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Carbonic anhydrase inhibitors. X-ray crystal studies of the carbonic anhydrase II–trithiocarbonate adduct—An inhibitor mimicking the sulfonamide and urea binding to the enzyme ☆

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ABSTRACT

Trithiocarbonate (CS_3^{2-}) inhibits with low micromolar affinities several mammalian carbonic anhydrases, CAs, EC 4.2.1.1 [Innocenti et al., *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1855]. Here we report the X-ray crystal structure of the hCA II–trithiocarbonate adduct. Trithiocarbonate is monodentately bound to the Zn(II) ion and makes several hydrogen bonds with Thr199 and two water molecules from the enzyme active site. Its binding is different from that of ureate, another small inhibitor isosteric with trithiocarbonate but somehow mimicks the binding of the SO_2NH moiety present in the sulfonamide inhibitors and is similar to that of bicarbonate. Compounds incorporating this new zinc-binding group, CS_2^-, may thus lead to new classes of potent inhibitors.

In a recent work[1] we demonstrated that trithiocarbonate, CS_3^{2-}, an inorganic anion rarely investigated for its interaction with metallo-enzymes, shows low micromolar inhibitory activity against the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1). This is generally unexpected for such a simple chemical species, even if inorganic anions constitute a well-known class of inhibitors of most metallo-enzymes, including CAs, but with few exceptions they show activity in the millimolar range.[2–5] Our group investigated[1–3] the interaction between all mammalian CA isozymes (i.e., CA I–XV) with many such simple inorganic anions, including the physiological ones (such as chloride, bicarbonate, carbonate, sulfate), as well as 'metal poisons' (cyanide, cyanate, thiocyanate, azide, hydrogen sulfide, bisulfite, nitrite, etc.), but also anions with less affinity for metal ions in solution, such as tetrafluoroborate, perchlorate, nitrate, fluoride and heavier halides, among others.[1–5]

The mammalian α-CA family comprises many members: 16 isoforms were described so far, CA I–CA XV, of which 13 show catalytic activity, that is, CA I–CA VII, CA IX, CA XII–CA XV (there are two type 'V' isoforms, CA VA and CA VB).[4,5] The physiological role of some CA isoforms which may work in tissues where high concentrations of inorganic anions are present,[5] is poorly understood at this moment. Thus, investigating the interactions between such chemical species and diverse CA isoforms may shed some light regarding the role of some of these enzymes in the cell, but also be helpful for the design of novel zinc-binding groups to be incorporated in new classes of tight-binding CA inhibitors (CAIs), a highly investigated topic in the last years.[1–6] Indeed, inorganic anions are usually weak CAIs, with affinities in the millimolar–submillimolar range, with few anions arriving to be low micromolar inhibitors for some isoforms (e.g., cyanide, azide, hydrogen sulfide).[1–6] Still, some of these anions have been used to design much better CAIs incorporating a zinc-binding group (ZBG) attached to an organic scaffold, which usually lead to enhanced affinity for the enzyme active site and thence much better inhibitory activity as compared to the starting inorganic anion. For example, considering HS^- as lead, some organic thiols (of the type Ar-SH or Het-SH where Ar is an aromatic, substituted benzene, and Het a heterocyclic 1,3,4-thiadiazole or 1,3,4-triazole scaffold) were shown to act as potent CAIs, with low nanomolar affinity being detected in some cases.[7]

One of the recent interesting findings regarding this class of CAIs deals with the strong inhibitory activity against several cytosolic CA isozymes of the simple anion trithiocarbonate (K_Is of 8.7–9.9 μM against hCA I, II and III).[1] In order to explain this property, we hypothesized that trithiocarbonate binds to the Zn(II) ion within the enzyme active site most probably in a bidentate manner (Fig. 1A), although the monodentate binding shown in Figure 1B has also been considered as possible.[1] As this strong anion inhibitor represents a new ZBG for obtaining CAIs, which has not been considered before for such a purpose,[1] we decided to understand

☆ The coordinates of the hCA II–trithiocarbonate adduct have been deposited in PDB, ID code 3K7K.

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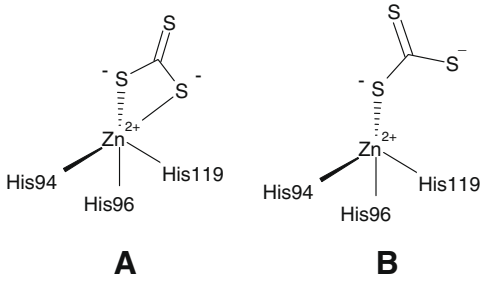


Figure 1. Putative, proposed[1] binding modes of trithiocarbonate to the CA active site: (A), bidentate; (B) monodentate binding.

in detail its binding mode to the enzyme by means of X-ray crystallography, a technique of great interest for exploring protein–ligand interactions.[8,9] In fact, the mono- or bidentate binding of the inhibitor to the metal ion is of crucial importance for designing more complex, and presumably also better CAIs possessing this new ZBG found in trithiocarbonate. In this letter we report the high resolution X-ray crystal structure of trithiocarbonate bound to the active site of the ubiquitous, physiologically dominant isoform hCA II.

Crystals of the hCA II–trithiocarbonate[10–15] adduct were isomorphous with those of the native protein,[11] allowing for the determination of the crystallographic structure by difference Fourier techniques. The refined structure presented a good geometry with r.m.s.d. from ideal bond lengths and angles of 0.009 Å and 1.42°, respectively (Table 1). The overall quality of the model was good with all residues in the allowed regions of the Ramachandran plot (data not shown). Refinement statistics are summarized in Table 1. Inspection of the electron density maps at various stages of the refinement, showed features compatible with the presence of one molecule of inhibitor bound within the active site (Figs. 2 and 3). As shown in Figure 2 and Table 2, trithiocarbonate is monodentately bound to the Zn(II) ion within the CA II active site, with the Zn–S distance of 2.1 Å. Thus, our initial hypothesis[1] that trithiocarbonate may be a bidentate zinc ligand (due to its dinegative charge 'neutralizing' the Zn^{2+} ion) when bound to CAs, as presented in Figure 1A, has not been confirmed.

Trithiocarbonic acid (an unstable compound, similarly to carbonic acid) is a much stronger acid compared to H_2CO_3, with a

Table 1
Crystallographic parameters and refinement statistics of the hCA II–trithiocarbonate adduct

Parameter	Value
Crystal parameter	
Space group	$P2_1$
Cell parameters	a = 42.0Å
	b = 41.4 Å
	c = 72.2 Å
	β = 104.4°
Data collection statistics (70.0–1.9 Å)	
No. of total reflections	101,898
No. of unique reflections	37,400
Completeness (%)[a]	95.2 (90.6)
$I/\sigma(I)$	13.7 (2.5)
R-sym (%)	17.0 (38.0)
Refinement statistics (10.3–1.9 Å)	
R-factor (%)	19.4
R-free (%)[b]	24.8
Rmsd of bonds from ideality (Å)	0.009
Rmsd of angles from ideality (°)	1.24

[a] Values in parentheses relate to the highest resolution shell (2.02–1.9 Å).
[b] Calculated using 5% of data.

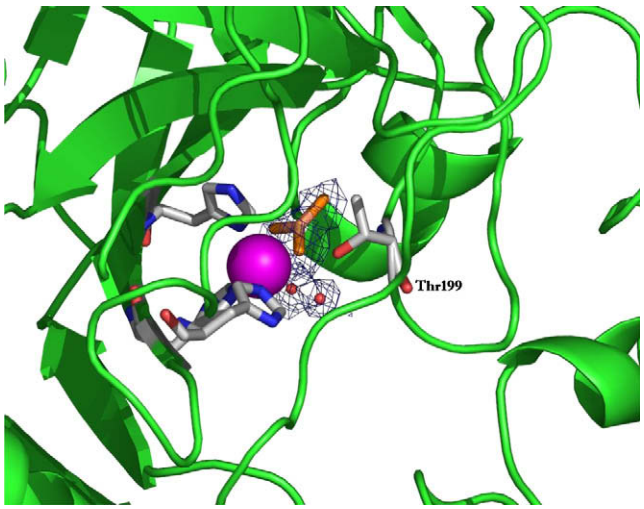


Figure 2. Electron density at 1σ of trithiocarbonate (yellow and gold) bound within the hCA II active site. The electron density of the two water molecules (w162 and w179) making hydrogen bonds with the bound inhibitor are also presented. The catalytically critical Zn(II) ion is shown as the violet sphere, and its three protein ligands, His94, His96 and His119 in CPK colors. Thr199, a conserved amino acid residue involved in catalysis and binding of inhibitors is also shown, whereas the protein backbone is in green.

pK_{a1} of 2.7 and pK_{a2} of 8.1,[16] compared to pK_{a1} of 6.36 and pK_{a2} of 10.33, respectively, for carbonic acid.[17] As a consequence, at the pH at which the experiments have been performed, of 8.1, the only species of trithiocarbonate present in solution is CS_3^{2-}, the one we observed in the X-ray structure reported here. Bitritiocarbonate (HCS_3^-), the analogous species to bicarbonate should presumably be present in very acidic solutions (pH <2.7) which is not the case in our experiments. Furthermore, literature data show it to be of low stability, whereas trithiocarbonate is reported to be highly stable in solution and solid phase.[16] Thus, we can rule out the hypothesis that another species than trithiocarbonate, such as bitrithiocarbonate or bicarbonate are being modeled in the electron density presented in Figure 2, where the two water molecules interacting with the inhibitor are also shown (w162 and w179). The fact that we have indeed three sulfur atoms in the inhibitor molecule is also confirmed by the C–S distance in the hCA II–trithiocarbonate adduct. All these three bonds have the same distance of 1.56 Å (whereas the C–O distance, for example, in the urea–hCA II adduct is of 1.27 Å, whereas the C–N one, in the same adduct, of 1.34 Å).[18]

It should be noted that even if the three sulfur atoms from tritiocarbonate are equivalent, when the anion is bound to the enzyme, these three atoms experience different contacts and interactions, becoming non-equivalent. As a consequence they have been crystallographically labeled as S_A, S_B and S_D as shown in Table 2. S_D, the atom coordinated to the zinc ion, also participates in a hydrogen bond (of 2.9 Å) with the OH group of Thr199, an amino acid residue conserved in all α-CAs, which plays an important role in catalysis and inhibition of these enzymes.[4] It should be noted that in all other hCA II–inhibitor adducts in which the inhibitor atom directly bound to the metal ion has not a proton, such a hydrogen bond has not been yet evidenced, also because Thr199 makes a hydrogen bond (as a H-bond donor) with the carboxylate of Glu106.[11,18,19] Our finding thus either unravels a completely new binding mode in a CA–inhibitor adduct, never evidenced before for non-protonated inhibitors, or evidenced for the first time a breaking of the gate-keeper residues (Thr199–Glu106)[11] hydrogen bonding pattern. This may also explain the unexpectedly potent inhibitory effects of this small anion inhibitor compared to other such anions (bicarbonate, carbonate, which are 100–100 times less effective CA inhibitors). All atoms of the inhibitor

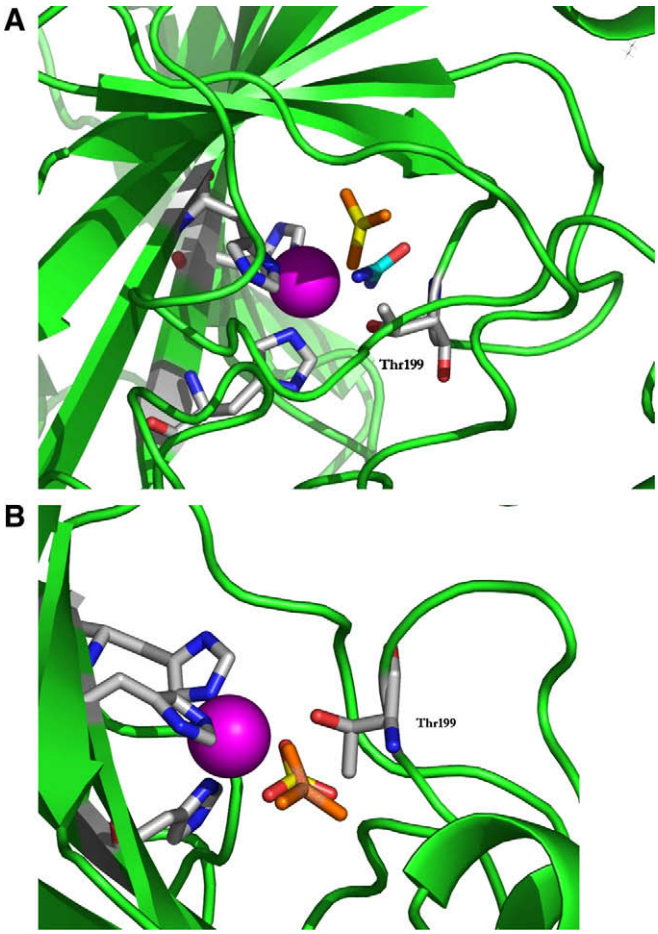
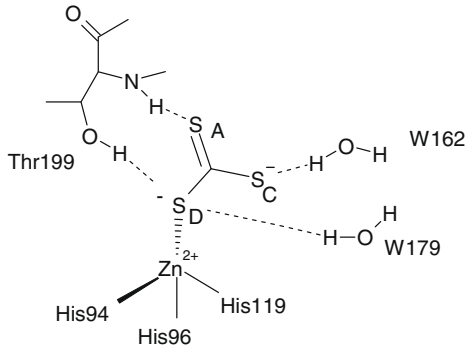


Figure 3. (A) Superposition between hCA II–trithiocarbonate (yellow and gold, PDB code 3K7K) and hCA II–ureate (sky, PDB code 1BV3) adducts. The Zn(II) ion (violet sphere), its three protein ligands (His94, His96 and His119, in CPK colors) and Thr199, are also evidenced, whereas the protein backbone is in green and all these elements are completely superposable between the two adducts. B. Superposition between hCA II–trithiocarbonate (orange, PDB code 3K7 K) and hCA II–bicarbonate (yellow-red, PDB file 2vvb[20]). Trithiocarbonate is not superposable on the bicarbonate structure, but the two anions bind in a rather similar manner to the enzyme.

Table 2
Contacts and distances between various atoms of trithiocarbonate and the metal ion, amino acid residues and water molecules in the hCA II–trithiocarbonate adduct



K_2CS_3	hCA II residue	Distance (Å)
S_D	Zn	2.1
S_C	Zn	3.7
S_D	Oγ1 Thr199	2.9
S_D	w179	2.8
S_D	Nε2 His94	3.2
S_A	N Thr199	3.2
S_C	w162	3.0

The 3 equiv sulfur atoms of the anion are non-equivalent crystallographically and are denominated S_A, S_C and S_D, as shown schematically below. The three C–S bonds in trithiocarbonate bound to the enzyme are all of 1.56 Å (data not shown), ruling out the hydrolysis of the anion to carbonate or thiocarbonate.

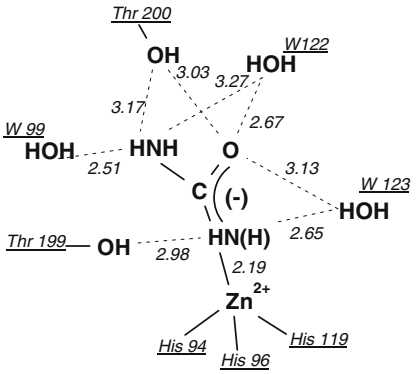


Figure 4. Hydrogen bond network of the hCA II–ureate complex. Adapted from Ref. 16.

(as well as the Zn(II) ion, and the waters near it—w162 and w179—have B factors of 1), proving that the occupancy is of 100%. This is different, for example, from the hCA II–bicarbonate complex reported recently by Sjoblom et al.,[20] in which only 50% of the hCA II adduct is with bicarbonate, the remaining 50% being sulfate bound to the Zn(II) ion. This is obviously due to the fact that sulfate and bicarbonate are very weak CA II inhibitors compared to trithiocabonate.[1]

The same sulfur atom, S_D, makes a second strong hydrogen bond (of 2.8 Å) with a water molecule (W179) and a van der Waals contact with the Nε2 atom of the imidazole ring of His94, one of the Zn(II) ligands. Whether the hydrogen bond with Thr199 is observed in all adducts of hCA II with inhibitors binding to the metal ion,[8,9,11,16] the second interaction described above, has rarely been evidenced before in other hCA II–inhibitor adducts. In fact, only in the hCA II–ureate complex, reported by us earlier (Fig. 3A and Fig. 4), the nitrogen atom of urea coordinated to the Zn(II) ion was observed to form a hydrogen bond (of 2.65 Å) with a water molecule.[18] However, urea binding to hCA II is a special case, since neutral urea does not inhibit (and does not bind) to the enzyme, even when very high concentration of urea are incubated with the enzyme for prolonged periods of time.[18] The urea(te) adduct that we have crystallized in complex with hCA II has been obtained from cyanamide incubated with hCA II and it has been formed by the CA-catalyzed hydrolysis of cyanamide bound within the enzyme cavity.[19] The formed ureate was subsequently firmly fixed within the active site by coordination to the metal ion and the network of eight hydrogen bonds (with Thr199, Thr200 and several water molecules) in which it participates (Fig. 3B).[16] On the other hand, the van der Waals interaction between the trithiocarbonate sulfur atom coordinated to Zn(II) and His94 has never been observed earlier in any hCA II–inhibitor adduct except the present one.

A second sulfur atom of trithiocarbonate, labeled S_C, is at 3.7 Å from the Zn(II), similarly with the oxygen of the SO_2NH_2 moiety, found in many classical CAIs of the sulfonamide, sulfamate or sulfamide type.[8,9,11,18] For such CAIs, the inhibitors coordinates as sulfonamidate anion to the metal ion, with the nitrogen atom the the SO_2NH^- moiety bound to Zn(II), at a distance of 2.0–2.1 Å, comparable to the Zn–S distance in the tritiocarbonate adduct reported here (2.1 Å, see Table 2), whereas one oxygen of the SO_2 moiety is at 3.6–3.8 Å away from the metal ion (again comparable with ther distance of the S_C atom from zinc, of 3.7 Å). However, for

sulfonamide/sulfamate/sulfamides adduct, this oxygen does not participate in any other interactions, whereas S_C makes a strong hydrogen bond (of 3.0 Å) with another water molecule from the enzyme active site (Table 2). S_A, the third sulfur of trithiocarbonate, on the other hand, makes a hydrogen bond of 3.2 Å with the backbone NH of Thr199, similarly with the other oxygen atom of the SO_2 moiety present in sulfonamide/sulfamate/sulfamide CAIs.[8,9,11,18] Thus, trithiocarbonate is fixed within the enzyme cavity by coordination to the metal ion and participation in a network of four strong hydrogen bonds as well as several van der Waals contacts with amino acid residues and water molecules in the neighborhood of the Zn(II) ion.

Even if trithiocarbonate and ureate share the same planar geometry and some common features of binding to the CA II active site, as shown in Figure 3A, their orientation when complexed to the enzyme cavity is quite different. In fact ureate is somehow tilted and orientated towards the hydrophilic part of the bottom of the CA II active site, whereas trithiocarbonate binds in a more central part of the active site, extending its three arms towards the metal ion and then symmetrically in both the hydrophilic and hydrophobic halves of the enzyme cavity. Furthermore, this simple inorganic molecule shares some similarity with both sulfonamide/sulfamate/sulfamide CAIs.[8,9,11] as well as with urea(te), a particularly strange but very potent CAI, formed in situ by cyanamide hydrolysis within the enzyme cavity.[18,19] In fact, trithiocarbonate combines both the sulfonamide as well as the ureate positive features for binding to the active site of these enzymes with high affinity, but also leading to reversible inhibition (ureate produces an irreversible inhibition, as mentioned earlier).[18,19] However, as shown in Figure 3B, trithiocarbonate binds in a more similar manner to bicarbonate to hCA II active site, although the two inhibitors are not superposable, as the sulfur containing anion is much bulkier compared to bicarbonate (and obviously, their protonation state is different, due to the important differences in pK_a between the two acids, discussed above).

But what are the consequences of these findings for the development of better CAIs? In fact we have already shown,[1] that the CS_2^- ZBG is a very valid alternative to the sulfonamide-based ones for designing interesting CAIs. The widely used reagents in analytical chemistry dialkyldithiocarbamates[21] (of which sodium *N,N*-diethyl-dithiocarbamate is a well-known example) were already tested for the inhibition of cytosolic CAs, confirming that they behave as even better CAIs compared to trithiocarbonate. Thus, *N,N*-diethyl-dithiocarbamate was a more potent CAI as compared to trithiocarbonate, inhibiting hCA I with a K_I of 0.79 μM (11 times better than trithiocarbonate which had a K_I of 8.7 μM); hCA II with a K_I of 3.1 μM (2.8 times better than trithiocarbonate); hCA III with a K_I of 6.5 μM (1.5 times better than trithiocarbonate), hCA VII with a K_I of 1.47 mM (24.6 times better than trithiocarbonate); and mCA XIII with a K_I of 56 μM (7.6 times better than trithiocarbonate), respectively.[1] Thus, again a simple modification of the lead molecule had important consequences for obtaining tighter-binding CAIs. Work is in progress to evaluate the way in which the dithiocarbamates bind to the enzyme and to design more potent such inhibitors, based on the present X-ray crystal data of the hCA II–trithiocarbonate complex reported here.

In conclusion, we report the X-ray crystal structure of the human hCA II adduct with trithiocarbonate. The many interactions leading to the high affinity of this small anion for the enzyme active site have been evidenced and explained at molecular level. Trithiocarbonate is monodentately bound to the Zn(II) ion and makes several hydrogen bonds with Thr199 and two water molecules from the enzyme active site. Its binding is different from that of ureate, another small inhibitor isosteric with trithiocarbonate but somehow mimicks the binding of the SO_2NH moiety present in the sulfonamide inhibitors. Compounds incorporating this new zinc-binding group found in trithiocarbonate, CS_2^-, may thus lead to new classes of potent CA inhibitors.

Acknowledgments

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10. Crystals of the complex between hCA II and potassium trithiocarbonate (Sigma–Aldrich, Milan, Italy) were obtained by using the hanging-drop method for co-crystallizing the protein with the ligand, as previously described.[11] A monochromatic experiment at the Cuα wavelength was performed on a crystal of hCA II grown in the presence of the ligand by the rotation method on a PX-Ultra sealed-tube diffractometer (Oxford Diffraction) at 100 K. The crystal diffracted up to 1.90 Å resolution and belonged to space group *P*21 (a = 42.0 Å, b = 41.4 Å, c = 72.2 Å and $\alpha = \gamma = 90°$, $\beta = 104.4°$). Data were processed with CRYSALIS RED (Oxford Diffraction 2006).[12] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2[11e] as starting model. The refinement was carried out with the program REFMAC5;[13] model building and map inspections were performing using the COOT program.[14] The final model of the complex hCA II–trithiocarbonate had an *R*-factor of 19.4% and *R*-free 24.8%, with a rms deviation from standard geometry of 0.009 Å in bond lengths and 1.24° in angles. The correctness of stereochemistry was finally checked using PROCHECK.[15]
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