zn^{2+} binding site and the key hydrogen bonds between the sulfonamide moiety of the inhibitor **8** and enzyme active site were all retained with respect to other hCA II–sulfonamide complexes for which X-ray structures have been reported.[1–6] In particular, the nitrogen atom of the sulfonamide moiety of the inhibitor coordinated the catalytic Zn^{2+} ion and displaced the hydroxide ion present in the native enzyme. In addition, this nitrogen was also involved in a hydrogen bond with the OH group of Thr199 (2.76 Å), which was in turn hydrogen bonded to the carboxylate of Glu106 (2.56 Å). One of the oxygen atoms (O1) of the sulfonamide moiety accepted a hydrogen bond with the backbone amide of Thr199 (ThrN–O1 = 2.90 Å), whereas the other one (O2) was semi-coordinated to the catalytic Zn^{2+} ion (Zn^{2+}–O2 = 3.04 Å), as observed in other hCA II–sulfonamide adducts.[7–15] The phenyl ring of the inhibitor filled the active site channel of the enzyme wherein its atoms established strong van der Waals interactions (distance <4.5 Å) with the side chains of Gln92, Val121, Leu198, Thr200 and Pro202 (Fig. 2). The sugar moiety of **8,** which is the most characteristic part of this inhibitor, as no other sugar sulfonamide adduct has been crystallized up to now with any CA isoform, as a consequence of its highly polar nature was oriented towards the hydrophilic part of the active site funnel (Fig. 3), where it was stabilized by several polar interactions with amino acid residues/water molecules. Thus, a strong hydrogen bond was observed between the O7

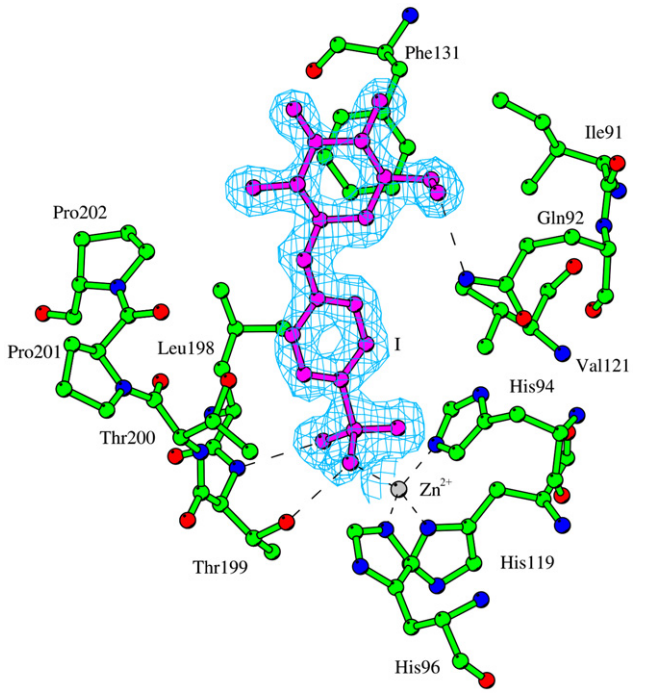


Figure 1. Active site region in the hCA II–**8** complex. The inhibitor (labelled I) is shown associated with simulated annealing omit $2|F_o| - |F_c|$ electron density map,[20] computed at 1.55 Å and contoured at 1.0σ.

atom of the glycopyranosyl ring and the Gln92NE2 atom (O7–Gln92NE2 = 2.88 Å). Besides this interaction, several hydrogen bonds involving the other hydroxyl groups of the glycopyranosyl ring and water molecules located in the active site were also observed (Fig. 2). Among these, the most notable one was the hydrogen bond network involving the O4 hydroxyl group of **8**, the water molecule W454, the N2 nitrogen atom of the inhibitor and the Pro201 carbonyl group. Specifically, a strong hydrogen bond (of 2.75 Å) was evidenced between the Pro201 carbonyl group and the water molecule W454, which in turn interacted both with the N2 (3.00 Å) and O4 (3.04 Å) atoms of the inhibitor **8**. Finally a number of van der Waals interactions with residues Asn67, Ile91, Gln92, Phe131 and Pro202 also contributed to stabilize the binding of the glycopyranosyl ring in this region of the enzyme active site. All these interactions were never before observed in any CA–inhibitor adduct characterized by means of X-ray crystallography.

As the most structurally related compound to **8** among the CA inhibitors investigated crystallographically up to now is the sugar sulfamate topiramate, **9**, we compared the binding of compounds **8** and **9** (both of them being strong CA II inhibitors)[9b,17] to this enzyme. Figure 4 shows a structural overlay of topiramate **9**, a sulfamate CA inhibitor,[9b] and the sulfonamide inhibitor **8** investigated here, both of them bound within the hCA II active site, as determined by the superposition of the enzyme active site residues. It may be observed that these two sugar-containing CA inhibitors show a very different binding mode to the enzyme. Whereas topiramate **9** compactly binds within the active site in the close vicinity of zinc (almost filling it completely due to its quite bulky tricyclic ring structure), and interacting with residues Asn62, Gln92 and Thr200 (and a water molecule),[9b] the binding of sulfonamide **8** is quite different (Fig. 4), with the phenylsulfonamide part of the

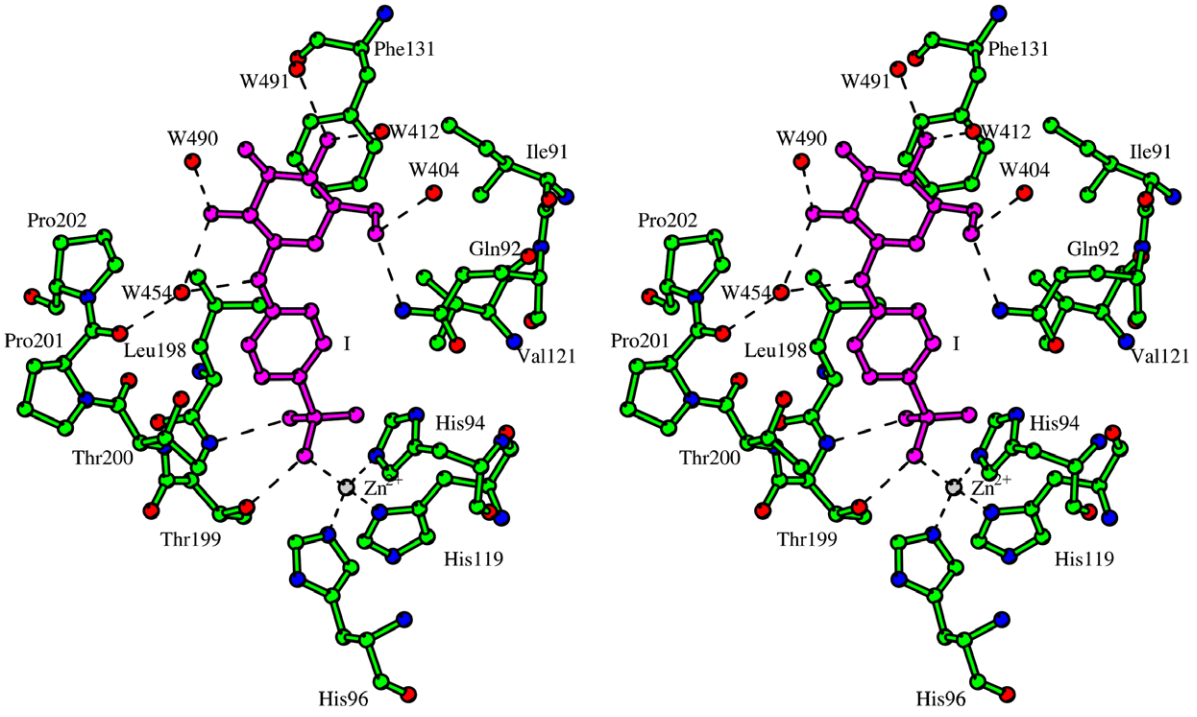


Figure 2. Stereo view of the active site region in the hCA II–**8** complex showing the residues participating in recognition of the inhibitor molecule (in magenta). Hydrogen bonds and the active site Zn^{2+} ion coordination are also shown (dotted lines).

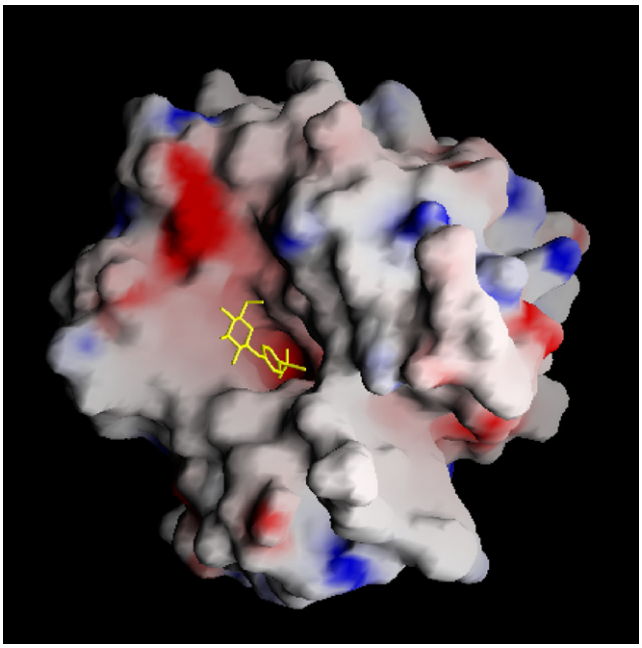


Figure 3. Electrostatic surface potential of hCA II in its complex with sulfonamide **8**. Polar atoms are coloured in red (negatively charged) and blue (positively charged). The inhibitor molecule is shown in yellow.

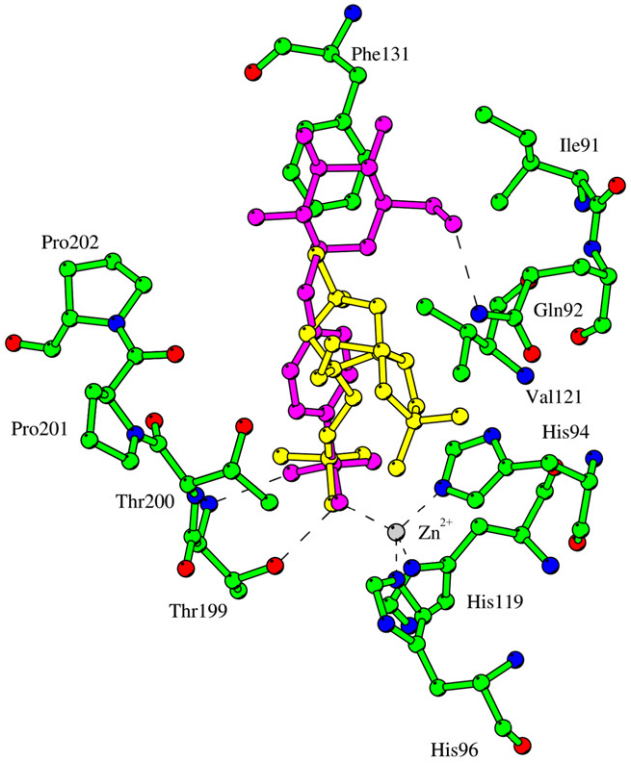


Figure 4. Superimposition of hCA II–inhibitor adducts: topiramate **9** is shown in yellow, whereas **8** is shown in magenta. Amino acid residues important for binding of these inhibitors, the Zn^{2+} ion and its three protein ligands are also shown.

molecule occupying the central channel of the enzyme, but the glucosyl moiety extending towards the hydrophilic half of the active site. In this region, very few inhibitors were observed to bind up to now, after analyzing most of the CA–inhibitor adducts deposited in PDB,[14] and this may lead to important consequences for the drug design of such agents. Indeed, the type of interactions observed in the hCAII–**8** adduct has never been reported up to now in other hCA II–inhibitor complexes, and they may explain the potent inhibitory activity of **8** towards this isozyme on one hand, and on the other one, they can be used for the rational drug design of other sugar-based CA inhibitors. In fact, exploiting the binding of inhibitors in the hydrophilic half of the active site, eventually correlated with the presence in their molecule of other moieties which may extend towards the hydrophobic part (where most of the other sulfonamide CA inhibitors investigated earlier were shown to bind),[7–15] may lead to compounds with an even better inhibitory activity. It must also be mentioned that the sulfonamide/sulfamate zinc binding groups of the two compounds **8** and **9** are very much superimposable in the two structures (Fig. 4) proving once again that potent CA inhibitors may be obtained both from the sulfonamide, sulfamate (and of course sulfamide) classes of derivatives.

In conclusion, *N*-(4-sulfamoylphenyl)-α-D-glucopyranosylamine, a potent inhibitor of several CA isozymes, was also shown to be a promising antiglaucoma agent with topical activity in an animal model of the disease. In order to understand the strong enzyme inhibitory activity of this sugar derivative, the high resolution X-ray crystal structure of its adduct with the target isoform involved in glaucoma, CA II, was determined and reported in this work. This sugar sulfanilamide derivative bound to the enzyme in a totally new manner as compared to other CA–inhibitor adducts investigated up to now. Thus, the sulfonamide zinc binding anchor and the phenyl ring of the inhibitor bound in the expected way, that is, being coordinated to the metal ion, and filling the channel of the enzyme cavity, respectively. However, the glycosyl moiety responsible for the high water solubility of the compound was oriented towards the hydrophilic region of the active site, where other inhibitors were never observed to be bound up to now. A relay of seven hydrogen bonds with four water molecules and the amino acid residues Pro201, Pro202 and Gln92 further stabilized this enzyme–inhibitor adduct. The only other sugar derived CA inhibitor for which the X-ray crystal structure was reported in adduct with CA II, the antiepileptic sulfamate topiramate, was shown earlier to bind in a completely different manner to the enzyme as compared to the sulfonamide investigated here. These findings may be useful for the design of tight-binding, sugar-derived enzyme inhibitors with applications in therapy.

Acknowledgments

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19. Eriksson, A. E.; Jones, T. A.; Liljas, A. *Proteins Struct. Funct.* **1988**, *4*, 274, The hCA II–**8** complex was obtained by adding a five molar excess of the inhibitor to a 10 mg/mL protein solution in 100 mM Tris–HCl, pH 8.5. The hanging drop vapour diffusion method was used for the crystallization. The drop consisted of 2 μL of the complex solution and 2 μL of the precipitant solution containing 2.5 M $(NH_4)_2SO_4$, 0.3 M NaCl, 100 mM Tris–HCl (pH 8.4) and 5 mM of 4-(hydroxymercurybenzoate) to improve the crystal quality. Crystals appeared after 2–3 days at 22 °C and were isomorphous to those of the native enzyme belonging to the space group $P2_1$ with unit cell parameters of $a = 42.07$ Å, $b = 41.12$ Å, $c = 72.06$ Å and $\beta = 104.51°$. X-ray diffraction data were collected at Synchrotron source Elettra in Trieste, using a Mar CCD detector, at 100 K. A cryoprotectant solution was made by inclusion of 15% (v/v) glycerol in the reservoir solution. Data were indexed and reduced with DENZO and SCALEPACK modules of the HKL suite.[21] Native hCA II crystallized in the $P2_1$ space group containing the active-site zinc ion without any water molecule (1CA2.pdb)[19] was used as the starting model for rigid body refinement followed by energy minimization and isotropic thermal factor (*B*-factor) refinement within CNS 1.0.[20] Clear density for the inhibitor was observed in the difference map after this single round of refinement (*R*-free = 0.335; *R*-factor = 0.311). Introduction of the inhibitor and alternating cycles of addition of water, manual rebuilding and energy minimization and *B*-factor refinement gave a final model with *R*-free = 0.197 and *R*-factor = 0.176. The statistics for refinement are summarized in Table 1. Coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (Accession code 2HL4).
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