Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 14 (2004) 4563–4567
Bioorganic & Medicinal Chemistry Letters

Carbonic anhydrase inhibitors. Inhibition of the beta-class enzyme from the methanoarchaeon *Methanobacterium thermoautotrophicum* (Cab) with anions

Alessio Innocenti,[a] Sabrina Zimmerman,[b] James G. Ferry,[b] Andrea Scozzafava[a] and Claudiu T. Supuran[a,*]

[a]*Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy*
[b]*Department of Biochemistry and Molecular Biology, Eberly College of Science, Pennsylvania State University, University Park, PA 16802-4500, USA*

Received 29 April 2004; revised 31 May 2004; accepted 7 June 2004

Abstract—The first inhibition study of the β-class carbonic anhydrase (CA, EC 4.2.1.1) from the methanoarchaeon *Methanobacterium thermoautotrophicum* (Cab) 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 and the γ-class enzyme from another archaeon, *Methanosarcina thermophila* (Cam) with a large number of anionic species such as halides, pseudohalides, bicarbonate, carbonate, nitrate, nitrite, hydrosulfide, bisulfite, sulfate, etc., are also provided for comparison. The best Cab anion inhibitors were thiocyanate and hydrogen sulfide, with inhibition constants in the range of 0.52–0.70 mM, whereas cyanate, iodide, carbonate, and nitrate were weaker inhibitors (K_i's in the range of 7.8–13.2 mM). Fluoride, chloride, and sulfate do not inhibit this enzyme appreciably, whereas the CA substrate bicarbonate, or other anions, such as bromide, nitrite, bisulfite, or sulfamate behave as weak inhibitors (K_i in the range of 40–45 mM). It is interesting to note that the metal poison, coordinating anions cyanide and azide are also rather weak Cab inhibitors (K_i in the range of 27–55 mM), whereas sulfamide is a very weak Cab inhibitor (K_i of 103 mM), although it strongly inhibits Cam (K_i of 70 μM). Surprisingly, phenylboronic and phenylarsonic acids, which have been investigated for the inhibition of all these CAs for the first time, showed very weak activity against the α-CA isozymes, but were effective Cab and Cam inhibitors. The best Cab inhibitors were just these two compounds (K_i's of 0.20–0.33 mM), whereas the best Cam inhibitor was sulfamic acid (K_i of 96 nM). These major differences of behavior between the diverse CAs investigated here toward anion inhibitors can be difficultly explained considering the convergent evolution of so diverse enzymes for the binding and turnover of small molecules such as carbon dioxide and anions.

1. Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) are excellent examples of convergent evolution of catalytic function.[1] Indeed, these ubiquitous metalloenzymes are present in prokaryotes and eukaryotes, being encoded by at least five distinct, evolutionarily unrelated gene families: the α-CAs (in prokaryotes from the *Bacteria* domain, algae, cytoplasm of green plants and vertebrates—with 14 isozymes presently known in humans), the β-CAs (predominantly in *Bacteria*, algae, and chloroplasts of both mono- as well as dicotyledons), the γ-CAs (mainly in *Archaea* and some *Bacteria*), the δ-CAs, found so far only in a marine diatom (*Thalassiosira weisflogii*)—which were the first cadmium-enzymes ever reported, and the very recently described ε-CAs, isolated in cyanobacteria and some chemolithoautotrophic bacteria, respectively.[1–9] Genes encoding enzymes from all classes have been identified in the prokaryotes with the β- and γ-classes predominating.[1] These enzymes catalyze the reversible hydration of carbon dioxide to bicarbonate, but at least the α-CAs possess other catalytic activities.[2] CAs are important to many eukaryotic physiological processes such as respiration, CO_2 transport, electrolyte secretion, and photosynthesis among others.[1–3] Although ubiquitous in highly evolved organisms from

* Corresponding author. Tel.: +39-055-457-3005; fax: +39-055-457-3385; e-mail: claudiu.supuran@unifi.it

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.06.073

the *Eukarya* domain, these enzymes have received scant attention in prokaryotes from the *Bacteria* and *Archaea* domains and CA has been purified from only five such species since it was first identified in *Neisseria sicca* in 1963.[1,7] Recent work has shown that CAs are widespread in metabolically diverse species from both the *Archaea* and *Bacteria* indicating that the enzyme has a more extensive and fundamental role in prokaryotic biology than originally recognized.[1–9] Interestingly, many prokaryotes contain CA genes from more than one class, some even containing genes from three of the known classes (i.e., α, β, and γ). In addition, some prokaryotes contain multiple genes encoding CAs from the same class. The presence of multiple CA genes within a species underscores the importance of this enzyme in prokaryotic physiology; however, the role(s) played by these CAs are still largely unknown.[5,6] Even though most of the information known about the function(s) of CA primarily relates to its role in cyanobacterial CO_2 fixation, some prokaryotic enzymes have also been shown to function in cyanate degradation and the survival of intracellular pathogens within their host.[5,6]

The active site of hCA II, one of the best studied α-CAs is shown in Figure 1A. It may be seen that the metal ion (essential for catalysis) is coordinated by three histidine residues and a water molecule/hydroxide ion that participates in a hydrogen bond network involving residues Thr 199 and Glu 106.[1–4] Many diverse organisms contain CAs belonging to the β-class, such as, for example, the methanoarchaeon *Methanobacterium thermoautotrophicum*.[10] The principal difference between these enzymes and the α-CAs investigated in detail since they were discovered in the 30-s,[1–4] consists in the fact that usually the β-CAs are oligomers, generally formed of 2–6 monomers of molecular weight of 25–30 kDa. The X-ray crystal structure of four β-CAs is available at this moment: the enzyme isolated from the red alga *Porphyridium purpureum*,[11] the enzyme from chloroplasts of *Pisum sativum*,[12] another prokariotic enzyme, from *Escherichia coli*[13] and Cab, the enzyme described in the archaeon *M. thermoautotrophicum*.[10,14]

The *P. purpureum* CA monomer is composed of two internally repeating structures, being folded as a pair of fundamentally equivalent motifs of an α/β domain and three projecting α-helices. The motif is very distinct from that of either α- or γ-CAs. This homodimeric CA appears like a tetramer with a pseudo 2–2–2 symmetry.[11] β-CAs are thus very different from the α-class enzymes (Fig. 1A). The Zn(II) ion is essential for catalysis in both families of enzymes, but its coordination is different and rather variable for the β-CAs: thus, in the red alga β-CA the Zn(II) ion is coordinated by two cysteinate residues, an imidazole from a His residue and a carboxylate belonging to an Asp residue (Fig. 1B), whereas the chloroplast enzyme has the Zn(II) ion coordinated by two cysteinates, the imidazole belonging to a His residue, and a water molecule (Fig. 1C).[11–14] The polypeptide chain folding and active site architecture is obviously very different from those of the CAs belonging to the α-class. Cab exists as a dimer with a subunit fold similar to that observed in plant-type β-CAs.[14] The active site zinc ion was shown to be coordinated by the amino acid residues Cys 32, His 87, and Cys 90, with the tetrahedral coordination completed by a water molecule (Fig. 1C). The major difference between plant- and Cab-type β-CAs is in the organization

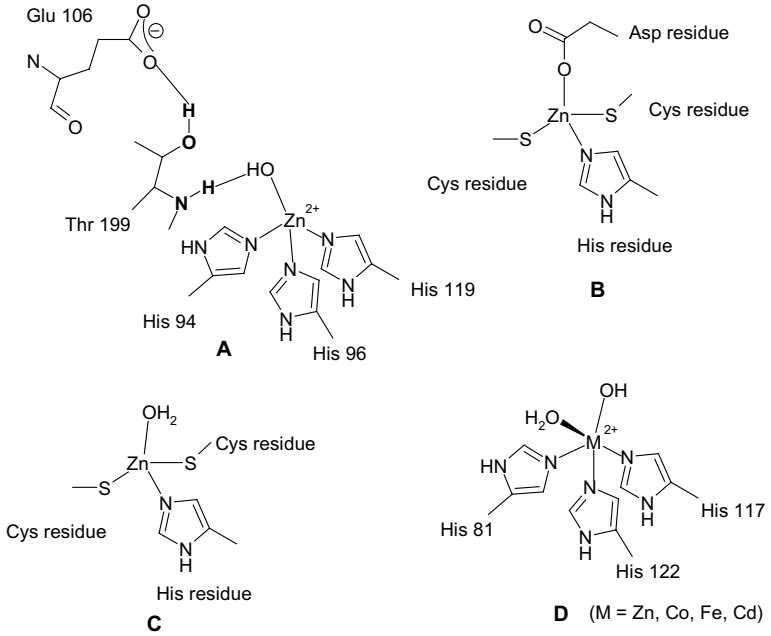

Figure 1. Metal ion coordination sphere in the five classes of CAs: **A**—α-CAs (hCA II numbering); **B**—*P. purpureum* and *E. coli* β-CAs; **C**—*P. sativum* chloroplast and *M. thermoautotrophicum* β-CAs; **D**—γ-class CA from *M. thermophila* (M is presumably Zn(II), Co(II), or Fe(II)—which can be either tetra-, penta-, or hexacoordinated by additional water molecules; Cam numbering of the His residues) and δ-class CA from *Thalassiosira weissflogii* (M = Cd(II) or Zn(II)—the exact geometry of the metal ion is not known). No information is available yet regarding the metal ion coordination for the ε-class CAs.

of the hydrophobic pocket. The X-ray crystal structure of Cab also revealed a Hepes buffer molecule bound 8 Å away from the active site zinc, which suggests a possible proton transfer pathway from the active site to the solvent.[14]

The prototype γ-CA, Cam, has several features that differentiate it from the α- and β-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.[15] The metal ion within the active site is coordinated by three histidine residues (Fig. 1D), as in α-CAs, but relative to the tetrahedral coordination geometry seen at the active site of α-CAs, the active site of this γ-CA 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. 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.[15] Little is known so far regarding the coordination of the metal ion and the metal ion requirement in the δ- and ε-class of CAs.

In recent work from our laboratories, we investigated the inhibition of various α-CAs (the mitochondrial isozyme CA V[16] and the tumor associated isozyme CA IX[17]) or the γ-CA from *Methanosarcina thermophila* (Cam)[18] with anions. Here we extend this type of study and report the first anion inhibition study of the β-CA from *M. thermoautotrophicum*, a CO_2-reducing methanoarchaeon.[10]

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. Sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid were from Sigma–Aldrich. Recombinant Cab and Cam were obtained as previously reported.[10,14,15] The recombinant α-CA isozymes used for comparison in this study were obtained as previously reported.[19]

3. CA inhibition

In a previous work[18a] we have investigated the inhibition of the γ-class enzyme Cam with anions, whereas more recently, we also reported the inhibition of both Cam as well as Cab with sulfonamides,[18b] the main class of inhibitors of these metalloenzymes.[4] Here we report the first detailed anion inhibition study of Cab, but in addition to the common inorganic anions we also included in our study sulfamide and sulfamic acid, as these are the simplest compounds incorporating a sulfonamide moiety, as well as phenylboronic acid and phenylarsonic acid, since it is well known that some of these compounds act as inhibitors of other metalloenzymes, such as, for example, the arginases, and they have not been investigated for their interaction with the CAs.[18c]

Inhibition data against three α-class CA isozymes (hCA I, II, and IV), Zn(II)-Cam and Cab with anions, sulfamide, sulfamic acid, phenylboronic, and phenylarsonic acid are shown in Table 1.

As seen from data of Table 1, Cab has an inhibition profile by anions very different from that of the α-CA isozymes CA I, II, or IV, and the γ-class enzyme Cam (the zinc-containing Cam has been used for comparison, since Cab and the other vertebrate isozymes employed in the experiments are all zinc enzymes). The best Cab inhibitors were unexpectedly, phenylboronic acid, phenylarsonic acid, thiocyanate, and hydrogen sulfide, which showed inhibition constants in the range of 0.20–0.70 mM. Surprisingly, phenylboronic acid and phenylarsonic acid, which were not tested previously for their interaction with any CA, behave as strong Cam inhibitors (K_i's in the range of 0.15–0.23 mM) but are quite weak inhibitors of the human isozymes hCA I and hCA II (K_i's in the range of 23.1–58.6 mM). For example, phenylboronic acid is almost 300 times a more potent Cab inhibitor than a hCA I inhibitor, whereas this ratio for Cam is around 250. It may be thus considered that this compound is a specific inhibitor for prokaryotic over eukaryotic CAs. It should also be noted that thiocyanate is a stronger inhibitor only against hCA I (K_i of 0.2 mM), whereas it behaves as a weaker inhibitor of hCA II or Cam. Hydrogen sulfide on the other hand is a much stronger inhibitor for hCA I, hCA II, and Cam than for Cab.

The binding of these potent inhibitors to Cab may be hypothesized to be similar to that observed for the enzymes belonging to the α- and γ-classes.[2,14,15] Thus, thiocyanate is probably directly coordinated to Zn(II) as shown in Figure 2A, replacing the water molecule/hydroxide ion present in the uninhibited enzyme. Phenylboronic acid and phenylarsonic acid probably also bind as anions (the experiments have been done at pH 7.4, at which these compounds are presumably ionized), coordinating the zinc ion as shown schematically in Figure 2B and C, in analogy to the binding of some of them (i.e., the boronic acids) to the Mn(II) ion of the metalloenzyme arginase, recently investigated by Christianson's group.[18c] These hypothesis are in the course of being verified, as work is in progress for resolving the X-ray crystal structure of adducts of these inhibitors with Cab (and Cam).

The next best inhibitors of Cab were iodide, cyanate, carbonate, and nitrate, which showed inhibition constants in the range of 7.8–13.2 mM (Table 1). Iodide is a much better Cab inhibitor than a Cam inhibitor (K_i of 160 mM), whereas this anion has rather similar potency against hCA II and hCA IV as for Cab, but is a much stronger hCA I inhibitor (this is generally the α-CA isozyme with the highest affinity for anions).[2–4]

Table 1. Inhibition constants of anionic inhibitors against α-isozymes hCA I, II, IV, γ isozyme Zn-Cam and β-isozyme Cab, for the CO_2 hydration reaction, at 20 °C[25]

Inhibitor	K_i (mM)[a]				
	hCA I	hCA II	hCA IV	Zn-Cam	Cab
F^-	>300[b]	>300[b]	nt	>200[c]	>1000
Cl^-	6[b]	200[b]	36[b]	>200[c]	152
Br^-	4[b]	63[b]	52[b]	160[c]	42.1
I^-	0.3[b]	26[b] (35)[d]	11[b]	160[c]	13.2
CNO^-	0.0007[b]	0.03[b]	0.03[b]	0.09[c]	11.2
SCN^-	0.2[b]	1.6[b]	nt	7.0[c]	0.52
CN^-	0.0005	0.02	nt	0.68[c]	27.8
N_3^-	0.0012	1.5[b]	nt	5.8[c]	55.7
HCO_3^-	12[b]	85[b]	44[b]	42[c]	44.9
CO_3^{2-}	15[b]	73[b]	nt	6.7[c]	9.6
NO_3^-	7[b]	35[b]	nt	36.5[c]	7.8
NO_2^-	8.4[b]	63[b]	nt	6.8[c]	44.8
HS^-	0.0006[b]	0.04[b]	nt	0.05[c]	0.70
HSO_3^-	18[b]	89[b]	nt	11.7[c]	45.1
SO_4^{2-}	63[b]	>200[b]	44	>200[c]	950
$H_2NSO_2NH_2$	0.31[e]	1.13[e]	nt	0.07	103
H_2NSO_3H[f]	0.021[e]	0.39[e]	nt	9.6×10^{-5}	44.0
Ph-$B(OH)_2$	58.6	23.1	nt	0.23	0.20
Ph-AsO_3H_2[f]	31.7	49.2	nt	0.15	0.33

[a] Errors were in the range of 3–5% of the reported values, from three different assays.
[b] From Ref. 16.
[c] From Ref. 18a.
[d] From Ref. 26.
[e] From Ref. 27.
[f] As sodium salt.

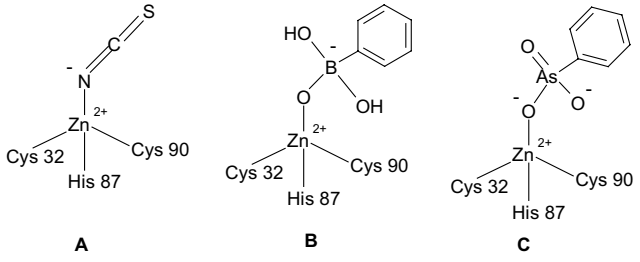

Figure 2. Proposed schematic binding of anion inhibitors to Cab: **A** thiocyanate; **B** phenylboronic acid; **C** phenylarsonic acid.

Cyanate on the other hand is a much stronger inhibitor for all these α- and γ-CAs than for Cab, whereas carbonate has very similar affinities for the two prokaryotic CAs, Cab, and Cam, being a weaker CA I and II inhibitor. Nitrate has similar affinities for hCA I and Cab, being a much weaker hCA II and Cam inhibitor. Another group of anions, including bromide, cyanide, azide, bicarbonate, nitrite, bisulfite, and sulfamate acted as weak Cab inhibitors, showing K_i's in the range of 27.8–55.7 mM (Table 1). Among them, bromide is a four times better inhibitor of Cab than of Cam, whereas its affinity for hCA II and hCA IV is in the same range as for Cab. Only hCA I has a much higher affinity for this anion. On the contrary, cyanide, azide, nitrite, and sulfamate are much better Cam than Cab inhibitors. Noteworthy is sulfamate, which is one of the most potent Cam inhibitors detected so far,[18] with an affinity of 96 nM for this enzyme. Sulfamate, whose X-ray crystal structure with hCA II has been reported,[28] is a much weaker inhibitor of these α-CAs, and it may be truly considered a Cam-specific inhibitor. It is hard to explain this very interesting behavior at this point, without an X-ray crystal structure of the Cam-sulfamate adduct. Another unexpected feature was the rather low inhibitory activity toward Cab of the metal poisons cyanide and azide, known to possess strong coordinating properties toward metal ions present within the active site of many metalloenzymes.[1–4] Thus, these anions are micromolar inhibitors of hCA I, also appreciably inhibiting hCA II, but they act as rather weak Cab and Cam inhibitors. Bicarbonate, which may also act as a substrate for CAs is itself a weak Cab inhibitor, showing a very similar K_i as for Cam and hCA IV. Sulfamide is a weak Cab inhibitor (K_i of 103 mM), but it acts as a much more potent Cam, hCA I and hCA II inhibitor. Finally, a very weak inhibitor was chloride, whereas sulfate and fluoride are practically devoid of inhibitory properties toward this enzyme (these two anions are generally the weakest CA inhibitors against enzymes from all classes investigated so far) (Table 1).

In conclusion, we report the first inhibition study with anions of the β-class CA from the methanoarchaeon *M. thermoautotrophicum*. This enzyme, Cab, showed an inhibition profile by anions very different from that of enzymes belonging to the α-class (hCA I, II, and IV) or γ-class (Zn-Cam) investigated earlier. Furthermore, Cam has also been tested for the inhibition with some compounds (sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid) for the first time. The best Cam inhibitor ever reported has been detected, which is sulfamic acid (sulfamate), showing a K_i of 96 nM against this enzyme, whereas it acts as a much weaker inhibitor of Cab and α-CAs. Surprisingly, the best Cab inhibitors were phenylboronic acid and phenylarsonic

acid (K_i's of 0.20–0.33 mM), whereas the best anions inhibiting it were thiocyanate and hydrogen sulfide. Thus, probably potent Cab inhibitors may be designed considering phenylboronic acid and phenylarsonic acid as leads molecules, whereas for Cam, such inhibitors should be designed considering sulfamic acid as lead molecule. CA inhibitors specific for the prokaryotic over the eukaryotic CAs may have important pharmacological and environmental applications. It should also be mentioned that additional studies are needed for explaining the very different behavior of these evolutionarily convergent enzymes toward small molecules, such as the substrate and the anion inhibitors.

Acknowledgements

Supported by National Institutes of Health Grant GM44661 and NASA-Ames Research Laboratory Cooperative Agreement (NCC21057) to J.G.F.

References and notes

1. Tripp, B. C.; Smith, K. S.; Ferry, J. G. *J. Biol. Chem.* **2001**, *276*, 48615–48618.
2. *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton (FL), USA, 2004; pp 1–363, and references cited therein.
3. (a) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146–189; (b) Supuran, C. T. Carbonic Anhydrases: Catalytic Mechanism, Distribution and Physiological Roles. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton (FL), USA, 2004; pp 1–24.
4. (a) Supuran, C. T.; Scozzafava, A. *Expert Opin. Ther. Pat.* **2002**, *12*, 217–242; (b) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Pat.* **2004**, *14*, 667–702.
5. Smith, K. S.; Ferry, J. G. *FEMS Microbiol. Rev.* **2000**, *24*, 335–366.
6. Smith, K. S.; Jakubzick, C.; Whittam, T. S.; Ferry, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 15185–15189.
7. Chirica, L. C.; Elleby, B.; Jonsson, B. H.; Lindskog, S. *Eur. J. Biochem.* **1997**, *244*, 755–760.
8. (a) Cox, E. H.; McLendon, G. L.; Morel, F. M.; Lane, T. W.; Prince, R. C.; Pickering, I. J.; George, G. N. *Biochemistry* **2000**, *39*, 12128–12130; (b) Lane, T. W.; Morel, F. M. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 4627–4631.
9. So, A. K.; Espie, G. S.; Williams, E. B.; Shively, J. M.; Heinhorst, S.; Cannon, G. C. *J. Bacteriol.* **2004**, *186*, 623–630.
10. (a) Smith, K. S.; Ferry, J. G. *J. Bacteriol.* **1999**, *181*, 6247–6253; (b) Smith, K. S.; Cosper, N. J.; Stalhandske, C.; Scott, R. A.; Ferry, J. G. *J. Bacteriol.* **2000**, *182*, 6605–6613; (c) Smith, K. S.; Ingram-Smith, C.; Ferry, J. G. *J. Bacteriol.* **2002**, *184*, 4240–4245.
11. Mitsuhashi, S.; Mizushima, T.; Yamashita, E.; Yamamoto, M.; Kumasaka, T.; Moriyama, H.; Ueki, T.; Miyachi, S.; Tsukihara, T. *J. Biol. Chem.* **2000**, *275*, 5521–5526.
12. Kimber, M. S.; Pai, E. F. *EMBO J.* **2000**, *19*, 1407–1418.
13. Cronk, J. D.; Endrizzi, J. A.; Cronk, M. R.; O'Neill, J. W.; Zhang, K. Y. *Protein Sci.* **2001**, *10*, 911–922.
14. Strop, P.; Smith, K. S.; Iverson, T. M.; Ferry, J. G.; Rees, D. C. *J. Biol. Chem.* **2001**, *276*, 10299–10305.
15. (a) Iverson, T. M.; Alber, B. E.; Kisker, C.; Ferry, J. G.; Rees, D. C. *Biochemistry* **2000**, *39*, 9222–9231; (b) Kisker, C.; Schindelin, H.; Alber, B. E.; Ferry, J. G.; Rees, D. C. *EMBO J.* **1996**, *15*, 2323–2330; (c) Tripp, B. C.; Ferry, J. G. *Biochemistry* **2000**, *39*, 9232–9240.
16. Franchi, M.; Vullo, D.; Gallori, E.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2857–2861.
17. Vullo, D.; Franchi, M.; Gallori, E.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *J. Enzym. Inhib. Med. Chem.* **2003**, *18*, 403–406.
18. (a) Innocenti, A.; Zimmerman, S.; Ferry, J. G.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3327–3331; (b) Zimmerman, S.; Innocenti, A.; Ferry, J. G.; Scozzafava, A.; Supuran C. T. *Bioorg. Med. Chem. Lett.*, in press; (c) Cama, E.; Shin, H.; Christianson, D. W. *J. Am. Chem. Soc.* **2003**, *125*, 13052–13057.
19. Human CA I, CA II, and CA IV cDNAs were expressed in *E. coli* strain BL21 (DE3) from the corresponding plasmids described by Lindskog's group Lindskog, S.; Behravan, G.; Engstrand, C.; Forsman, C.; Jonsson, B. H.; Liang, Z.; Ren, X.; Xue, Y. In *Carbonic Anhydrase—From Biochemistry and Genetics to Physiology and Clinical Medicine*; Botrè, F., Gros, G., Storey, B. T., Eds.; VCH: Weinheim, 1991; pp 1–13. Cell growth conditions were those described in Ref. 20 and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[21] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of $49\,mM^{-1}cm^{-1}$ for CA I and $54\,mM^{-1}cm^{-1}$ for CA II, respectively, based on $M_r = 28.85$ kDa for CA I, and 29.3 kDa for CA II, respectively.[22–24].
20. Behravan, G.; Jonsson, B. H.; Lindskog, S. *Eur. J. Biochem.* **1990**, *190*, 351–357.
21. Khalifah, R. G.; Strader, D. J.; Bryant, S. H.; Gibson, S. M. *Biochemistry* **1977**, *16*, 2241–2247.
22. Lindskog, S.; Coleman, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **1964**, *70*, 2505–2508.
23. Steiner, H.; Jonsson, B. H.; Lindskog, S. *Eur. J. Biochem.* **1975**, *59*, 253–259.
24. Baird, T. T., Jr.; Waheed, A.; Okuyama, T.; Sly, W. S.; Fierke, C. A. *Biochemistry* **1997**, *36*, 2669–2678.
25. An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA 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 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength—this anion is not inhibitory anyhow, see Table 1), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10–200 mM (in the assay buffer) and dilutions up to 0.01 μM done with the assay buffer mentioned above. Enzyme concentrations were 0.09 μM for hCA I and hCA IV, 0.01 μM for hCA II, Zn-Cam and 1 μM for Cab. Inhibition constants were calculated as described by Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573, and are the mean form three different determinations.
26. Tibell, L.; Forsman, C.; Simonsson, I.; Lindskog, S. *Biochim. Biophys. Acta* **1984**, *789*, 302–310.
27. Briganti, F.; Pierattelli, R.; Scozzafava, A.; Supuran, C. T. *Eur. J. Med. Chem.* **1996**, *31*, 1001–1010.
28. Abbate, F.; Supuran, C. T.; Scozzafava, A.; Orioli, P.; Stubbs, M. T.; Klebe, G. *J. Med. Chem.* **2002**, *45*, 3583–3587.