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Purification and inhibition studies with anions and sulfonamides of an α-carbonic anhydrase from the Antarctic seal *Leptonychotes weddellii*

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ABSTRACT

A high activity α-carbonic anhydrase (CA, EC 4.2.1.1) has been purified from various tissues of the Antarctic seal *Leptonychotes weddellii*. The new enzyme, denominated lwCA, has a catalytic activity for the physiologic CO_2 hydration to bicarbonate reaction, similar to that of the high activity human isoform hCA II, with a k_{cat} of 1.1×10^6 s^{-1}, and a k_{cat}/K_m of 1.4×10^8 M^{-1} s^{-1}. The enzyme was highly inhibited by cyanate, thiocyanate, cyanide, bicarbonate, carbonate, as well as sulfamide, sulfamate, phenylboronic/phenylarsonic acids (K_Is in the range of 46–100 μM). Many clinically used sulfonamides, such as acetazolamide, methazolamide, dorzolamide, brinzolamide and benzolamide were low nanomolar inhibitors, with K_Is in the range of 5.7–67 nM. Dichlorophenamide, zonisamide, saccharin and hydrochlorothiazide were weaker inhibitors, with K_Is in the range of 513–5390 nM. The inhibition profile with anions and sulfonamides of the seal enzyme was rather different from those of the human isoforms hCA I and II. The high sensitivity to bicarbonate inhibition of lwCA, unlike that of the human enzymes, may reflect an evolutionary adaptation to the deep water, high CO_2 partial pressure and hypoxic conditions in which Weddell seals spend much of their life.

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1. Introduction

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) is involved in pH buffering of extra- and intracellular spaces by catalyzing the interconversion of CO_2 to HCO_3^- with generation of protons.[1–4] The equilibration between these chemical species is assured steadfastly by the catalytic activity of these enzymes, present in organisms all over the phylogenetic tree, in prokaryotes and eukaryotes.[5–9] CAs are in fact encoded by five distinct, evolutionarily unrelated gene families: the α-, β-, γ-, δ- and ζ-CAs.[1,5–9]

As this reaction is involved in many physiological processes,[1–4] it is not surprising that in mammals 16 different α-CA isozymes were described, with very different catalytic activity, sub-cellular localization, tissue distribution, physiological/pathological roles and susceptibility to inhibitors.[1,3,5–9] Among them, there are five cytosolic catalytically active forms (CA I–III, CA VII and CA XIII), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial ones (CA VA and CA VB), a secreted CA isozyme, CA VI (in the saliva and milk), as well as three acatalytic proteins, CA VIII, X and XI, denominated also CA-related proteins (CARPs).[1,3] CA XV is not present in primates but is found in other vertebrates, such as rodents, birds and fish.[3a] These different isoforms are involved in physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (e.g., gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, epileptogenesis, tumorigenesis, etc.[1–9]

Vertebrate genomes encode only for α-CAs (which are zinc enzymes), but generally a rather high (and variable) number of isoforms are present in these organisms, and their precise number is not known for most orders.[1–3,8] Except humans and rodents (for which 15 and respectively 16 CAs were described and characterized in detail, as outlined above),[1,3] few other vertebrates were thoroughly investigated regarding the number of isoforms, their catalytic activity, and inhibition susceptibility with various classes of inhibitors.[1,10,11] Indeed, some literature data are available for several fish species[10] (such as the model animal zebra fish, *Danio rerio*;[11] the rainbow trout *Oncorhyncus mykiss*;[12a] the sea bass *Dicentrarchus labrax*[12b] or the haemoglobinless Antarctic icefish *Chionodraco hamatus*).[13] Among birds, the red blood cell enzyme from the ratid *Struthio camelus* (ostrich)[14] has been investigated in some detail, but as outlined above, these data for enzymes which are of non human or non rodent origin, are rather scarce.

There are mammals which are adapted to live in extreme conditions, such as the Antarctic seals (Phocidae), among which the Weddell seal ((*Leptonychotes weddellii*) is one of the best examples. In addition to being adapted to the harsh environment of the

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Antarctic winter, with the annual average temperature of −50 °C, these animals are among the best known divers, being able to remain under water at depth of up to 720 m, for 1 h.[15] The gas exchange processes are very much dependent on the CA activity (in the blood, kidneys, lungs, etc.), both through the Bohr–Haldane effect (O_2 loading and unloading of hemoglobin, a process dependent on pH, which is regulated by the CAs),[10] and through their direct involvement in the CO_2 excretion pathways (in kidneys, as bicarbonate ion, in the lungs as gaseous CO_2).[1,10,16] It appears thus of interest to investigate these enzymes in one of the organism adapted to live in extreme condition, that is, the Weddell seal. These animals support low temperatures, high pressure (in the depth of the ocean where they usually forage, at 720 m under the sea, the pressure is 72 times higher than at the sea level),[15] and probably also hypoxia, due to the rather long periods spent in immersion (up to 1 h).[15] It should be noted that there are literature data reporting that animals adapted to hypoxia, such as the beluga whale and the llama, possess low activity CA in the red blood cell, as an adaptation mechanism to hypoxia.[17,18]

In this paper, we report the purification, characterization and inhibition studies of a high activity α-CA isoform from the Weddell seal (*L. weddellii*), denominated here lwCA. We investigated the kinetic properties of this enzyme for the physiologic reaction, that is, CO_2 hydration to bicarbonate and protons, and its inhibition with two of the most relevant classes of CA inhibitors (CAIs),[1] the inorganic anions and the sulfonamides.

2. Results and discussion

2.1. Enzyme purification and catalytic activity

Samples of various organs (liver, kidney, intestine, heart, lung, and spleen) from two adult female Weddell seals found dead and frozen in Antarctica have been used for the isolation and purification of the enzyme, as described in detail in Section 4. From all these tissues, only one lwCA isoform, with a molecular weight of around 30 kDa (the same as those of the human (h) CA isoforms hCA I and II)[1] has been isolated and purified by sulfonamide affinity chromatography (Fig. 1).[19] The catalytic activity of each individual preparation has been measured by using a stopped flow spectrophotometric method,[20] monitoring the physiologic reaction catalyzed by these enzymes, that is, CO_2 hydration to bicarbonate and protons. All enzyme preparations from the seal had the same catalytic activity for the physiologic reaction (data not shown) and this is the reason why we shall denominate all of them with the same name, that is, lwCA (without isoform numbering, as in the case of the primate or rodent CAs). Data of Table 1 show the kinetic parameters (k_{cat} and K_m) for the physiologic reaction catalyzed by various vertebrate CA isoforms, such as the primate ones hCA I–hCA IV,[1] the bird enzyme from *Struthio camelus* (scCA)[14] as well as for the newly purified seal enzyme, lwCA, determined in strictly identical conditions (see Section 4).

Data of Table 1 show the seal enzyme lwCA to possess a very high catalytic activity as CO_2 hydrase, with a k_{cat}/K_m of 1.4×10^8 M^{-1} s^{-1}, similar to that of the most active human isoform, hCA II (the physiologically dominant one),[1–4] which is also one of the most effective catalysts known in nature (with a k_{cat}/K_m of 1.5×10^8 M^{-1} s^{-1}).[1,10] The seal enzyme has a first order rate constant of 1.1×10^6 s^{-1}, slightly lower than hCA II (k_{cat} of 1.4×10^6 s^{-1}), whereas the Michaelis–Menten constants (K_m) of the two enzymes are also rather similar, of 7.5 mM for the seal enzyme and 9.3 mM for hCA II (Table 1). It should be observed that lwCA is catalytically much more active than other vertebrate enzymes investigated earlier, such as the human ones hCA I, III and IV or the ratid one scCA, which have turnover numbers in the range of 2.5×10^5–5.1×10^7 M^{-1} s^{-1} (Table 1). The seal enzyme, similar to all other CAs explored to date apart CA III,[21] was also significantly inhibited by the clinically used sulfonamide CAI acetazolamide (1,3,4-thiadiazole-2-sulfonamide)[1] with an inhibition constant of 63 nM (Table 1). Considering the methodology used for the enzyme purification (see Section 4) and the similar activities of lwCA and hCA II (a cytosolic isoform), we speculate that the seal enzyme is also cytosolic.

2.2. Seal CA inhibition with anions and small molecules

Metal-complexing anions represent a well-known class of inhibitors for metalloenzymes, including CAs, and such compounds usually bind to the metal ion from the enzyme cavity, coordinating to it or adding to the metal coordination sphere (which in all α-CAs is constituted by three His residues and a water molecule/hydroxide ion coordinated to a catalytically crucial Zn(II) ion).[1,22] We thus investigated the inhibition of the seal enzyme with a range of such anions and small molecules reported earlier to act as CAIs,[1] such as sulfamide, sulfamic acid, phenylboronic- and phenylarsonic acid, Table 2). Again, the enzymes purified from various seal tissues had a similar behavior to the anion inhibitors (data not shown). Inhibition data of the human isoforms hCA I and II with these anions is also provided in Table 2, for comparison reasons, as they were reported earlier by this group.[23]

Data of Table 2 show that the only anion not inhibiting significantly these enzymes (K_I >200 mM) was perchlorate, known for its low propensity to bind metal ions in solution or in metalloenzymes.[23] Iodide and nitrate were also rather ineffective lwCA inhibitors, with inhibition constants in the range of 20.0–55.6 mM. Most of the investigated anions (fluoride, chloride, bromide, azide, nitrite, hydrogensulfide, bisulfite, and sulfate) were on the other hand low millimolar lwCA inhibitors, with inhibition con-

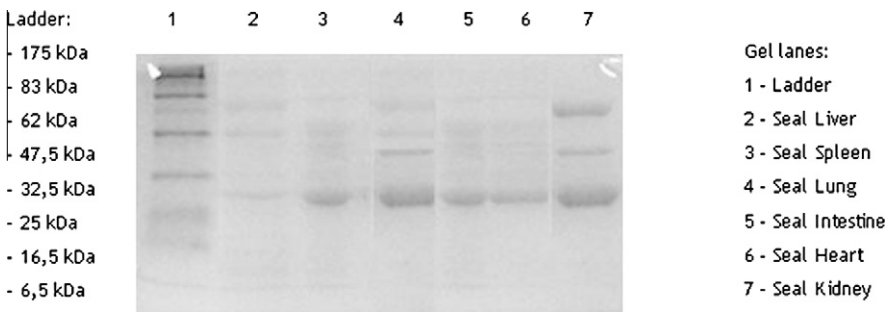


Figure 1. SDS–PAGE of lwCA (at ≈30 kDa) isolated from various seal organs. The purified enzymes, from various organs were thereafter used for kinetic and inhibition studies.

Table 1
Kinetic parameters and inhibition with acetazolamide of CAs from various vertebrates (hCA = human isoforms; scCA = *Struthio camelus* blood enzyme; lwCA = seal enzyme), by a CO_2 hydrase assay, at 20 °C and pH 7.5[20]

Enzyme	Species	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	K_i (AAZ) (nM)	Localization	Ref.
hCA I	Human	2.0×10^5	4.0	5.0×10^7	250	Cytosol	1
hCA II	Human	1.4×10^6	9.3	1.5×10^8	12	Cytosol	1
hCA III	Human	1.3×10^4	52.0	2.5×10^5	240,000	Cytosol	1
hCA IV	Human	1.1×10^6	21.5	5.1×10^7	74	Membrane-bound	1
scCA	Ostrich	1.2×10^6	1.5	1.8×10^7	303	Cytosol	14
lwCA[a]	Seal	1.1×10^6	7.5	1.4×10^8	63	Cytosol?	[a]

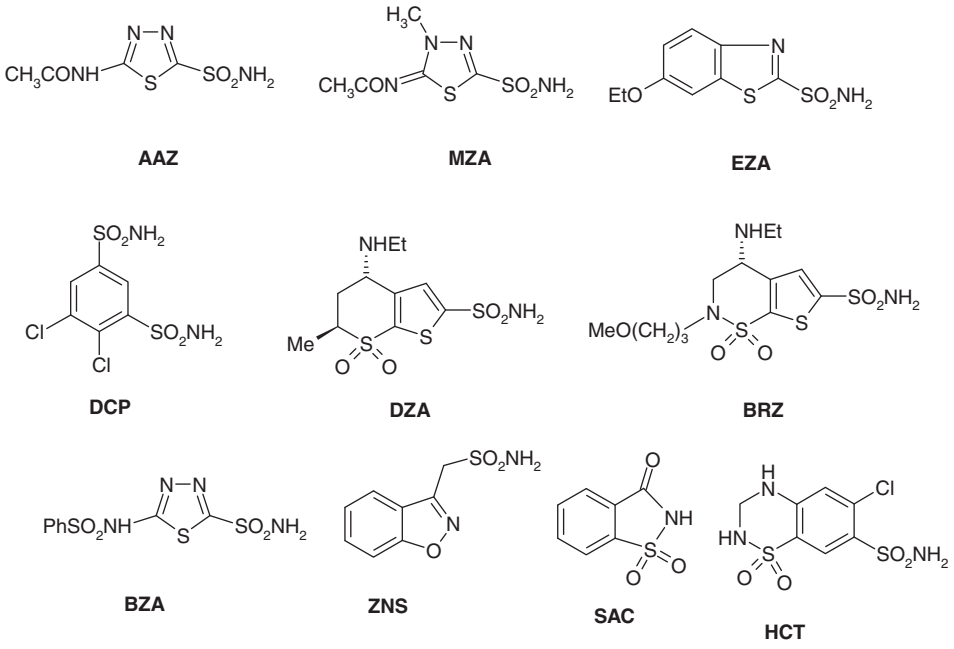
[a] This work. Errors of the kinetic/thermodynamic parameters were in the range of ±5–10% of the reported value, from at least three different assays.

stants in the range of 0.59–1.01 mM. However, several anions such as the 'metal poisons' cyanate, thiocyanate, cyanide and bicarbonate/carbonate, were much stronger inhibitors of the seal enzyme, with K_Is in the range of 60–100 μM (Table 2). Whether the data for the complexing anions (cyanate, thiocyanate, and cyanide) are not at all surprising, the bicarbonate/carbonate strong inhibition is unexpected, also considering the fact that the human enzymes hCA I and II have inhibition constants of 12–85 mM (for bicarbonate) and of 15–73 mM (for carbonate).[23] Thus, the fact that just one of the substrates of these enzymes (bicarbonate) acts as such a strong lwCA inhibitor, points to the fact that the seal enzyme, unlike the human ones, might play physiological roles connected to the adaptation of the seal to hypoxia and high CO_2 partial pressure when foraging in the deep sea. According to the Bohr–Haldane effect, an increase in the CO_2 partial pressure (or a decrease of the pH), leads to a reduced oxygen affinity for haemoglobin.[10] The high catalytic activity of lwCA may generate both more protons (decreasing thus the pH) and more bicarbonate (consuming excess CO_2) which may have as a result the liberation of O_2 from oxyhemoglobin. Thus, the strong bicarbonate inhibition of lwCA may be a feed-back mechanism useful in regulating tissue oxygenation, in an animal which has to adapt to large variations of CO_2 partial pressure when emerging to high depth and returning back to the sea level.

Other small molecules such as sulfamic acid (as sulfamate), sulfamide, phenylboronic and phenylarsonic acids, also act as potent inhibitors of lwCA, with inhibition constants of 46–70 μM, being thus much more inhibitory against the seal than the human enzymes (Table 2).

2.3. Seal CA inhibition with sulfonamides

Sulfonamides constitute the most important class of CAIs, with many representatives used as drugs (diuretics, antiglaucoma, antiobesity, antiepileptics, etc).[1,24] It appeared thus of interest to investigate inhibition of the seal enzyme lwCA with some of these compounds, many of which are widely used drugs.[1,24]



We have included 10 clinically used compounds: acetazolamide **AAZ**, methazolamide **MZA**, dichlorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, zonisamide **ZNS**, saccharin **SAC** and hydrochlorothiazide **HCT**. They include both aromatic and heteroaromatic derivatives. Inhibition data of the two human enzymes hCA I and II[1,24] are also provided in Table 3, for comparison reasons. Dorzolamide and brinzolamide, two antiglaucoma agents,[1] were low nanomolar inhibitors of lwCA, with K_Is in the range of 5.7–6.4 nM. **AAZ**, **MZA**, **EZA** and **BZA** were medium potency inhibitors, with K_Is in the range of 51–67 nM, whereas **DCP**, **ZNS**, **SAC** and **HCT** were weaker inhibitors, with K_Is in the range of 513–5390 nM (Table 3). It may be observed that the inhibition profiles of the three enzymes with these sulfonamides are rather diverse, with hCA II being the most inhibited one, followed by the seal enzyme, whereas hCA I was less prone to be inhibited potently by most of the sulfonamides (except benzolamide and ethoxzolamide). It may be thus observed that even if the catalytic

Table 2
Inhibition constants of anionic inhibitors against the cytosolic human isozymes hCA I, II, and lwCA, for the CO_2 hydration reaction, at 20 °C and pH 7.5[20]

Inhibitor	K_I[c] (mM)		
	hCA I[a]	hCA II[a]	lwCA[b]
F^-	>300	>300	0.74
Cl^-	6	200	0.89
Br^-	4	63	1.01
I^-	0.3	26	20.0
CNO^-	0.0007	0.03	0.087
SCN^-	0.2	1.6	0.097
CN^-	0.0005	0.02	0.070
N_3^-	0.0012	1.5	0.83
HCO_3^-	12	85	0.10
CO_3^{2-}	15	73	0.060
NO_3^-	7	35	55.6
NO_2^-	8.4	63	0.90
HS^-	0.0006	0.04	1.00
HSO_3^-	18	89	0.59
SO_4^{2-}	63	>200	0.82
ClO_4^-	>200	>200	>200
H_2NSO_3H[d]	0.021	0.39	0.055
$H_2NSO_2NH_2$	0.31	1.13	0.070
$PhB(OH)_2$	58.6	23.1	0.046
$PhAsO_3H_2$[d]	31.7	49.2	0.057

[a] Human recombinant isozyme, data from Ref. 23.
[b] Seal enzyme, this work.
[c] Errors were in the range of 3–5% of the reported values, from three different assays.
[d] As sodium salt.

Table 3
Human (h) hCA I, II, and seal CA inhibition data with sulfonamides **AAZ–HCT**, by a stopped flow CO_2 hydrase assay method at 20 °C and pH 7.5[20]

Inhibitor	K_I[a] (nM)		
	hCA I[b]	hCA II[b]	lwCA
AAZ	250	12	63
MZA	50	14	61
EZA	25	8	51
DCP	1200	38	513
DZA	50,000	9	5.7
BRZ	45,000	3	6.4
BZA	15	9	67
ZNS	56	35	517
SAC	18,540	5950	5390
HCT	328	290	630

[a] From Ref. 1,24.
[b] Errors were in the range of 5–10% of the reported values, from three different assays.

activity of the seal enzyme is more similar to that of the high activity cytosolic human isoform, hCA I, the sulfonamide inhibition data of the two enzymes are rather different, with the seal enzyme being more similar to hCA I in its behavior to this class of inhibitors.

3. Conclusions

There are few literature data regarding the characterization, catalytic activity and inhibition of vertebrate CAs other than the human and rodent enzymes. Here we report a high activity such enzyme which has been purified from various tissues of the Antarctic seal *L. weddellii*. The new enzyme, lwCA, has a catalytic activity for the physiologic CO_2 hydration to bicarbonate reaction, similar to that of the high activity human isoform hCA II, with a k_{cat} of $1.1 \times 10^6\ s^{-1}$, and a k_{cat}/K_m of $1.4 \times 10^8\ M^{-1}\ s^{-1}$. The enzyme was highly inhibited by cyanate, thiocyanate, cyanide, bicarbonate, carbonate, as well as sulfamide, sulfamate, phenylboronic/phenylarsonic acids (K_Is in the range of 46–100 μM). Many clinically used sulfonamides, such as acetazolamide, methazolamide, dorzolamide, brinzolamide and benzolamide were low nanomolar inhibitors, with K_Is in the range of 5.7–67 nM. Dichlorophenamide, zonisamide, saccharin and hydrochlorothiazide were weaker inhibitors, with K_Is in the range of 513–5390 nM. The inhibition profile with anions and sulfonamides of the seal enzyme was rather different from those of the human isoforms hCA I and II. The high sensitivity to bicarbonate inhibition of lwCA, unlike that of the human enzymes, may reflect an evolutionary adaptation to the deep water, high CO_2 partial pressure and hypoxic conditions in which Weddell seals spend much of their life.

4. Experimental

4.1. Chemicals

Sulfonamides **AAZ–HCT**, buffers, inorganic sodium salts, sulfamide, sulfamic acid, phenylboronic acid and phenylarsonic acid were the highest purity, commercially available compounds, from Sigma-Aldrich (Milan, Italy) and were used without further purification. Human CA I and II were from Sigma-Aldrich (Milan, Italy).

4.2. Enzyme purification

Samples of liver, kidney, intestine, heart, lung, and spleen were taken from two adult female Weddell seals (*L. weddellii*) found dead during the XX Expedition of the Italian Antarctic Research Program (PNRA) in Terra Nova Bay, Antarctica, during the Austral summer months. Samples were stored at −20 °C until analysis. Seal samples were washed twice with NaCl (0.9%). Cells were lysed by immersion in liquid nitrogen (at −163 °C). The lysed samples underwent three freeze–thaw cycles in dry ice with the addition of five times their volume of ice-cold distilled water, and then sonicated, in order to solubilize all cytosolic enzymes. The pH of the homogenate was adjusted to 8.7 using solid Tris, and the supernatant was applied to an activated CH Sepharose 4B-4-(2aminoethyl)-benzensulfonamide affinity column (1.36 × 30 cm) I order to separate CA. The CA enzyme was purified by using this affinity gel chromatography according to the published method.[19] All procedures were performed at 4 °C. Protein concentrations in the column effluents were determined at 280 nm spectrophotometrically.

4.3. CA catalytic activity and inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[20] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes buffer (pH 7.5) and 20 mM $NaClO_4$ for maintaining constant ionic strength, following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s, at 20 °C. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water and dilutions down to 0.01 nM were made thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes were obtained from

Lineweaver–Burk plots, as reported earlier,[20–23] and represent the mean from at least three different determinations.

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