Articles

Nonaromatic Sulfonamide Group as an Ideal Anchor for Potent Human Carbonic Anhydrase Inhibitors: Role of Hydrogen-Bonding Networks in Ligand Binding and Drug Design

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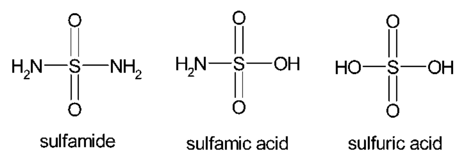
X-ray crystal structures of the adducts of human carbonic anhydrase (hCA) isozyme II with derivatives incorporating a sulfamide or sulfamic acid moiety are reported. The absence of a $C-SO_2NH_2$ bond in the first type of compound can be exploited for the design of more potent and selective CA inhibitors. This study also explains why sulfate is a several-orders-of-magnitude weaker CA inhibitor compared to derivatives incorporating sulfonamide/sulfamide moieties.

Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc enzymes, present in *Archaea*, prokaryotes, and eukaryotes, encoded by three distinct and evolutionarily unrelated gene families: the α-CAs (present in vertebrates, eubacteria, algae, and cytoplasm of green plants), the β-CAs (predominantly in eubacteria, algae, and chloroplasts of both mono- as well as dicotyledons), and the γ-CAs (mainly in *Archaea* and some eubacteria).[1−3] In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) have been described, which possess different subcellular localization and tissue distribution.[1−3] These enzymes catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, 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, and many other physiologic or pathologic processes.[1−3] Thus, it is not surprising that many of these isozymes have been discovered as important targets for inhibitors with clinical applications.[2,3] Almost all of the most potent inhibitors of CAs contain a terminal sulfonamide as the anchoring group to coordinate the catalytic zinc. These sulfonamides are widely used clinically, mainly as antiglaucoma agents but also for the therapy of other diseases, e.g., increased intracranial pressure, various neurological/neuromuscular pathologies such as epilepsy, genetic hemiplegic migraine, and ataxia, tardive diskinesia, hypokalemic periodic paralysis, essential tremor and Parkinson's disease, and mountain sickness. Accordingly, drugs of this pharmacological class are under constant development.[2,3]

Chart 1



The apparent importance of this class of inhibitors calls upon an important question: what makes the sulfonamide group such a unique and prominent moiety to coordinate Zn in CAs, since for other zinc-containing enzymes a wide variety of functional groups such as hydroxamates, carboxylates, thiols, and phosphates have been proven to be successful?[1−4] In this study, we report a comparative crystallographic study of hCA II complexes with the two simplest archetypal sulfonamides (Chart 1): sulfamide ($H_2NSO_2NH_2$) and sulfamic acid (H_2NSO_2OH). The results help elucidate the intrinsic stabilizing factors for the binding of the sulfonamide moiety and provide a rationale for the unique, ideally tailored properties of this anchoring group for CAs.

Two classes of CA inhibitors (CAIs) have been studied crystallographically: the metal-complexing anions and the sulfonamides with a terminal SO_2NH_2 group that coordinates to the Zn(II) ion. Simple anions such as HS^-, CN^-, NCO^-, N_3^-, HSO_3^-, I^-, and $HCOO^-$ may bind with (distorted) tetrahedral geometry or form (distorted) trigonal-bipyramidal coordination,[2−9] whereas the sulfonamides replace the water coordinated to zinc and the

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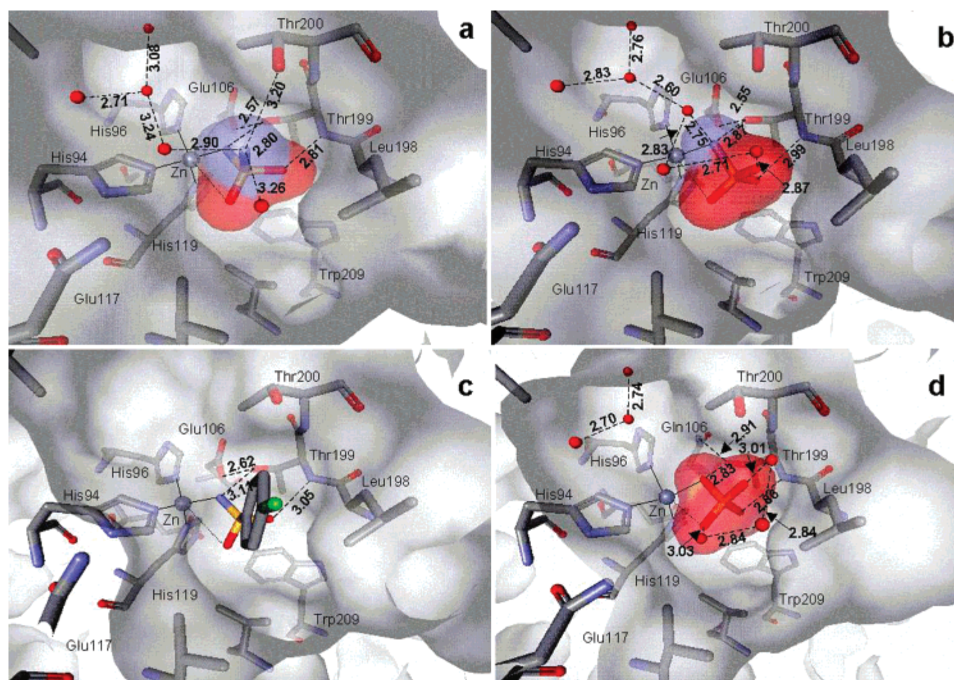


Figure 1. (a) Binding mode of sulfamide, presumably present as $(NH_2)SO_2NH^-$: direct contacts to zinc (solid lines); hydrogen bonds (dashed lines); extracoordination (dotted line). All distances are in angstroms. Water molecules are shown as red spheres, and the transparent solvent-accessible surface is indicated (produced by WebLabViewer). (b) Binding mode of sulfamate, supposedly binding as OSO_2NH^{2-} anion to hCA II. (c) Binding mode of *p*-fluorophenylsulfonamide to hCA II taken from 1if4 (pdb code). (d) Binding mode of sulfate, presumably present as SO_4^{2-}, to hCA II (E106Q) taken from 1cak (pdb code).

"deep-water" hydrogen-bonded to Thr199NH. Both waters are present in the uncomplexed state.[10] Similarly to the zinc-bound water that experiences a significant pK_a shift to about 7[11] and thus binds in a negatively charged deprotonated form, the sulfonamides are also present in the negatively charged form with one single proton at nitrogen, as evidenced by NMR.[12] They give rise to a tetrahedral geometry at Zn, with the nitrogen atom of the sulfonamide coordinating to the Zn(II) ion.[4,8]

It has recently been shown that sulfamide and sulfamic acid act as moderate hCA II inhibitors, with inhibition constants of 1130 μM (for sulfamide) and 390 μM (for sulfamic acid) at pH = 7.4, respectively.[14] This contrasts remarkably to the strongly reduced affinity of the sulfate ion (1−2 M) toward hCA II. In agreement, no zinc-bound sulfate has been observed in crystals of native hCA II, even when equilibrated with high sulfate concentrations at pH values as low as 5.7.[10,16] To better understand the deviating properties of these isostructural zinc anchoring groups, we performed a comparative crystallographic study of hCA II adducts with sulfamide and sulfamic acid, the simplest derivatives incorporating a sulfonamide moiety. The relevance of these data for the drug design of novel CA inhibitors is also discussed, considering the fact that new such inhibitors with modified zinc-anchoring moieties were reported ultimately and some of them might possess interesting pharmacological applications.

Results

Crystallographic properties and details about the data collection of the studied complexes are shown in Table 1, whereas the spatial arrangements around the Zn(II) ion in the complexes of hCA II with sulfamide, sulfamate, 4-fluorobenzenesulfonamide, and sulfate are shown in Figure 1a−d.

Table 1. Statistics of Data Collection and Refinement for hCA II−Sulfamate and hCA II−Sulfamide

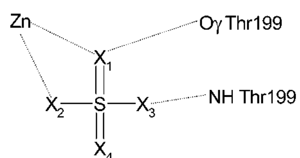
	hCA II−sulfamate	hCA II−sulfamide
space group	$P2_1$	$P2_1$
a (Å)	42.32	42.70
b (Å)	41.48	41.70
c (Å)	72.44	73.00
β (deg)	104.0	104.6
temp (K)	100	r.t.
wavelength (Å)	1.00	1.5418
resolution (Å)	1.4	1.6
refln collected	93 047	112 997
refln unique	39 027	30 369
redundance	2.4	3.7
completeness (%)	83.1	91.2
R_{merge} (%)	6.9	6.7
refinement *R* (%)	16.5	17.3
R_{free} (%)	18.1	19.9

Discussion

Suitable crystals with sulfamide and sulfamic acid were grown and used for data collection. In Table 1, the crystallographic properties and details about the data collection of the studied complexes are summarized.[17−19] After structure refinement, the spatial arrangement in the neighborhood of the catalytic zinc is revealed (Figure 1).

Both complexes have been determined with high resolution. However, a proper discussion of the ligand binding modes to hCA II requires a unique assignment of oxygen and nitrogen to the four terminal sites of the tetrahedral ligands and some assumptions about their protonation states in the binding pocket. Both can only be concluded indirectly from structural evidence and from an appreciation of elaborate H-bond networks. First, we assign the tetrahedral vertex X1 next to zinc and Thr199Oγ (Chart 2). As is usually assumed for sulfonamide/CA complexes,[4a] the sulfamide binds to the

Chart 2



zinc ion (Figure 1a) most likely via its amide group (Zn−N:, 1.76 Å). In coordination chemistry, the zinc ion is considered to be on the borderline between hard and soft; thus, ligand binding through nitrogen is frequently observed.[4a] Furthermore, this nitrogen donates an H bond to Oγ of Thr 199 via its remaining hydrogen, similar to the catalytic water coordinated to Zn in the active enzyme.[4a] Thus, Oγ acts as an acceptor for this NH group. In turn, the hydroxyl group of Thr199 donates a hydrogen bond to one of the terminal carboxylate oxygens of the adjacent Glu106. Via its second oxygen, this carboxylate mediates a hydrogen-bond network as acceptor to the backbone NH of Arg246 and via a water molecule to the hydroxyl oxygen of Tyr7.

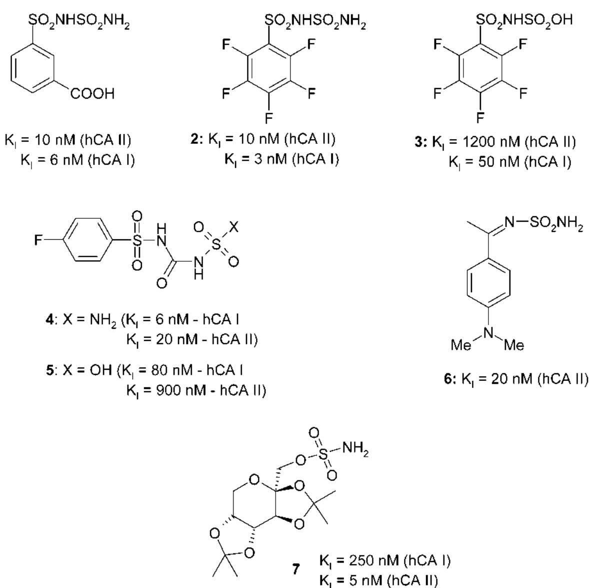
Next, we assign the vertex X2 of the sulfamide moiety at 3.14 Å (dotted line) from the zinc to an oxygen. This extended extracoordination results in a distorted tetrahedral arrangement around the metal ion, the remaining three ligands of zinc being His 94, His 96, and His 119. We then attribute the tetrahedral site X3 close to the backbone NH of Thr199 (2.81 Å) to the second oxygen of the SO_2 group, which accordingly accepts an H bond from Thr199NH. This oxygen replaces the "deep water" in the ligand-free enzyme. This situation is similarly discussed in other sulfonamides complexes of CA.[4,8,19,20] Finally, the remaining site X4 is allocated to the second, presumably uncharged, NH_2 group of sulfamide, in accordance with the binding mode observed for other sulfonamide inhibitors where this group is usually replaced by an aromatic moiety (Figure 1c).[21] It forms a hydrogen bond of 2.90 Å to an adjacent water molecule, which in turn is involved in an H-bond network to three other water molecules in the binding pocket. In addition, this latter sulfamide nitrogen forms a second weak hydrogen bond to the OH group of Thr 200 (3.20 Å) and a third contact is found to another water molecule (3.26 Å). In summary, the simple sulfamide, supposedly present as the negatively charged $(NH)SO_2NH_2^-$ ion, shows a large number of favorable contacts in the binding pocket of CA, preserving the hydrogen-bond network of the active enzyme and replacing the zinc-bound and "deep" waters. [4,8,19,20]

In the hCA II−sulfamate complex, the bound tetrahedral species shows a different spatial orientation resulting in a deviating H-bond network (Figure 1b). As for the sulfamide ion, the site X1 coordinating zinc and facing Thr199Oγ is assigned to a monoprotonated nitrogen. Interestingly, the Zn−N distance is noticeably extended by about 0.31 Å while the bond to Oγ of Thr 199 (2.87 Å) is similar to that in the sulfamide complex. Because of a tilt of the tetrahedral ion compared to that of the former complex, the NH becomes more accessible. Thus, an additional third hydrogen bond is formed from this NH to an adjacent water molecule at 2.75 Å. This further contact probably extends the coordinative bond to zinc compared to that in the sulfamide complex. Upon assignment of N to one of the vertexes of a tetrahedron (X1), the remaining three positions can only be oxygens. The second-nearest contact (X2) of the ligand to Zn, leading to extracoordination, is about the same length as in the previous complex (3.07 Å, dotted line). The remaining two oxygens of the SO_3 moiety are involved in two other hydrogen bonds. One (X3) is with the backbone NH group of Thr 199 at 2.99 Å (comparable to the sulfamide complex where this bond is 0.18 Å shorter). The other (X4) is bound to a water molecule at 2.87 Å. This water molecule bridges a network to the three solvent molecules also present in the previously described complex in conserved positions. Furthermore, it is in contact with another water molecule located above the zinc. Estimating the protonation state of sulfamic acid in the binding pocket is difficult. Monoprotonation of the NH_2 group is very likely, however, the OH group replacing the second NH_2 in the sulfamide exhibits a pK_a of 1.2 in water.[11] Thus, the formation of a binegatively charged $(NH)SO_3^{2-}$ sulfamate ion, analogous to a sulfate ion, might occur.

Why is the presumably binegatively charged sulfate ion a much weaker hCA II ligand (1−2 M)?[22] Sulfonic acids, isostructural to sulfonamide inhibitors (besides N/O replacement), do not bind to the enzyme.[25] Liljas et al.[19] studied mutants of hCA II crystallographically that show sulfate ions in the binding site. The E106Q mutant (Figure 1d) shows the sulfate (SO_4^{2-}) in an orientation very similar to that of the $(NH)SO_3^{2-}$ ion. Here, all sites of the tetrahedral ligand are oxygens and full deprotonation is supposedly given. The oxygen (X1) coordinating zinc (2.12 Å) also interacts with Oγ of Thr 199 through a normal H bond (2.83 Å). However, in contrast to the two previous complexes and the situation with the bound catalytic water, Oγ must now donate a hydrogen bond. The conformational flexibility of a Thr OH group allows for such a donor/acceptor switching. The former acceptor site for the Thr199 γOH group, the carboxylate of Glu106, has been mutated to Gln. Thus, reversal of the H-bonding network to accommodate an acceptor between zinc and Thr199Oγ is induced by the O to NH_2 exchange upon switching from Glu to Gln. The remaining carboxamide oxygen of Gln106 accepts, as in the wild type, an H bond from the backbone NH of Arg246. The additional contacts of the remaining sulfate oxygens (X2−X4) found for the E106Q mutant are comparable to those in the $(NH)SO_3^{2-}$ complex.

Interestingly, the E106A and E106D mutants also show a bound sulfate. In the Ala mutant, removal of the side chain makes space for a newly bound water molecule that now serves as a hydrogen-bond partner for Thr199Oγ. This allows for a similar reverse of the H-bonding network concomitant with full sulfate occupancy.[19] Sulfate binding to the Asp mutant is surprising. The carboxylate of the truncated acid preserves the role of Glu106 in the wild type with less optimal H-bonding geometry. In contrast to the E106Q and E106A mutants, the sulfate is only partially occupied, thus indicating a reduced binding affinity due to the nonoptimal H-bonding network.[19] The complexes with the present archetypal ligands clearly explain the intrinsic stabilizing interactions of the sulfonamide moiety. They provide some insight into why this functional group appears to have unique properties for CA inhibition: (i) it exhibits a negatively charged, most

Chart 3



likely monoprotonated nitrogen coordinating to the Zn ion; (ii) simultaneously this group forms a hydrogen bond as donor to the oxygen Oγ of the adjacent Thr 199, and (iii) a hydrogen bond is formed by one of the SO_2 oxygens to the backbone NH of Thr 199.

In the binding of the less potent isostructural inhibitor sulfate, a strong Zn−N bond is replaced by a weak Zn−O interaction. Furthermore, the inability of sulfate to donate an H bond to Thr 199Oγ is not in accordance with an optimal hydrogen-bond network, and thus, it results in a significantly reduced binding affinity. However, it can still maintain the interaction via one of its oxygens (X3) to Thr 199NH, as for the other two ions.

An important aspect of this study arises from the fact that the two adducts of hCA II described here differ substantially from other complexes previously investigated by Liljas', Lindskog's, and Christianson's groups[4,8,9,11] with respect to one critical portion of the molecule: all compounds investigated up to now display aromatic or heterocyclic sulfonamides, and as such, they possess a $C_{ar}-SO_2NH_2$ bond. Despite the apparent resemblence between the ligands described in this study and those investigated previously, our compounds do not possess such carbon−sulfur bonds. Instead, an additional sulfur−heteroatom bond is given, more precisely a supplementary S−N bond in the case of the sulfamide and an S−O bond in the case of sulfamic acid. In fact, these moieties are able to participate in an intricate hydrogen-bonding network that differs entirely from that formed by the aromatic/heterocyclic sulfonamides (compare Figure 1a,b with Figure 1c). As a consequence, the terminal part of these two archetypal inhibitors (the H_2NSO_2 moiety) binds to Zn(II) similarly to the well-established aromatic/heterocyclic sulfonamides, but the additional heteroatom involves new and not yet observed interactions (precisely the three new hydrogen bonds). This interesting observation could be exploited for the design of novel classes of CA inhibitors.

In agreement with this design concept, some new types of inhibitors derived from sulfamide and sulfamic acid, e.g., types **1**−**6** (Chart 3), have been developed recently.[27] These compounds (sulfonylated sulfamides **1** and **2**, sulfonylated sulfamate **3**, the arylsulfonylureidosulfamide/sulfamic acids **4** and **5**, or the sulfamide Schiff base **6**) exhibit new functional groups to provide zinc binding. They show high-affinity binding to some isozymes, and they are at least 2−3 orders of magnitude more effective inhibitors compared to the aromatic sulfonamides $ArSO_2NH_2$ comprising the same Ar moiety.[3] Furthermore, some of them show an enhanced affinity toward hCA I compared to hCA II, thus leading to the possibility of developing isozyme-specific CA inhibitors. This aspect is highly desired for designing drugs devoid of side effects.[1,2]

Another inhibitor recently investigated in great detail is topiramate **7**, a clinically used antiepileptic drug, which is a nanomolar hCA II inhibitor and formally corresponds to a sulfamic acid derivative.[28] Its binding to the CA II active site has not been characterized up to now, but Figure 1b provides a good approximation for the interaction of the terminal part of this molecule with the enzyme active site. Thus, our study may support the design of novel types of CA inhibitors possessing different zinc-binding functionalities and possibly improved isozyme specificity that is superior to the properties of the classical sulfonamides.

In conclusion, the simplest parent structures for sulfonamides, the sulfamide and sulfamate anions, combine the most prominent stabilizing yet affinity-discriminating interactions with the enzyme active site, revealing the secret behind the sulfonamide/sulfamate-type inhibitors as the presently most successful and potent class of lead structures for carbonic anhydrases. Furthermore, these structures may be used for the design of new types of inhibitors that may potentially discriminate among the large number of CA isozymes presently known.

Experimental Protocols

Crystals of hCA II (from Sigma-Aldrich) were obtained by the hanging drop technique, using 0.3 mM solution of protein in 100 mM Tris-HCl buffer (pH 8.2). The drops consisted of 6 μL of the enzyme solution and 6 μL of the precipitant solution containing 2.5 M $(NH_4)_2SO_4$ in 100 mM Tris-HCl (pH 8.2) and 5 mM 4-(hydroxymercury)benzoate to promote the growth of highly oriented crystals. The drops were equilibrated by vapor diffusion against the precipitant solution at 4 °C, and crystals appeared after 10−12 days. The complexes of hCA II with inhibitors were obtained by soaking the crystals of the protein for 5 days in a solution of 2.5 M $(NH_4)_2SO_4$ in 100 mM Tris-HCl (pH 8.2) and about 1 M inhibitor at 4 °C. The soaked crystals were isomorphous to the native enzyme, being monoclinic $P2_1$ with the following cell parameters: $a = 42.32$ Å, $b = 41.48$ Å, $c = 72.44$ Å, and $\beta = 104.0°$ for the hCA II−sulfamate; $a = 42.70$ Å, $b = 41.70$ Å, $c = 73.00$ Å, and $\beta = 104.6°$ for the hCA II−sulfamide. For each crystal, the Fourier maps $2F_o - F_c$ and $F_o - F_c$ were calculated, where F_c and phases were obtained from the native hCA II model from which all the water molecules have been omitted. For each experiment, the difference Fourier maps were inspected to detect evidence for inhibitor binding before the assignment of water molecules. The hCA II−sulfamide complex data were collected on an R-Axis IV imaging plate detector (Molecular Structure Corporation) mounted on a Rigaku RU-300 rotating anode generator, at room temperature using Cu Kα X-ray radiation. The crystal was mounted in a capillary and sealed with epoxy resin. The hCA II−sulfamate complex data were collected at Elettra Synchrotron in Trieste (Italy) on a 165 mm MarCCD detector at 50 mm from the crystal, using radiation of 1.00 Å

wavelength and 15 s exposure at 100 K. The data collection parameters and the refinement statistics are reported in Table 1. All calculations were done with SHELX97[17] and O.[18,29] These programs were used to build the model and to compute the Fourier maps. The last refinement cycle yielded a final R factor of 0.178 (R_{free} = 0.184) for the hCA II−sulfamate and 0.174 (R_{free} = 0.204) for the hCA II−sulfamide complex. The final temperature factor of the sulfamate atoms ranged between 4.2 and 10.3 Å^2, and that of the sulfamide atoms ranged between 15.2 and 34.3 Å^2. The final number of water molecules was 311 for the hCA II−sulfamate and 184 for the hCA II−sulfamide complex. The final rmsd's from ideal geometry for bond lengths and angles are 0.014 Å and 0.029° (sulfamate) and 0.008 Å and 0.024° (sulfamide).

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