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Carbonic anhydrase inhibitors. Inhibition of the zinc and cobalt γ-class enzyme from the archaeon *Methanosarcina thermophila* with anions

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Abstract—Anions represent the second class of inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), in addition to sulfonamides, which possess clinical applications. The first inhibition study of the zinc and cobalt γ-class enzyme from the archaeon *Methanosarcina thermophila* (Cam) with anions is reported here. Inhibition data of the α-class human isozymes hCA I and hCA II (cytosolic) as well as the membrane-bound isozyme hCA IV with a large number of anionic species such as halides, pseudohalides, bicarbonate, carbonate, nitrate, nitrite, hydrosulfide, bisulfite, and sulfate, etc., are also provided for comparison. The best Zn-Cam anion inhibitors were hydrogen sulfide and cyanate, with inhibition constants in the range of 50–90 μM, whereas thiocyanate, azide, carbonate, nitrite, and bisulfite were weaker inhibitors (K_Is in the range of 5.8–11.7 mM). Fluoride, chloride, and sulfate do not inhibit this enzyme appreciably up to concentrations of 200 mM, whereas the substrate bicarbonate behaves as a weak inhibitor (K_i of 42 mM). The best Co-Cam inhibitor was carbonate, with an inhibition constant of 9 μM, followed by nitrate and bicarbonate (K_Is in the range of 90–100 μM). The metal poisons were much more ineffective inhibitors of this enzyme, with cyanide possessing an inhibition constant of 51.5 mM, whereas cyanate, thiocyanate, azide, iodide, and hydrogen sulfide showed K_Is in the range of 2.0–6.1 mM. As for Zn-Cam, fluoride, chloride, and sulfate are not inhibitors of Co-Cam. These major differences between the two γ-CAs investigated here can be explained only in part by the different geometries of the metal ions present within their active sites.

1. Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metallo-enzymes, present in prokaryotes and eukaryotes, being encoded by at least five distinct, evolutionarily unrelated gene families: the α-class CAs (present in prokaryotes from the *Bacteria* domain, algae, cytoplasm of green plants and vertebrates), the β-class CAs (predominantly in *Bacteria*, algae and chloroplasts of both mono- as well as dicotyledons), the γ-class CAs (mainly in *Archaea* and some *Bacteria*), the δ-class, found in a marine diatom (*Thalassiosira weisflogii*), and the very recently isolated ε-CAs, found in cyanobacteria and some chemolithoautotrophic bacteria, respectively.[1–9] These enzymes are very efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate, but at least the α-class CAs possess a high versatility, being able to catalyze different other hydrolytic processes such as the hydration of cyanate to carbamic acid, or of cyanamide to urea; the aldehyde hydration to *gem*-diols; the hydrolysis of carboxylic, or sulfonic acids esters, as well as other less investigated hydrolytic processes, such as hydrolysis of halogeno derivatives, arylsulfonyl halides, and other hydrolyzable substrates.[1,2,4,6] It is not known whether reactions catalyzed by CAs other than the hydration of CO_2 or dehydration of HCO_3^- may have physiological relevance in organisms where these enzymes are present. The catalytic mechanism of the α-CAs is understood in great detail. The

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active site consists of a Zn(II) ion co-ordinated by three histidine residues and a water molecule or hydroxide ion. The latter is the catalytically active species, acting as a potent nucleophile attacking CO_2.[1,2,4,6] For β- and γ-CAs, the zinc hydroxide mechanism is valid also, although at least some β-class enzymes do not have water directly coordinated to the metal ion.[2] CAs possess two main classes of inhibitors: the inorganic anions (such as cyanide, cyanate, thiocyanate, azide, and hydrogen sulfide) and the unsubstituted sulfonamides with the general formula RSO_2NH_2 (R = aryl, hetaryl, perhaloalkyl).[1,4] Several important physiological and physiopathological functions are dependent on the CA isozymes present in organisms over the entire phylogenetic tree. In mammals, CAs are essential for respiration and transport of CO_2 and bicarbonate between metabolizing tissues and the lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues and organs. CAs are also important in mammalian biosynthetic pathways such as lipogenesis, gluconeogenesis, and ureagenesis, among others. In plants, algae and prokaryotes, CAs are essential for fixation of CO_2.[1,2,4] The presence of these ubiquitous enzymes in so many tissues and in so many different isoforms, and in all domains of life, represents an attractive goal for the design of inhibitors or activators with biomedical and environmental applications.[1,4]

The prototype of the γ-class CAs, 'Cam' has been isolated from the methanogenic archaeon *Methanosarcina thermophila*.[2,5,6] The crystal structures of zinc-containing and cobalt-substituted Cam were reported in the unbound form and co-crystallized with sulfate and bicarbonate.[10,11]

Cam has several features that differentiate it from the α-class and β-class CAs. Thus, the protein fold is composed of a left-handed β-helix motif interrupted by three protruding loops and followed by short and long α-helixes. The Cam monomer self-associates in a homotrimer with the approximate molecular weight of 70 kDa.[2,5,6,10,11] The Zn(II) ion within the active site is coordinated by three histidine residues, as in α-class CAs. However, relative to the tetrahedral coordination geometry seen at the active site of α-class CAs, the active site of Cam contains additional metal-bound water ligands, so that the overall coordination geometry is trigonal bipyramidal for the zinc-containing Cam and octahedral for the cobalt-substituted enzyme.[2,5,6,10,11] Two of the His residues coordinating the metal ion belong to one monomer (monomer A) whereas the third one is from the adjacent monomer (monomer B). Thus, the three active sites are located at the interface between pairs of monomers.[2,5,6,10,11] The catalytic mechanism of γ-class CAs was proposed to be similar with the one presented for the α-class enzymes.[2] Still, the finding that Zn(II) is not tetracoordinated as originally reported[11] but pentacoordinated,[10] with two water molecules bound to the metal ion, demonstrates that much is still to be understood regarding these enzymes. At this moment, the zinc hydroxide mechanism is accepted as being valid for γ-class CAs, as it is probable that an equilibrium exists between the trigonal bipyramidal and the tetrahedral species of the metal ion from the active site of the enzyme (Eq. 1).

$$(His)_3Zn^{2+}(OH_2)_2 \leftrightarrow (His)_3Zn^{2+}(OH_2) \leftrightarrow (His)_3Zn^{2+}OH^- + H^+ \quad (1)$$

Bicarbonate and sulfate bound to the active site were shown to make contacts with the side chain of Glu 62 in a manner that suggests this side chain to be protonated.[10] In the uncomplexed zinc-containing Cam, the side chains of Glu 62 and Glu 84 appear to share a proton; additionally, Glu 84 exhibits multiple conformations.[10] This suggests that Glu 84 may act as a proton shuttle, which is an important aspect of the reaction mechanism of α-CAs, for which a histidine active site residue generally plays this function (usually His 64).[1,2,4] Anions, such as bicarbonate or sulfate were reported to bind to Cam,[10,12,13] but no inhibition data were provided.[10] Here we report the first detailed inhibition study of zinc- and cobalt-substituted Cam with anions to further characterize Cam and the γ-class CAs.

2. Chemistry

Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, hydrogen sulfite and sulfate) were of highest purity available, and were used without further purification. Recombinant Cam isozymes were obtained as previously reported.[10–12] The recombinant α-CA isozymes used for comparison in this study were obtained as previously reported.[14–20]

3. CA inhibition

Inhibition data against three α-class CA isozymes, and Zn(II)- and Co(II)-Cam are shown in Table 1.

Although CA inhibition by anions has been discovered quite early,[23] very few quantitative and accurate data on this subject are presently available in the literature, and they all regard the α-class CA isozymes (CA I, II, IV, V, and IX).[21,22,24]

As seen from data of Table 1, the γ-class enzymes Zn-Cam and Co-Cam are inhibited by anions, their affinity for this class of inhibitors being very different from that of the α-class CA isozymes. As shown in Table 1, both the γ-class and α-class enzymes are inhibited by anions; however, the pattern of inhibition is remarkably different among all the enzymes indicating that the active site environment exerts a profound influence on the effectiveness of this suite of inhibitors. The hCA I is quite susceptible to this class of inhibitors, whereas the hCA II or hCA IV isozymes are more resistant to inhibition by anions and very susceptible to inhibition by sulfonamides.[1,4,21] For Zn-Cam, the strongest inhibition has been observed with the poisonous, metal complexing anions hydrosulfide and cyanate, which showed inhibition constants in the range of 50–90 μM, whereas cya-

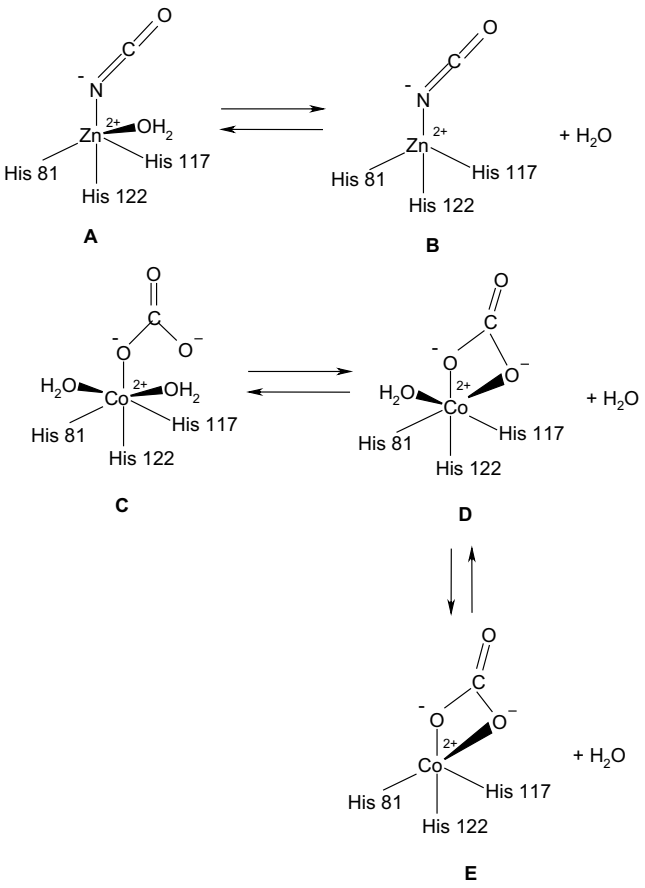
Table 1. Inhibition constants of anionic inhibitors against α-isozymes hCA I, II, IV and γ isozymes Zn-Cam and Co-Cam, for the CO_2 hydration reaction, at 20 °C[20]

Inhibitor	K_i (mM)[a]				
	hCA I	hCA II	hCA IV	Zn-Cam	Co-Cam
F^-	>300[b]	>300[b]	—	>200	>200
Cl^-	6[b]	200[b]	36[b]	>200	>200
Br^-	4[b]	63[b]	52[b]	160	22.2
I^-	0.3[b]	26[b] (35)[c]	11[b]	160	5.3
CNO^-	0.0007[b]	0.03[b]	0.03[b]	0.09	4.7
SCN^-	0.2[b]	1.6[b]	—	7.0	6.1
CN^-	0.0005	0.02	—	0.68	51.5
N_3^-	0.0012	1.5[b]	—	5.8	4.9
HCO_3^-	12[b]	85[b]	44[b]	42	0.10
CO_3^{2-}	15	73	—	6.7	0.009
NO_3^-	7[b]	35[b]	—	36.5	0.09
NO_2^-	8.4	63	—	6.8	7.3
HS^-	0.0006	0.04	—	0.05	2.0
HSO_3^-	18	89	—	11.7	1.8
SO_4^{2-}	63	>200	44	>200	>200

[a] Errors were in the range of 3–5% of the reported values, from three different assays.
[b] From Ref. 21.
[c] From Ref. 22.

nide was slightly less effective an inhibitor, with a K_i value of 0.68 mM (Table 1). These anions are on the other hand much stronger hCA I inhibitors, whereas their affinity for hCA II and hCA IV is of the same order of magnitude as that for the γ-class Cam except cyanide, which is a much stronger hCA II inhibitor. The next potent inhibitors of Zn-Cam were thiocyanate, azide, nitrite, and hydrogen sulfite, with inhibition constants in the range of 5.8–11.7 mM. Thiocyanate acts as a stronger hCA I and II inhibitor, whereas carbonate, nitrite, and hydrogen sulfite are more potent Zn-Cam inhibitors. This may be explained by the fact that these last three anions are able to act as bidentate ligands to the active site metal ion, whereas thiocyanate is unable to act in such a manner. Thus, considering the preference of the Zn(II) ion for trigonal bipyramidal geometries in Zn-Cam and tetrahedral geometries in α-class CA isozymes, it may be hypothesized that the bidentate binding of carbonate, nitrite, and hydrogen sulfite to the Zn(II) ion of the Zn-Cam active site explains these inhibition data. But it is also interesting to note that bicarbonate, nitrate, or sulfate, which could also act as bidentate ligands, showed much weaker inhibition (bicarbonate and nitrate, with K_i values in the range of 36.5–42 mM) or no inhibition (sulfate, with a K_i value >200 mM) of the γ-class enzyme. Very weak inhibitory effects were shown with bromide and iodide, although these anions appreciably inhibit hCA I, and to a less extent hCA II. Fluoride and chloride showed no detectable inhibition of Zn-Cam, similarly to sulfate. A striking result was the rather large difference of inhibition observed with carbonate and bicarbonate, the former being more than seven times a stronger inhibitor than the latter. This result is in contrast to isozymes hCA I and II for which the two anions possess inhibition constants in the same range (12–15 mM against hCA I, and 73–85 mM, against hCA II, respectively). It is difficult to explain these results presently, especially considering the fact that carbon dioxide, bicarbonate, and carbonate are also substrates of these enzymes. The unexpected behavior for Zn-Cam inhibition by carbonate and bicarbonate extends to Co-Cam; however, Co-Cam has the further distinction that carbonate is a much more potent inhibitor, with a K_i value of 9 μM, followed by bicarbonate and the isostructural nitrate, which showed K_i values of 90–100 μM. The difference between these data for Co-Cam and the corresponding data against Zn-Cam are remarkable and suggests that the identity of the metal determines the strength of inhibition. It must also be noted that the metal poisons cyanate, thiocyanate, cyanide, azide, or hydrogen sulfide are much weaker Co-Cam inhibitors as compared to their behavior against the Zn-Cam and α-class isozymes, with K_i values in the range of 2.0–51.5 mM, whereas bisulfite or iodide act as efficient inhibitors, with K_i values in the range of 1.8–5.3 mM. A weaker inhibitor was bromide (K_i of 22 mM), whereas fluoride, chloride, and sulfate do not inhibit this enzyme, similarly with their behavior against Zn-Cam.

Trying to rationalize the differences of inhibitory power of anions toward the two metal-substituted Cam enzymes investigated here, by considering the geometric preferences of Zn(II) and Co(II), may be rather speculative, but we propose the following explanation (Scheme 1). Thus, for the zinc enzyme: it is well known that this metal ion prefers tetrahedral and trigonal bipyramidal geometries in metallo-proteins.[1,2] This means that the anion adducts investigated here, such as, for example, the cyanate complex, may possess either the trigonal bipyramidal geometry **A**, in which a water molecule is also bound to zinc, or the tetrahedral geometry **B**, when this coordinated water is lost. An equilibrium between these two forms is also probable, as shown in Scheme 1. This proposal explains why the monodentate anions such as cyanate, cyanide, and hydrogen sulfide are much stronger Zn-Cam inhibitors as compared to the bidentate anions such as bicarbon-



Scheme 1.

ate, carbonate, nitrate, nitrite, hydrogen sulfide, etc. Cobalt on the other hand has a preference for higher coordination numbers (five and six) in many proteins, including CAs.[2,28] Considering the adduct of Co-Cam with carbonate, it is rather improbable that the monocoordinated, octahedral structure **C** is stable. On the other hand, carbonate can be bidentately bound to Co(II), either in an octahedral geometry of the metal ion (as in **D**) or in a trigonal bipyramidal geometry (as in **E**), after losing a water molecule. It is rather probable that complex equilibria take place between these species presented here. Such a behavior would explain the higher affinity of bidentate ligands (such as bicarbonate, carbonate, nitrate, or hydrogen sulfite) for Co-Cam as compared to the monodentate anions, as well as the difference of inhibition with these anions between the two metal-substituted enzymes investigated here. A much more precise insight regarding the binding of these anions to Zn- and Co-Cam will be obtained by resolving the X-ray crystal structures of some of these adducts.

In conclusion, Zn(II)- and Co(II)-substituted Cam show a very different behavior against anion inhibitors, both by comparing the two enzymes between themselves, or by comparing them with the α-class isozymes hCA I, II, and IV. The best Zn-Cam anion inhibitors were hydrogen sulfide and cyanate, with inhibition constants in the range of 50–90 μM, whereas thiocyanate, azide, carbonate, nitrite, and bisulfite were weaker inhibitors (K_i values in the range of 5.8–11.7 mM). The inhibition of Zn-Cam by sulfide is puzzling since Cam is proposed to be located outside the cytoplasmic membrane where it is exposed to high levels of sulfide in the anaerobic environments where *M. thermophila* and other *Methanosarcina* species are found.[25] On the other hand, the identity of the metal ion in Cam isolated directly from *M. thermophila* has not been determined since only Cam overproduced in *Escherichia coli* has been characterized. Thus, if cobalt is the physiologically relevant metal, sulfide would have less of an inhibitory effect based on the results presented here. Fluoride, chloride, and sulfate do not inhibit either Zn-Cam or Co-Cam appreciably up to concentrations of 200 mM. This lack of inhibition may represent an adaptation to marine environments high in chloride and sulfate where *M. thermophila* and other *Methanosarcina* species are able to proliferate.[26,27] Whereas the substrates bicarbonate and carbonate behave as weak inhibitors of Zn-Cam, the best Co-Cam inhibitor was carbonate, with an inhibition constant of 9 μM, followed by nitrate and bicarbonate (K_i values in the range of 90–100 μM). The metal poisons were generally much less effective inhibitors of Co-Cam compared to Zn-Cam, with cyanide possessing an inhibition constant of 51.5 mM for Co-Cam, whereas cyanate, thiocyanate, azide, iodide, and hydrogen sulfide showed K_i values in the range of 2.0–6.1 mM for Co-Cam. These major differences between the two γ-class CAs investigated here can be explained only in part by the different geometries of the metal ions present within their active sites. Regardless of the various mechanisms of inhibition of either the α-class or γ-class CAs, the results presented here demonstrate that both the identity of the metal and the active site environment adjacent to the metal profoundly influence the ability of the anions to inhibit these enzymes. The results further indicate that, although all CAs catalyze the same reaction, the active sites can be significantly different. This proposal is reinforced by the diverse active site architectures of the five independently evolved classes as revealed by the crystal structures available up to now for enzymes from the α-, β-, and γ-classes.[1,2]

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