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Inhibition of the R1 fragment of the cadmium-containing ζ-class carbonic anhydrase from the diatom *Thalassiosira weissflogii* with anions

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ABSTRACT

We investigated the catalytic activity and inhibition of both the zinc and cadmium-containing R1 fragment of the ζ-class carbonic anhydrase (CA, EC 4.2.1.1) from the marine diatom *Thalassiosira weissflogii*. Our data prove that these enzymes are not only very efficient catalysts for the physiological reaction, but also sensitive to sulfonamide and anion inhibitors, with inhibition constants from the nanomolar to millimolar range. Acetazolamide inhibited the two enzymes with K_Is in the range of 58–92 nM. The best anion inhibitors of Cd-R1 were thiocyanate, sulfamate and sulfamide, with K_Is of 10–89 μM, whereas the best Zn-R1 anion inhibitors were sulfamate and sulfamide with K_Is of 60–72 μM. These enzymes were only weakly inhibited by chloride, bromide or sulfate, main anion components of sea water, with inhibition constants in the range of 0.24–0.85 mM. Thus, similarly to CAs belonging to other classes, the ζ-class CA (with either cadmium or zinc ions at the active site) was inhibited by both anions and sulfonamides.

The carbonic anhydrase (CA, EC 4.2.1.1.) superfamily of metalloenzyme evolved independently at least five times, with five genetically distinct enzyme classes known to date: the α-, β-, γ-, δ-, and ζ-CAs.[1–6] The α-, β-, and δ-CAs use Zn(II) ions at the active site,[1,4] the γ-CAs are probably Fe(II) enzymes (but they are active also with bound Zn(II) or Co(II) ions),[5] whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis.[6] The 3D fold of the five enzyme classes are very different from each other, as it is their oligomerization state: α-CAs are normally monomers and rarely dimers; β-CAs are dimers, tetramers or octamers; γ-CAs are trimers, whereas the δ- and ζ-CAs are probably monomers but in the case of the last family, three slightly different active sites are present on the same protein backbone which is in fact a pseudotrimer, at least for the best investigated member of the class, the enzyme from the marine diatom *Thalassiosira weissflogii*.[6] Many representatives of all these enzyme classes have been crystallized and characterized in detail, except the δ-CAs.[1–6] The best investigated class is the α-CA one, as it encodes for enzymes present in mammals, some of which are established drug targets for the design of pharmacological agents such as diuretics, antiglaucoma, antiobesity, and anticancer agents/diagnostic tools.[1–3] Recently the β-CAs, widely spread in bacteria, fungi, and plants have also started to be investigated in more detail, as they are present in some widespread human pathogens belonging to the bacteria or fungi domains, and their inhibition may lead to novel antiinfectives.[1,7–11] However, CAs are key enzymes also involved in the acquisition of inorganic carbon for photosynthesis in phytoplankton, as they catalyze efficiently the interconversion between carbon dioxide and bicarbonate.[1,6] Most of the phytoplankton operates a carbon concentrating mechanism (CCM) to increase the CO_2 concentration at the site of fixation by RuBisCO several folds over its external concentration, allowing the enzyme to function efficiently.[6] Marine diatoms possess both external and internal CAs.[12] It has been hypothesized in the model diatom *T. weissflogii* that the external CA catalyzes the dehydration of HCO_3^- to CO_2 to increase the gradient of the CO_2 diffusion from the external medium to the cytoplasm, and the internal CA in the cytoplasm catalyzes the rehydration of CO_2 to HCO_3^- to prevent the leakage of CO_2 to the external medium again.[13] One of the most remarkable findings regarding the ζ-CA from *T. weissflogii* was that this is a Cd(II)-containing enzyme, which can however also work with Zn(II) bound at the active site, and that there is a rather rapid metal exchange between zinc and cadmium, depending on the availability of metal ions in the marine environment.[6] It is not known yet where this enzyme, (denominated CDCA1) is localized in *T. weissflogii* or other diatoms.[6]

Xu et al. reported the X-ray crystal structures of two of the three repeats of CDCA1.[6] It has been thus observed that in Cd(II)-bound CDCA1 repeat 2, the metal ion resides at the bottom of a

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funnel-shaped active site pocket, and is tetrahedrally coordinated by three conserved residues, Cys263, His315, and Cys325, and a water molecule (Fig. 1),[6] which is the same metal ion coordination observed in β-CAs, but quite different from that of α-class enzymes.[1–6] A second water molecule also contributes to binding of Cd(II), and a third water molecule forms hydrogen bonds to these two water molecules.[6] In Zn-bound CDCA1 repeat 2, Zn(II) is coordinated in the same manner except the distances between zinc and the metal-binding atoms of the three coordinating residues are reduced by approximately 0.16–0.19 Å. The Cd(II)-bound CDCA1 repeat 1 had identical metal coordination as in repeat 2.[6] As the three repeats have a rather high sequence homology (Fig. 2) it is quite probable that the metal ion coordination is similar also in CDCA1 repeat 3.

The anion acetate present in high amounts in the crystallization buffer, has been found bound to CDCA1 repeat 1 and repeat 2, replacing one of the two water molecules that were hydrogen-bonded to the metal ion.[6] This finding shows that anion inhibitors probably bind in similar ways to the active site of these metalloenzymes, coordinating to the metal ion, irrespective to which CA family they belong.[1–6] However, there are no literature data regarding the interaction of ζ-CAs with inhibitors, except the X-ray crystallographic characterization of the acetate adducts mentioned above.[6] Such studies are relevant for better understanding the activity of these enzymes in their natural environment (sea water), rich in many anionic species among others. Here we report the first inhibition study of a ζ-CA, and more specifically of the first repeat (R1) of CDCA1 from *T. weissflogii* with both Cd(II) and Zn(II) bound to the active site.

The kinetic properties of full-length CDCA1 and of its repeats have been reported by Morel's group, as determined by an ^{18}O-exchange method.[6] Both enzymes showed pH dependence with higher catalytic efficiency at higher pH, as all investigated CAs to date.[1–6] The Zn-bound CDCA1 was a very efficient CA, with k_{cat}/K_m higher than CA II. k_{cat}/K_m of the Zn-bound full-length CDCA1 ranged from $3.2 \times 10^7\ M^{-1}\ s^{-1}$ to $8.6 \times 10^8\ M^{-1}\ s^{-1}$ for CO_2 hydration between pH 6.5 and 9.5, while k_{cat}/K_m of the Cd-bound full-length CDCA1 from $2.0 \times 10^6\ M^{-1}\ s^{-1}$ to $1.5 \times 10^8\ M^{-1}\ s^{-1}$. Here we used a stopped-flow assay to measure the catalytic activity of the R1 repeat of CDCA1 with both Cd(II) and Zn(II) bound to the active site, working at pH 7.5 (Table 1).[14,15]

Data of Table 1 show that both the Cd (abbreviated here Cd-R1) and the zinc (abbreviated here Zn-R1) containing enzymes investigated here show appreciable catalytic activity for the physiologic reaction, as determined by a stopped-flow assay method. Our data are thus in excellent agreement with what reported earlier by a diverse assay method.[6] Indeed, the k_{cat} of both enzymes were in the range of $(1.4–1.5) \times 10^6\ s^{-1}$ (close to the limit of diffusion controlled processes) basically identical with that of hCA II (Table 1). The k_{cat}/K_M of these enzymes were also a proof of their catalytic efficiency, with values in the range of $(1.4–1.6) \times 10^8\ M^{-1}\ s^{-1}$. Thus, together with hCA II, ζ-CAs are more efficient catalysts for CO_2 hydration compared to other isoforms, such as for example the human ones hCA I and IV (Table 1). Furthermore, the sulfonamide CA inhibitor (CAI) acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) inhibited in the nanomolar range both the Cd- and Zn-containing ζ-class enzymes, with inhibition constants of 58–82 nM (Table 1). The zinc-containing R1 had a higher affinity for acetazolamide compared to the Cd-containing enzyme. However, hCA II was the enzyme with the strongest inhibition by this sulfonamide (K_I of 12 nM for acetazolamide). As far as we know, this is the first study in which the sensitivity of ζ-CAs to sulfonamide inhibitors is investigated. Thus, similarly to enzymes belonging to the α-, β-, and γ-CA classes, the ζ-class enzymes are inhibited by the sulfonamides, the CAIs *par excellence*.

Data of Table 2 show the inhibition of Cd-R1 and Zn-R1 with a range of inorganic anions, some of which are abundant in sea water (chloride, sulfate, bromide, iodide, etc.). Other investigated anions belong to the so-called metal poisons (cyanide, thiocyanate, azide, hydrogen sulfide) whereas others (sulfamide, sulfamic acid, phenyl

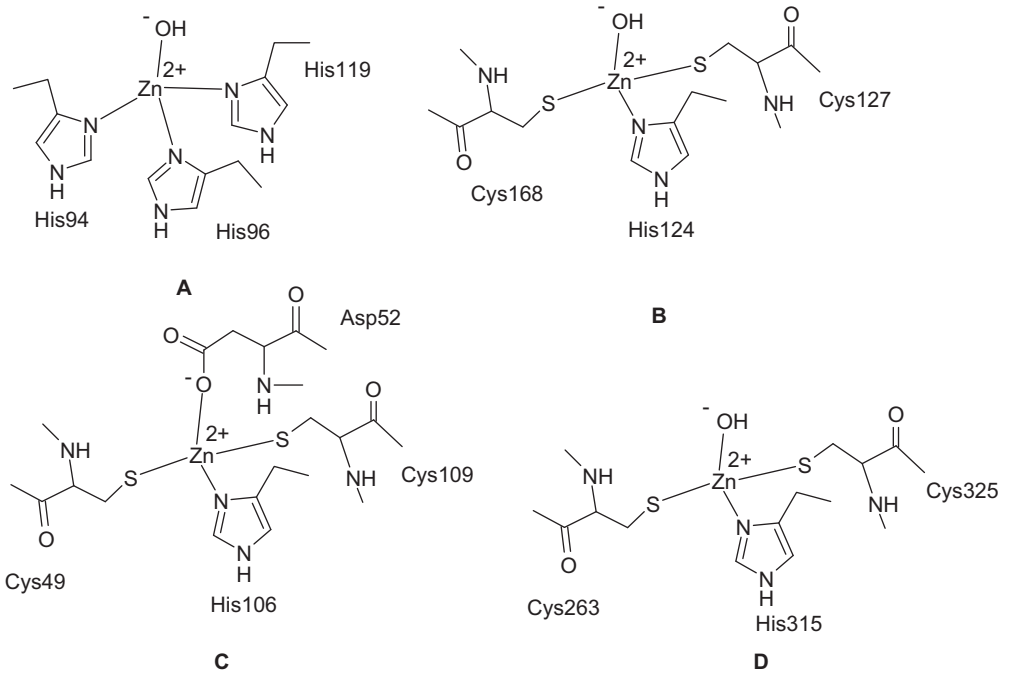

Figure 1. Metal ion coordination in CAs: (A) α-CA (hCA I numbering of amino acid residues); the γ- and δ-CAs have the same coordination of the metal ion, but for the γ-class the metal ion can also be Co(II) or Fe(II); (B) β-CAs, open active site, Can2 (the enzyme from *Cryptococcus neoformans*) numbering of amino acid residues; (C) β-CAs, closed active site, Rv3588c (an enzyme from *Mycobacterium tuberculosis*) numbering of amino acid residues; (D) ζ-CAs (Cd(II) can be also substituted by Zn(II), without loss of activity), amino acid residue numbering of the R1 fragment of the enzyme from *T. weissflogii* investigated here.

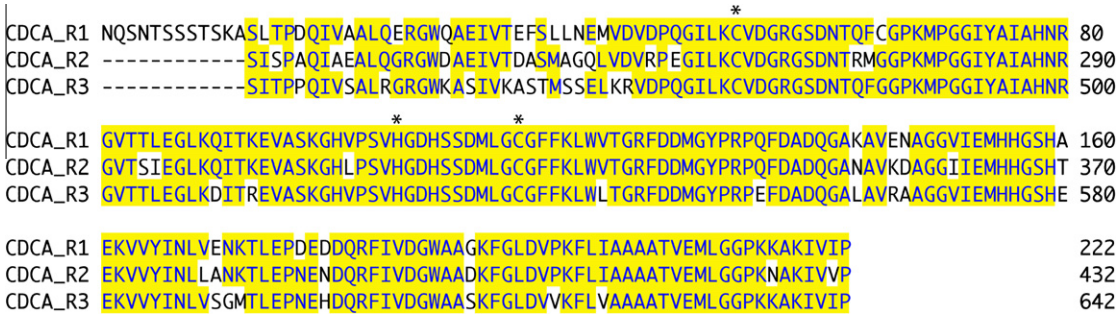

Figure 2. Sequence alignment of CDCA R1, R2, and R3 repeats. The metal-binding residues are indicated with asterisks.

Table 1
Kinetic parameters for the CO_2 hydration reaction catalyzed by α-isozymes hCA I, II, IV, and Cd/Zn-ζ-CAs

Isozyme	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I[a]	2.0×10^5	5.0×10^7	250
hCA II[a]	1.4×10^6	1.5×10^8	12
hCA IV[a]	1.1×10^6	5.1×10^7	74
Cd(II)-R1[b]	1.5×10^6	1.4×10^8	82
Zn(II)-R1 [b]	1.4×10^6	1.6×10^8	58

Experiments were performed at 20 °C and pH 7.5 in 10 mM HEPES buffer and 20 mM Na_2SO_4 (for hCA I and II) or 20 mM $NaClO_4$ (for hCA IV and Cd/Zn-ζ-CAs). Inhibition data with the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided.[15]

[a] Data from Ref. 1.

[b] This work.

Table 2
Inhibition constants of anionic inhibitors against human isozymes hCA I, II, and Cd/Zn-R1, for the CO_2 hydration reaction, at 20 °C

Inhibitor	K_I[a] (mM)			
	hCA I	hCA II	Cd-R1	Zn-R1
F^-	>300	>300	0.53	0.36
Cl^-	6	200	0.76	0.41
Br^-	4	63	0.85	0.53
I^-	0.3	26	1.12	0.61
CNO^-	0.0007	0.03	0.10	0.11
SCN^-	0.2	1.6	0.089	0.10
CN^-	0.0005	0.02	0.11	0.10
N_3^-	0.0012	1.5	0.84	0.11
HCO_3^-	12	85	0.12	0.10
CO_3^{2-}	15	73	0.13	0.11
NO_3^-	7	35	0.82	0.21
NO_2^-	8.4	63	0.88	0.58
HS^-	0.0006	0.04	0.70	0.15
HSO_3^-	18	89	0.63	0.34
SO_4^{2-}	63	>200	0.48	0.24
BF_4^-	>200	>200	>200	>200
ClO_4^-	>200	>200	>200	>200
H_2NSO_3H[b]	0.021	0.39	0.010	0.072
$H_2NSO_2NH_2$	0.31	1.13	0.065	0.060
$PhB(OH)_2$	58.6	23.1	0.69	0.61
$PhAsO_3H_2$[b]	31.7	49.2	0.60	0.52

[a] Errors were in the range of 3–5% of the reported values, from three different assays.

[b] As sodium salt.

boronic/arsonic acids) are known to inhibit CAs belonging to other classes, such as the α-, β- and γ-CAs or their metal-binding groups are present in the main class of CAIs, the sulfonamides and their bioisosteres (sulfamides, sulfamates).[1]

Cd-R1 was sensitive to all the investigated anions except tetrafluoroborate and perchlorate, which were not inhibitory up to 200 mM. This behavior was also seen for the inhibition of other enzyme classes (α-, β-, and γ-) with the two anions, which also show low affinity for metal ions in solution, not only when bound to metalloenzymes.[16] Most of the investigated anions as well as phenyl boronic/arsenic acids were millimolar inhibitors of Cd-R1, with inhibition constants in the range of 0.11–1.12 mM. Thus, halogenides were not very effective Cd-R1 inhibitors (except fluoride, the best inhibitor with an inhibition constant of 0.53 mM). Their inhibitory power decreased with the increase of the atomic weight of the halogen (opposite to the situation of hCA I or II inhibition, where iodide is a much more potent CAI compared to fluoride), which may be a positive feature for an enzyme which has to work in the marine environment rich in chloride, bromide, and iodide. Azide, nitrate, nitrite, hydrogen sulfide, bisulfite, and sulfate, as well as phenyl boronic/arsenic acids also showed inhibitory constants in the range of 0.48–0.88 mM, being thus rather weak Cd-R1 inhibitors. Better inhibition has been on the other hand observed with bicarbonate and carbonate, as well as the metal poisons cyanate and cyanide, with inhibition constants in the range of 0.10–0.13 mM. However, the best Cd-R1 inhibitors were thiocyanate, sulfamic acid (presumably the sulfamate anion) and sulfamide, which showed inhibition constants of 10–89 μM (Table 2).

The Zn(II)-containing R1 fragment, Zn-R1 showed a rather different inhibition profile compared to the corresponding cadmium-containing enzyme (Table 2). Thus, again tetrafluoroborate and perchlorate were not inhibitory, whereas the remaining anions/compounds showed K_Is in the range of 60 μM–0.61 mM. Except for cyanate, thiocyanate, and sulfamate, which were stronger Cd- than Zn-R1 inhibitors, the zinc-containing enzyme was generally more sensitive to inhibition by anions. In fact most of the investigated anions had K_Is in the range of 0.10–0.36 mM), with few of them (sulfamate and sulfamide) being more effective ones (K_Is of 60–72 μM) and few of them less effective inhibitors (heavy halogenides, nitrite, phenyl boronic and phenyl arsenic acid, with K_Is of 0.41–0.61 mM) (Table 2).

In conclusion, our data show that both the zinc and cadmium-containing R1 fragment of the ζ-class CA from a marine diatom are not only very efficient catalysts for the physiological reaction, but also sensitive to sulfonamide and anion inhibitors, with inhibition constants from the nanomolar to millimolar range. Acetazolamide inhibited these two enzymes with K_Is in the range of 58–92 nM. The best anion inhibitors of Cd-R1 were thiocyanate, sulfamate, and sulfamide, with K_Is of 10–89 μM, whereas the best Zn-R1 anion inhibitors were sulfamate and sulfamide with K_Is of 60–72 μM. These enzymes were only weakly inhibited by chloride, bromide or sulfate, the main anion components of sea water, with inhibition constants in the range of 0.24–0.85 mM.

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14. The first repeat of CDCA (R1) was cloned into pET15b expression vector (Novagen), from the plasmid originally reported by Morel's group.[6] 100 ng of vector was transformed in *E. coli* BL21-star(DE3) cells (Novagen). Recombinant colonies were grown in 1.5 mL of LB medium up to 0.6 OD/mL at 37 °C (OD was monitored at 600 nm) and expression was then induced with 0.5 mM IPTG for 16 h at 22 °C (for the Zn-bound form). For the expression of the Cd-bound protein, 0.5 mM $CdCl_2$ was added to the medium just before the IPTG induction. Bacterial cultures were harvested, lysed in 20 mM Tris–HCl, 500 mM NaCl, 1 mM PMSF, 0.05% TritonX-100, 1 mg/mL lysozyme, pH 8.0 buffer and soluble fractions were recovered after centrifugation for 20 min at 4 °C at 15 krpm and loaded on a 1 mL His-trap column (GE Healthcare). Unbound proteins were washed with 20 mM Tris–HCl, 500 mM NaCl, 40 mM imidazole, pH 8.0 buffer and the His-tagged proteins were eluted in 50 mM Tris–HCl, 200 mM NaCl, 300 mM imidazole, pH 8.0 buffer. Proteins were further purified by gel filtration chromatography, using a Superdex 200 column (GE Healthcare) in 20 mM Tris–HCl, 150 mM NaCl, pH 8.0 buffer. An LC–MS analysis of the proteins, performed using a LCQDECA XP Ion Trap spectrometer (ThermoElectron), confirmed their identity. By atomic absorption spectrometry it has been checked (and confirmed) that the corresponding metal ions were present in the protein.
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