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Carbonic Anhydrase and Heavy Metals

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1. Introduction

Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc metalloenzyme catalysing the reversible hydration of CO_2 to produce H^+ and HCO_3^-. Its activity is virtually ubiquitous in nature. The fundamental role of this biochemical reaction in diverse biological systems has driven the evolution of several distinct and unrelated families of CAs. Five CA families, referred as α-, β-, γ-CA, δ, and ζ-CAs have been identified in animals, plants and bacteria (Hewett-Emmett and Tashian, 1996; Supuran, 2010). These are the α-CAs, present in vertebrates, bacteria, algae and plants; the β-CAs, predominantly in bacteria, algae and plants; the γ-CAs, mainly present in archaea and some bacteria; the δ-CAs and ζ-CAs only found in some marine diatoms (Supuran, 2010).

The monomeric α-carbonic anhydrases are by far the best studied, being found in animals. In mammals at least 16 different CA isoforms were isolated and several novel isozymes have also been identified in non-mammalian vertebrates. The α-CA isoenzymes differ in their kinetic properties, their tissue distribution and subcellular localization, and their susceptibility to various inhibitors. In general, there are three distinct groups of CA isozymes within the α-CA gene family. One of these groups contains the cytoplasmic CAs, which includes mammalian CA I, II, III, V, VII and XIII. These isozymes are found in the cytoplasm of various tissues, with the exception of the mitochondrial confined CA V. Another group of isozymes, termed the membrane-bound CAs, consists of mammalian CA IV, IX, XII, XIV and XV (Esbaugh and Tufts, 2006). These isozymes are associated with the plasma membranes of many different tissue types. The final group contains several very intriguing isozymes, CA VIII, X and XI, which are termed the CA-related proteins (CA-RP; Tashian et al., 2000). These isozymes have lost classical CA activity – the hydration/dehydration of CO_2 – and have no known physiological function; however, their highly conserved nature does suggest a very important role in vertebrates (Tashian et al., 2000).

The β-carbonic anhydrases are dimers, tetramers, or octamers and include the majority of the higher plant CA isoforms (Kimber and Pai, 2000). The γ-carbonic anhydrase is a homotrimer that has been reported for the bacterium *Methanosarcina thermophila* (Alber and Ferry, 1994). The δ class has its prototype in the monomeric CA TWCA1 from the marine diatom *Thalassiosira weissflogii* (Roberts et al., 1997; Tripp et al., 2001). The ζ-CAs are probably monomer with three slightly different active sites on the same protein backbone (Xu et al 2008).

All CAs are metalloenzymes but whereas α-, β-, and δ-CAs use Zn(II) ions at the active site, the γ-CAs are probably Fe(II) enzymes (Ferry et al., 2010), but they are active also with bound Zn(II) or Co(II) ions, and the ζ-class uses also Cd(II) to perform the physiologic reaction catalysis (Lane et al., 2000; Lane et al., 2005).

CA plays key roles in a wide variety of physiological processes involving CO_2 and HCO_3^-. In animals the various CA isozymes are found in many different tissues and are involved in a number of different physiological processes, including bone resorption, calcification, ion transport, acid–base transport, and a number of different metabolic processes such as biosynthetic reactions (gluconeogenesis, lipogenesis, and ureagenesis). In algae and plants they play an important role in photosynthesis (Ivanov et al, 2007; Zhang et al., 2010; Cannon Gordon et al., 2010).

Considerable advances towards a detailed understanding of the catalytic mechanism of the zinc enzyme carbonic anhydrase have been made during the past years as a result of the application of crystallographic and kinetic methods to wild-type and mutant enzymes. Moreover, a great amount of work has been performed on CA inhibitors, first of all sulfonamides, RSO_2NH_2, which represent the classical CA inhibitors (CAIs) and are in clinical use for more than 50 years as diuretics and systemically acting antiglaucoma drugs (Supuran, 2010).

The review focuses on one interesting but less investigated aspect of the biochemistry of this metalloenzyme, encompassing several areas of interest from human health to environmental science: the relationships between carbonic anhydrase and heavy metals. Heavy metals are chemical elements with a density higher than 5.0 g/cm^3, characterized by high reactivity, redox behaviour, and complex formation based on the characteristic of the outer *d* electron shell. In the scientific literature the following elements are normally ascribed to the heavy metal groups: aluminium, iron, silver, barium, beryllium, manganese, mercury, molybdenum, nickel, lead, copper, tin, titanium, tallium, vanadium, zinc. Some metalloids, such as arsenic, bismuthum, and selenium, are also included in the heavy metals groups.

Heavy metals generally regarded as essential for animals in trace amounts include zinc, the known cofactor of CAs, iron, copper, manganese, chromium, molybdenum and selenium. They are essential because they form an integral part of one or more enzymes involved in a metabolic or biochemical process. Besides essential metals, a number of other heavy metals, such as arsenic, lead, cadmium, mercury, have no known function in the body and are referred as toxic metals. However, also essential metals become toxic when their levels in the body exceed the homeostatic capacity of the organism. The intracellular levels of essential metals are regulated by transporters (which translocate metal across the plasma membrane) as well as by metallothionein and other metal binding proteins (Maret and Wolfgang, 2011). The toxicity of heavy metals is generally ascribed to their high affinity for nucleophilic groups like sulfhydryls. In fact they are soft donors and will therefore readily bind to soft acceptors such as sulphydryl groups.

Recently, a number body of evidence has emerged regarding the effect of several heavy metals on carbonic anhydrase catalytic activity and protein expression. These studies encompass a wide area of interest from human health to environmental sciences.

2. Heavy metals as carbonic anhydrase cofactors

CAs catalyze the reversible hydration of carbon dioxide to bicarbonate and protons by means of a metal-hydroxide ($Lig^3M^{2+}(OH)^-$) mechanism, although the α-CAs possess other catalytic activities such as esterase, phosphatase, cyanate/cyanamide hydrase, etc. (Supuran et al., 2003; Supuran and Scozzafava 2007; Innocenti et al., 2008). In the α-, γ, and δ-CA classes, Lig^3 is always constituted by three His residues. The metal (M) is ZnII for all classes. The zinc atom is in the +2 state and is located in a cleft near the center of the enzyme. The role of zinc in carbonic anhydrase is to facilitate the deprotonization of water with the formation of the nucleophilic hydroxide ion, which can attack carbonyl group of carbon dioxide to convert it into bicarbonate. This is obtained through the +2 charge of the zinc ion which attracts the oxygen of water, deprotonates water, thus converting it into a better nucleophile able to attack the carbon dioxide.

Water naturally deprotonates itself, but it is a rather slow process. Zinc deprotonates water by providing a positive charge for the hydroxide ion. The proton is donated temporarily to the surrounding amino acid residues, and then it is given to the environment, while allowing the reaction to continue. Zinc is able to help the deprotonation of water by lowering the pKa of water. Therefore, more water molecules are now able to deprotonate at a lower pH than normal, increasing the number of hydroxide ions available for the nucleophilic attack to carbon dioxide (Berg, 2007).

The affinity of carbonic anhydrase for zinc is in subpicomolar range, as assessed for studies on the α-class (Tripp et al., 2001). Cox et al. (2000) and Hunt et al. (1999) ascribed a role for hydrophobic core residues in human CA-II that are important for preorienting the histidine ligands in a geometry that favours zinc binding and destabilizes geometries that favour other metals. In particular, mutagenesis experiments demonstrated that substitutions of these amino acids at position 93, 95, and 97 decrease the affinity of zinc, thereby altering the metal binding specificity up to 10^4-fold. Furthermore, the free energy of the stability of native CAII, determined by solvent-induced denaturation, correlates positively with increased hydrophobicity of the amino acids at positions 93, 95, and 97 as well as with zinc affinity (Hunt et al., 1999).

β-CAs, present in green plants and cyanobacteria, contain also Zn^{2+} in the active site but are differentiated from α-CAs by virtue of the fact that the active site is coordinated by a pair of cysteine residues and a single histidine residue, whereas the fourth ligand may be either a water molecule/hydroxide ion, or a carboxylate from a conserved aspartate residue in some β-CAs (Type II β-CAs) [Trip et al., 2001; Xu et al., 2008]. The metal hydroxide catalytic mechanism seems to be also valid for these enzymes [Supuran, 2008].

Besides zinc, other metals have demonstrated to be physiologically relevant cofactor for some CAs. In fact, in the γ-CAs metal may also be FeII (Ferry et al., 2010). Cam, the prototypic γ-class carbonic anhydrase, from the anaerobic methane producing Archaea species *Methanosarcina thermophila,* contains zinc in the active site when overproduced in *Escherichia coli* and purified aerobically [Alber et al., 1996], while it has 3-fold greater carbonic anhydrase activity and contains Fe^{2+} in the active site (Fe-Cam) when purified anaerobically from *E. coli* or overproduced in the closely related species M. *acetivorans* and purified anaerobically. Soluble Fe^{2+} is abundant in oxygen free environments and available to anaerobic microbes. The different results obtained in aerobic and anaerobic conditions is

explained by the fact that in aerobic conditions Fe^{3+} is oxidized and rapidly loss from CAM enzyme, substituted by Zn^{2+} contaminating buffers not treated with chelating agents. These results indicate Fe^{2+} as the physiologically relevant metal [MacAuley et al., 2009; Tripp et al., 2004] in the active site for CAM enzyme. Interestingly, evidence for the role of ferrous ion in CA has been obtained also for the α class. In fact carbonic anhydrase activity from duck erythrocytes is increased in the presence of iron in the incubation medium suggesting a role for iron in the active site (Wu et al., 2007).

The ζ-CA naturally uses Cd^{2+} as its catalytic metal in marine diatoms (Lane and Morel, 2000; Lane et al., 2005; Park et al., 2008). This cdmium-CA (CDCA1) consists of three tandem CA repeats (R1–R3), which share 85% identity in their primary sequences (Lane et al., 2005). Although CDCA1 was initially isolated as a Cd enzyme, it is actually a "cambialistic" enzyme since it can use either Zn or Cd for catalysis—and spontaneously exchanges the two metals (Xu et al., 2008). Kinetic data show that the replacement of Zn by Cd results nonetheless in a decrease in catalytic efficiency (Xu et al., 2008). In the active site, Cd is coordinated by three invariant residues in CDCA of all diatom species (Park et al., 2007): Cys 263, His 315 and Cys 325. The tetrahedral coordination of Cd is completed by a water molecule. The use of Cd in CDCA is thought to explain the nutrient-like concentration profile of Cd in the oceans, where the metal is impoverished at the surface by phytoplankton uptake and regenerated at depth by remineralization of sinking organic matter (Lane and Morel 2000). It is cycled in the water column like an algal nutrient. It is thought that the expression of a CDCA in diatoms, which are responsible for about 40% of net marine primary production, represents an adaptation to life in a medium containing vanishingly small concentrations of essential metals (Xu et al., 2008). As suggested by Xu et al. (2008) the remarkable ability to make use of cadmium, an element known for its toxicity, gave presumably a significant competitive advantage to diatoms in the oceans, poor in metals, with respect to other species, and could have contributed to the global ratiation of diatoms during the Cenozoic Era and to the parallel decrease in atmospheric CO_2.

Moreover, Co(II) has been shown to replace Zn(II) in α-, β and γ-CA (Hoffmann et al., 2011). Cobalt ionic radius and polarizability are very similar to those of Zn(II). In contrast to Zn(II) (d^{10}), the d^7electron configuration of Co(II) is accessible to electronic spectroscopic methods (, yielding information about the interactions protein-metal. As a result, spectroscopy of Co(II) substituted CA isozymes has been used to probe the environment of the metal ions in the active sites and get information on the nature of the first coordination sphere of the metal (Hoffmann et al., 2011). The Co-containing form of the enzyme generally shows a marked decrease in activity compared with the native Zn form (Tu and Silverman, 1985). The demonstration that Zn can be extracted from a protein and replaced with Co *in vitro* does not demonstrate that such metal substitution takes place in vivo. The evidence for in vivo Co substitution in a CA was for the first time provided by Morel et al (1994) and Yee and Morel (1996) in the diatoms *T. weissflogii,* who demonstrated ^{65}Zn and ^{57}Co bands to co-migrate with a single band of CA activity on a native gel of diatom proteins.

3. Heavy metals as inhibitors of carbonic anhydrase activity

Several heavy metals were demonstrated to *in vitro* inhibit CA activity in a variety of organisms, including fishes, crabs, bovines, and humans.

The early work of Christensen and Tucker (1976) demonstrated carbonic anhydrase inhibition by heavy metals for the first time in fish. The study was carried out on red blood cells CA of the teleost *Oncorhynchus mykiss*. Erythrocyte CA, which represents the most abundant pool of the enzyme in fish, appeared significantly in *vitro* inhibited by several heavy metals cations, such as Cd^{2+}, Cu^{2+}, Ag^{+}, and Zn^{2+} (Tab1).

In the intestine and gills of the European eel, *Anguilla anguilla*, Lionetto et al. (1998; 2000) found cadmium to significantly inhibit carbonic anhydrase activity. The inhibition appeared tissue specific (Lionetto et al., 1998; Lionetto et al., 2000). The gill CA was much more sensitive to the heavy metal as compared to the enzyme activity in the intestine, as observed by comparing the IC50 values (Tab1). In particular in the intestine the inhibitory effect of cadmium was more pronounced on the cytosolic than the membrane-bound CA, which revealed only a partial inhibition at high concentrations. Moreover CA activity inhibition showed a certain time-dependence, with a delay of at least 10 min and 30 min for the cytosolic isoform and the membrane bound isoform respectively. The authors attributed this behaviour to the time required by cadmium for displacing the metal (zinc) associated with the enzyme, giving an inactive Cd-substituted carbonic anhydrase. Cadmium is a bivalent metal, similar in many respects to zinc: both are in the same group of the periodic table, contain the same common oxidation state (+2), and when ionized have almost the same size. Due to these similarities, cadmium can replace zinc in many biological systems. Moreover, the delayed inhibition of membrane-bound CA with respect to the cytosolic isoform was explained by a more difficult access of cadmium to the active site of the enzyme bound to the membrane. In fact, it has to be considered that the membrane-bound CA is stabilised by disulfide bonds (Whitney and Briggle, 1982) which could contribute to a less sensitivity of the membrane bound CA to cadmium.

As suggested by Lionetto et al (2000), the observed *in vitro* inhibition of cadmium on CA activity could be useful in the understanding of the toxic effects that the heavy metal can elicits on fish physiology *in vivo*. The inhibitory effect on gill CA activity suggests that the heavy metal might interfere with a number of physiological functions in which gill CA is involved as gas exchanges (Randall and Daxbaeck, 1984), acid–base balance (Heisler, 1984), osmoregulation (Henry, 1984) and clearance of the waste products from nitrogenous metabolism (Evans and Cameron, 1986). Morgan et al (2004) directly demonstrated in *in vivo* expoxure experiments on rainbow trout that inhibition of branchial CA was able to induce an early decline in the gill Na^{+} and Cl^{-} uptake. With regards to the intestine, the physiological role of the cytosolic CA is that of generating HCO_3^- from metabolic CO_2 while the role of the CA enzyme associated to the brush-border membrane should be that of mediating the environmental HCO_3^- uptake (Maffia et al., 1996). Therefore, the inhibitory effect of cadmium on intestinal CA isoforms should interfere with bicarbonate balance and in turn with systemic acid–base balance and osmoregulation in fish. In fact, as previously shown (Schettino et al., 1992), the HCO_3^- entry via the membrane-bound CA in the cell across the luminal membrane of the enterocytes seems to be essential for maintaining a steady intracellular HCO_3^- concentration and/or pHi; as a consequence the salt transport in eel intestine occurs at a highest rate and the passive water loss is recovered, so solving in part the osmoregulatory problem in marine fish. Therefore, inhibition of CA enzymes by cadmium could alter $[HCO_3^-]i$ and/or pHi leading to a reduction of salt absorption and consequently impairing the osmoregulation of marine fish.

More recently, Soyut et al (2008) demonstrated Co^{2+}, Cu^{2+}, Zn^{2+}, Ag^{+}, and Cd^{2+} to be potent inhibitor for brain CA enzyme activity in Rainbow trout (*Oncorhynchus mykiss),* with the following sequence Co^{2+} >Zn^{2+} >Cu^{2+}>Cd^{2+}>Ag^{+}. They also demonstrated that Co^{2+}, Ag^{+}, and Cd^{2+} inhibit the enzyme with competitive manner, Cu^{2+} inhibits with noncompetitive manner, and Zn^{2+} with uncompetitive manner.

Ceyhun et al., 2011 *in vitro* demonstrated Al^{+3}, Cu^{+2}, Pb^{+2}, Co^{+3}, Ag^{+1}, Zn^{+2} and Hg^{+2} to exert inhibitory effects on fish liver CA. Metal ions inhibited the enzyme activity at low concentrations. Al^{+3} and Cu^{2+} resulted the most potent inhibitors of the CA enzyme. All the metals inhibited CA in competitive manner and aluminium showed to be the best inhibitor for fish liver CA. Concerning the mechanism of inhibition, the authors argued a possible interaction of the metal with the histidines exposed on the surface of the molecule and/or other aminoacids around the active site.

In invertebrates Vitale et al (1999)demonstrated cadmium, copper and zinc to *in vitro* inhibit CA activity in the gills of the estuarine crabs *Chasmagnathus granulate* (Tab.1*). The* inhibitory potentials of the three metals on CA was in the following sequence: Cu^{2+} > Zn^{2+} > Cd^{2+}. The observed inhibitory effect *in vitro* was confirmed by a corresponding inhibitory effect *in vivo.*

In the euryhaline crabs *Callinectes sapidus and Carcinus maenas* Skaggs et al (2002) also documented a significant *in vitro* inhibition of gill CA by Ag^{+}, Cd^{2+}, Cu^{2+} and Zn^{2+}. The binding affinities of the metals were one thousand times weaker for cytoplasmic CA from the gills of *C. maenas* than that from *C. sapidus*. The large differences in Ki values (Tab.1) suggests the presence of two different CA isoforms in the gills of these species, with *Callinectes sapidus* possessing a highly metal-sensitive CA isoform and *Carcinus maenas* having a metal-resistant isoform. Interestingly, heavy metal inhibition of CA from the gills of another euryhaline crab, *Chasmagnathus granulata*, (as reported by Vitale et al., 1999, see above) appears to be intermediate between that found in the other two species. Moreover, in *Callinectes sapidus* CA isolated from the cytoplasmic pool of gill homogenates was much more sensitive to heavy metal inhibition than was CA from the microsomal fraction, which is believed to be anchored to the basolateral membrane, and as such, it exists within a lipid-rich environment. The authors argued that metal could be sequestered in the lipid component of the microsomal fraction and, therefore, higher amounts of metals are required to achieve an effective concentration of free metals available for CA inhibition. However, the authors did not considered the time-dependence of the inhibition, which can be an important aspect to be taken into account (see Lionetto et al., 2000) in the analysis of membrane bound vs cytosolic isoform CA inhibition.

In humans Ekinci et al (2007) demonstrated the inhibition of two human carbonic anhydrase isozymes *in vitro*, the cytosolic HCA I and II by lead, cobalt and mercury. Lead was a noncompetitive inhibitor for HCA-I and competitive for HCA-II, cobalt was competitive for HCA-I and noncompetitive for HCA-II and mercury was uncompetitive for both HCA-I and HCA-II. Lead was the best inhibitor for both HCA-I and HCA-II.

In tab.1 the Ki, IC50 values and the type of inhibition for several heavy metals on CA from different vertebrate and invertebrate species is summarized. A great variability among species, tissues and metals can be observed. This suggests that the inhibitory mechanisms through which heavy metals exert their effect on carbonic anhydrase activity could be different for different isoenzymes and that also small structural differences between CA isoforms could result in different metal binding affinities.

Metal	Average value of Ki (M)	IC50 (M)	Type of inhibition	Tissue	Species	Ref
Cd^{2+}	n.d.	$9.979\ 10^{-6}$	n.d.	gills	*Anguilla anguilla*	Lionetto et al 2000
	n.d.	$3.64\ 10^{-5}$	n.d.	Intestine (cytosolic isoform)	*Anguilla anguilla*	Lionetto et al 2000
	n.d.	$2.15\ 10^{-5}$	n.d.	gills	*Chasmagnathus granulata*	Vitale et al., 1999
	n.d.	$9.00\ 10^{-4}$	n.d.	Red blood cells	*Ictalurus punctatus*	Christensen and Tucker, 1976
	$94.16\ 10^{-3}$M	8.25 ± 10^{-2}	Competitive	brain	*Oncorhynchus mykiss*	Soyut et al., 2008
	$5.0\ 10^{-7}$	n.d.	n.d.	Gills (cytoplasmic isofom)	*Callinectes sapidus*	Skaggs and Hery, 2002
	$6.0\ -25.0\ 10^{-4}$	n.d.	n.d.	Gills (cytoplasmic isofom)	*Carcinus maenas*	Skaggs and Hery, 2002
Ag^{+}	$193.8\ 10^{-3}$M	$1.59\ 10^{-1}$	Competitive	brain	*Oncorhynchus mykiss*	Soyut et al., 2008
	$6.40\ 10^{-4}$	$3.79\ 10^{-4}$	Competitive	liver	*Dicentrarchus labrax*	Ceyhun et al., 2011
	n.d.	$3.50\ 10^{-5}$	n.d.	Red blood cells	*Ictalurus punctatus*	Christensen and Tucker, 1976
	$5.0–0.10\ 10^{-8}$	n.d.	n.d.	Gills (cytoplasmic isofom)	*Callinectes sapidus*	Skaggs and Hery, 2002
	$6.0\ -25.0\ 10^{-4}$	n.d.	n.d.	Gills (cytoplasmic isofom)	*Carcinus maenas*	Skaggs and Hery, 2002
Zn^{2+}	$2.15\ 10^{-3}$M	$3.10\ 10^{-4}$	Uncompetitive	brain	*Oncorhynchus mykiss*	Soyut et al., 2008
	$7.21\ 10^{-4}$	$3.90\ 10^{-4}$	Competitive	liver	*Dicentrarchus labrax*	Ceyhun et al., 2011
	n.d.	$7.00\ 10^{-4}$	n.d.	Red blood cells	*Ictalurus punctatus*	Christensen and Tucker, 1976
	n.d.	$1.62\ 10^{-5}$	n.d.	gills	*Chasmagnathus granulata*	Vitale et al., 1999
	$6.0\ -25.0\ 10^{-4}$	n.d.	n.d.	Gills (cytoplasmic isofom)	*Carcinus maenas*	Skaggs and Hery, 2002

Metal	Average value of Ki (M)	IC50 (M)	Type of inhibition	Tissue	Species	Ref
Cu^{2+}	27.6 10^{-3}M	3.00 10^{-2}	Non competitive	brain	*Oncorhynchus mykiss*	Soyut et al., 2008
	1.75 10^{-5}	7.15 10-5	Competitive	liver	*Dicentrarchus labrax*	Ceyhun et al., 2011
	n.d.	6.50 10^{-5}	n.d.	Red blood cells	*Ictalurus punctatus*	Christensen and Tucker, 1976
	n.d.	3.75 10^{-6}	n.d.	gills	*Chasmagnathus granulata*	Vitale et al., 1999
	3.60 10^{-7}	n.d.	n.d.	Gills (cytoplasmic isofom)	*Callinectes sapidus*	Skaggs and Hery, 2002
	6.0 -25.0 10^{-4}	n.d.	n.d.	Gills (cytoplasmic isofom)	*Carcinus maenas*	Skaggs and Hery, 2002
Co^{2+}	5 10^{-5}M	1.40 10^{-5}	competitive	brain	*Oncorhynchus mykiss*	Soyut et al., 2008
	5.32 10^{-4}	3.16 10^{-4}	competitive	liver	*Dicentrarchus labrax*	Ceyhun et al., 2011
	3.91 10^{-3}	n.d.	competititve	Erytrocytes (CAI)	*Homo sapiens*	Ekinci et al., 2007
	1.7 10^{-3}	n.d.	non competitive	Erytrocytes (CAII)	*Homo sapiens*	Ekinci et al., 2007
Al^{3+}	1.48 10^{-4}	6.92 10^{-5}	competitive	liver	*Dicentrarchus labrax*	Ceyhun et al., 2011
Pb^{2+}	2.42 10^{-4}	1.13 10^{-4}	competitive	liver	*Dicentrarchus labrax*	Ceyhun et al., 2011
	9.90 10^{-4}	n.d.	Non competitive	Erytrocytes (CAI)	*Homo sapiens*	Ekinci et al., 2007
	5.6 10^{-5}	n.d.	uncompetitive	Erytrocytes (CAII)	*Homo sapiens*	Ekinci et al., 2007
Hg^{2+}	7.68 10^{-4}	4.48 10^{-4}	competitive	liver	*Dicentrarchus labrax*	Ceyhun et al., 2011
	1.42 10^{-3}	n.d.	uncompetitive	Erytrocytes (CAI)	*Homo sapiens*	Ekinci et al., 2007
	3.12 10^{-4}	n.d.	uncompetitive	Erytrocytes (CAII)	*Homo sapiens*	Ekinci et al., 2007

Table 1. Ki, IC50 and type of inhibition for several heavy metals in different species and tissues as assessed in *in vitro* studies.

Concerning the mechanisms of inhibition some heavy metals are believed to bind to CA not at the specific catalytic site of CO_2 hydration but nearby in a pocket, the so called 'proton

shuttle' as demonstrated for human CAII (Tu et al., 1981). His-64 is a proton shuttle in catalysis, where it accepts the proton product (via the bridging solvent molecules) from zinc-bound water as zinc-bound hydroxide is regenerated; subsequently, the proton product is passed along to buffer (Liang et al, 1988; Tu et al., 1989; Vedani et al., 1989). The mechanism of inhibition of heavy metals on proton shuttle has been elucidated for copper on human CA II. Cu^{2+} is believed to competitively inhibit CAII by binding to the imidazole side chain of His-64, blocking its role in proton transfer from the zinc-bound water molecule to buffer molecules located outside of the active site region [Tu et al., 1981]. However, the knowledge of the mechanism of action of other metals on different CA isoforms is lacking. It cannot be excluded the CA binding to other different parts of the protein, possibly cysteine residues, as demonstrated in studies with other enzymes for silver and mercury.

4. Heavy metals as modulators of carbonic anhydrase activity and expression

If it has been widely demonstrated *in vitro* that heavy metals are able to inhibit CA activity in a variety of organisms, on the contrary little is known about the *in vivo* effects of trace metals on the activity and the expression of this metalloenzyme. The major information regards Zn^{2+}, while very few is known about other metals.

In humans early studies demonstrated that dietary zinc deficiency significantly reduces zinc concentrations of serum and in turn CA activity in erythrocytes (Hove,1940; Rahman et al., 1961; Kirchgessner et al., 1975) suggesting a possible influence of Zn^{2+} on CA protein expression. These early data have been more recently confirmed by Lukaski (2005) who demonstrated zinc concentration of serum and erythrocyte to be positively correlated to CA activity *in vivo*. Low dietary zinc decreases erythrocyte carbonic anhydrase activity and, in turn, impairs cardiorespiratory function in men during exercise (Lukaski et al., 2005). In ducks Zn^{2+} at a low level (up to 1.25 µM Zn) induced the rise of CA activity in erythrocytes (Wu et al., 2007). In parotid saliva of patients with CAVI deficiency Zn^{2+} treatment was able to stimulate synthesis/secretion of CAVI (Henkin et al., 1999), probably through stimulation of CAIV gene. In rats Zn^{2+} deficiency significantly reduced CAII protein expression in the submandibular gland (Goto et al., 2008).

As regards other metals Grimes et al (1997) reported the depression of CAIII mRNA and, in turn, CAIII protein in the mouse mutant 'toxic milk' (tx) liver following copper accumulation, Kuhara et al (2011) found CAIII suppression by copper accumulation during carcinogenesis, while Wu et al (2007) found iron at low levels to induce a rise in CA activity in duck erythrocytes.

Recently, Caricato et al (2010) demonstrated for the first time CA activity and protein expression to be enhanced by the exposure to the trace element cadmium in animals, opening new perspective in the comprehension of the functioning and regulation of this enzyme. Digestive gland CA activity showed a weak sensitivity to *in vitro* cadmium exposure since only high concentrations of $CdCl_2$ (from 10^{-5} to 10^{-3} M) were able to exert a significant inhibition. On the contrary digestive gland CA activity showed a significant increment in cadmium exposed animals (about 40% after two week of exposure). This was the first time that CA activity appears to be increased by cadmium in animals. Carbonic anhydrases from the microalgae *Chlamydomonas reinhardtii* (Wang at al., 2005) and *Thalassiosira weissflogii* (Morel et

al., 1994; Lee et al., 1995) are the only other examples reported in nature of CA activity increase induced by cadmium exposure. Evidence of *in vivo* utilization of Cd in CA has been found in microalgae (Price and Morel, 1990; Morel et al., 1994; Lee et al. 1995, Xu et al., 2008). In these organisms the ability of Cd to substitute for Zn at the active site of the enzyme is reflected in the regulation of the enzyme expression. In *Thalassiosira weissflogii* a cadmium-containing CA was found to be expressed during zinc limitation (Lane and Morel, 2000; Lane et al., 2005). This cadmium CA (CDCA1) which naturally uses Cd as its catalytic metal (Trip et al., 2001; Lane et al., 2005) has been ascribed to a novel ζ-CA class (see above). Genes coding for similar proteins have been identified in other cultured diatoms (Park et al., 2007). In mussel digestive gland western blotting analysis clearly demonstrated the enhancement of CA protein expression following cadmium exposure, according to the enzymatic activity data (Caricato et al., 2010). Laboratory experimental results were confirmed by a field experiment. Mussels exposed for 30 days to an anthropogenic impacted site showed a significant increase in CA activity and protein expression with respect to animals exposed for 30 days in a control site. If the new synthesized enzyme is a Cd-CA is not possible to say at the moment. If it was the case, then the increase in CA would not be a direct adaptive response to Cd pollution; rather, Cd could remove any limitations placed on CA synthesis by the availability of Zn. However, future studies will be needed to clarify this intriguing aspect of the research.

5. Carbonic anhydrase and heavy metals interactions: Potential applications

In the last years the interactions between carbonic anhydrase and heavy metals have found a number of applications in environmental and health fields, including the development of biomarkers of pollution exposure, in vitro bioassays, and biosensors.

5.1 Carbonic anhydrase sensitivity to heavy metals and development of biomarkers of pollution exposure

Pollution by trace metals is a world-wide problem due to the persistency and continuing accumulation of metals in the environment (de Mora et al. 2004; Hwang et al 2006). Heavy metals may enter the organisms through food, water, air, or absorption through the skin. As a result of mining, waste disposal and fuel combustion the environment is becoming increasingly contaminated with heavy metals.

In recent years the increasing sensibility to pollution problems has promoted the development of environmental "diagnostic" tools for early warning detection of pollution. Pollution monitoring has been increasingly concerned with the use of biological responses to pollutants at molecular and cellular level for evaluating biological hazard of toxic chemicals. Methods based on biological effects and their underlying mechanisms can complement the use of analytical chemistry in environmental monitoring. The major advantages of such biological, mechanism-based methods are their toxicological specificity, rapidity, and low cost. Toxicological specificity refers to the relationship between the assay response and the toxic potential rather than simply the contaminant concentrations (provided by chemical analysis) of the sample being analyzed. Moreover, biological assays provide rapid, sensitive, easily learnt and readily interpretable new useful tools for environmental biomonitoring and risk assessment. They include biomarkers, and *in vivo* and *in vitro* bioassays. It is known that the harmful effects of pollutants are typically first manifested at lower levels of biological organization before disturbances are realized at

population, community and ecosystem levels (Adams, 1990). This is the reason why in recent years the study of molecular and cellular effects of pollutants has given important advancement in the developing of biologically-based methodologies useful for environmental biomonitoring and risk assessment. Enzymatic inhibition studies have been a very fruitful field for environmental monitoring application as biomarker of exposure/effect. Biomarkers are defined as pollutant induced variation in cellular or biochemical components occurring in organisms as a result of natural exposure to contaminants in their environment (Depledge, 1994). As reported by several authors, the evaluation of biomarkers in bioindicator organisms sampled in one or more areas suspected of chemical contamination and their comparison with organisms sampled in a control area can allow the evaluation of the potential risk of toxicological exposure of the studied community (Lionetto et al., 2003; Lionetto et al., 2004).

Carbonic anhydrase sensitivity to heavy metal exposure has been recently explored for its possible applications as biomarker of exposure to heavy metal pollution (Lionetto et al. 2006; Caricato et al, 2010b.) in "sentinel" organisms. Lionetto et al., (2006) investigated CA activity inhibition by heavy metals in the filter feeding *Mytilus galloprovincialis*, widely used in pollution monitoring programs as sentinel organism (Jernelov et al., 1996). Following *in vitro* and *in vivo* exposure to cadmium, mantle CA activity was significant inhibited. The inhibitory effect of cadmium on mantle CA activity can explain results previously obtained by Soto et al. (2000), who observed a significant decreased in shell growth in *M. galloprovincialis* exposed to heavy metals. The sensitivity of CA to heavy metals in mussels appears to be tissue-specific. In fact, as reported above, in mussel's digestive gland CA activity and expression was found to increase following Cd exposure (Caricato et al., 2010). Because of the widely application of *M. galloprovincialis* in environmental quality monitoring and assessment, data on tissue specific sensitivity of carbonic anhydrase to heavy metals represent a starting point for future potential application of CA activity changes as biomarker of exposure to heavy metals in the sentinel organism *M. galloprovincialis*.

Other studies carried out on corals have suggested alteration in CA activity as potential biomarker of exposure to environmental chemical stress. CA activity has been demonstrated to be inhibited by heavy metal exposure in anemones and corals (Gilbert and Guzman, 2001), where the enzyme plays a key role in the calcification process. Coral growth has been shown to be an effective indicator of the overall health of a coral reef ecosystem and reduced growth can reflect impaired photosynthetic output of the zooxanthellae and/or changes in enzyme activity (Moya et al., 2008). In an era of climate change and ocean acidification, where factors impacting growth and resilience factors are becoming important, understanding the biological effects of metal exposure to these keystone tropical organisms may be critical (Bielmyer et al., 2010).

5.2 Carbonic anhydrase based bioassay

Bioassays use biological systems to detect the presence of toxic chemicals in the environmental matrices (water, sediment, sewage, soil, etc.). In recent years, *in vitro* bioassays, employing cultured cells or cellular extracts, are increasingly being developed and used to detect the presence of contaminants. Examples include assays that measure enzyme inhibition, receptor-binding, or changes in gene expression in *in vitro* systems. Although *in vitro* assay is not a substitute for biomarker approach, it can be used as an

adjunct model to whole-animal *in vivo* exposure and to ecotoxicological evaluation of the potential risk of trace pollutants in aquatic environments. They are rapid, low cost and simple tools to be utilized in combination with chemical analysis, for the pre-screening of the environmental samples that should be analyzed. Lionetto et al (2005; 2006) explored the possible application of heavy metal CA inhibition for the development of an *in vitro* bioassay applicable to the determination of the toxicity of environmental aqueous samples. They developed rapid and sensitive chemical hazard detection system for standardizing rapid, sensitive, and low cost CA based *in vitro* bioassay (Schettino et al., 2008).

6. Carbonic anhydrase-based biosensing of metal ions

In the last years the affinity of carbonic anhydrase for metal ions has been applied for the development of fluorescence based biosensors for determination of free metal ions in solution using variants of human carbonic anhydrase (apoCA). In particular, Cu^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+} have been determined at concentration down the picomolar range (Fierke and Thompson, 2001; Thompson and Jones, 1993; Mey et al., 2011) by changes in fluorescence emission (Thompson et al., 2000) and excitation wavelength ratios (Thompson et al., 2002), lifetimes (Thompson and Patchan, 1995), and anisotropy (polarization) (Elbaum et al., 1996; Thompson et al., 2000). The sensitivity, selectivity, analyte binding, kinetics and stability of the biosensors have been improved by subtle modification of the protein structure by directed mutagenesis (Kiefer et al., 1995; Hunt et al., 1999; DiTusa et al., 2001; McCall et al., 2004; Burton et al, 2000). These studies have hallowed the development of highly selective and sensitive fluorescence-based biosensors for Zn^{2+} e Cu^{2+}, which have been shown to be viable approach in some important applications. In fact, the CA-based Cu^{2+} biosensor has been used to obtain real-time measurement of free Cu(II) at picomolar concentrations in seawater (Zeng et al., 2003), while the CA-base Zn^{2+} biosensor has been used for measurement of free Zn ion at picomolar levels in cultured cells (Bozym et al, 2004).

7. Conclusions

Although carbonic anhydrase represents one of the most investigated metalloenzyme in nature, its interaction with heavy metals has been only partially elucidated to date and some issues still remains to be explored. An intriguing aspect that needs more investigation is the *in vivo* effect of heavy metals on CA expression. From the few studies available in literature some metals appear to be important modulator of the expression of this protein. The understanding of the underlying mechanisms could open new perspective in the comprehension of the functioning and regulation of this enzyme. Another intriguing aspect of the biochemistry of CA is the inhibition by heavy metals. It has been documented for some species and some metals, but the mechanisms behind the inhibition, its metal specificity and isoform specificity remains still unknown. These aspects merits in depth examination and open new perspective for drug design and biomarkers development.

8. References

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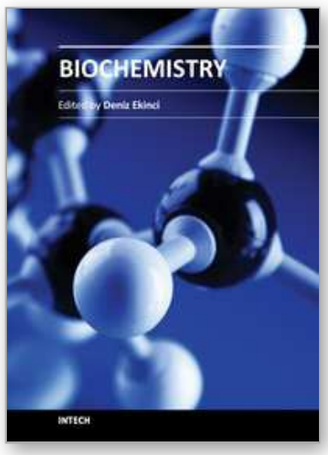

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Over the recent years, biochemistry has become responsible for explaining living processes such that many scientists in the life sciences from agronomy to medicine are engaged in biochemical research. This book contains an overview focusing on the research area of proteins, enzymes, cellular mechanisms and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in biochemistry. Particular emphasis is devoted to both theoretical and experimental aspect of modern biochemistry. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in biochemistry, molecular biology and associated areas. The book is written by international scientists with expertise in protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the book will enhance the knowledge of scientists in the complexities of some biochemical approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of biochemistry.

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