The Complex Relationship between Metals and Carbonic Anhydrase: New Insights and Perspectives

Maria Giulia Lionetto *, Roberto Caricato, Maria Elena Giordano and Trifone Schettino

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Abstract: Carbonic anhydrase is a ubiquitous metalloenzyme, which catalyzes the reversible hydration of CO$_2$ to HCO$_3^-$ and H$^+$. Metals play a key role in the bioactivity of this metalloenzyme, although their relationships with CA have not been completely clarified to date. The aim of this review is to explore the complexity and multi-aspect nature of these relationships, since metals can be cofactors of CA, but also inhibitors of CA activity and modulators of CA expression. Moreover, this work analyzes new insights and perspectives that allow translating new advances in basic science on the interaction between CA and metals to applications in several fields of research, ranging from biotechnology to environmental sciences.

Keywords: carbonic anhydrase; metals; inhibition; expression; biomarker; bioassay

1. Introduction

Carbonic anhydrase (CA) is a widely-distributed metalloenzyme, which catalyzes the reversible hydration of CO$_2$ to HCO$_3^-$ and H$^+$. This biochemical reaction plays a key physiological role in diverse biological systems. Six distinct and unrelated CA families (α-, β-, γ-CA, δ-, ζ and η-CAs) have been identified in animals, plants, algae and bacteria [1,2]. They all catalyze the same reaction of CO$_2$ hydration, but each family shows proper specific characteristics in primary amino acid sequence and 3D tertiary structure.

In animals, CA isoforms play a fundamental role in a number of physiological processes involving carbon dioxide and bicarbonate, such as transport of CO$_2$ and HCO$_3^-$ between body tissues and respiratory surfaces, pH homeostasis, electrolyte transport in various epithelia, biosynthetic reactions (gluconeogenesis, lipogenesis and ureagenesis), bone resorption and calcification. In algae, plants and some bacteria, CA isoforms are fundamental for photosynthesis [3,4].

The α-carbonic anhydrases are monomeric or dimeric and are found in animals, some fungi, bacteria, algae and green plants [5]. In mammals, at least 16 different α CA isoforms were isolated. Mammalian CA isoforms CAI, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV and CA XV (not expressed in humans) have catalytic activity, while the remaining three CAs (CARP VIII, CARP X and CARP XI) have lost the catalytic activity and are known as CA-related proteins [6].

The β-carbonic anhydrases are dimers, tetramers or octamers and are expressed mainly in fungi, bacteria, archaea, algae and chloroplasts of monocotyledons and dicotyledons [7] and some prokaryotes [8].

The γ-anhydrase class is a homotrimer that has been described in bacteria, Archaea and plants [9]. It also includes a number of non-catalytically-active homologs present in diverse species. δ- and ζ-CAs are present in several classes of marine phytoplankton. The δ class has been described in diatoms, and
its prototype is the CA TWCA1 from the marine diatom *Thalassiosira weissflogii* [10,11]. The ζ-CAs are probably monomers and have three slightly different active sites on the same protein molecule [12].

The η-CA was recently found in a number of species of the *Plasmodium* genus. These are a group of enzymes previously ascribed to the α family, but recently demonstrated to have a number of unique features, including their metal ion coordination pattern [2].

This review focuses on an interesting aspect of the research on CA, the relationships between carbonic anhydrase and metals, which play a fundamental role in the bioactivity of this metalloenzyme. The review points out the complexity and multi-aspect nature of these relationships, since metals can be cofactors of CA, but also inhibitors of CA activity and modulators of CA expression. New insights and perspectives are discussed encompassing several fields of research from biotechnological applications to environmental sciences.

2. Metals and CA Catalytic Site

All CA isoenzymes catalyze the reversible hydration of CO$_2$ to HCO$_3^-$ and H$^+$ through a metal-hydroxide [Lig$^3$M$^{2+}$(OH)$^-$] mechanism [13–15] (Figure 1). The central catalytic step involves the reaction between CO$_2$ and the OH$^-$ bound to the zinc ion, yielding a coordinated HCO$_3^-$ ion, which is subsequently displaced from the metal by H$_2$O. In the α, γ- and δ-CA classes, Lig$^3$ is represented by three key amino acid residues, which are three histidines in α-CA, γ-CA and δ-CA, one histidine and two cysteines in β-CA and ζ-CA and two His and one Gly residues in η-CA [16]. A fourth histidine, that is His 64 in human CAII (the most investigated CA isoform), not directly part of the active site, contributes to the catalytic process representing the so-called “proton shuttle”. This allows the H$^+$ transfer from the metal-bound water molecule to buffer molecules located outside the active site and ensures the reaction of the metal-bound OH$^-$ with CO$_2$ to produce HCO$_3^-$.

The metal (M) in the carbonic anhydrase metal-hydroxide [Lig$^3$M$^{2+}$(OH)$^-$] mechanism is Zn$^{2+}$ for all classes, but other transition metals have been demonstrated to bind to the catalytic site as physiologically-relevant metal cofactors or displacers of the native cofactor, producing in this case new CA metallovariants (Table 1).

![Figure 1](image-url)

**Figure 1.** The reversible hydration of carbon dioxide to bicarbonate catalyzed by CAs by means of a metal (M)-hydroxide mechanism. Modified from Berg [17]. (1) The release of a proton from the zinc-bound water generates the zinc-bound OH$^-$; (2) A CO$_2$ molecule binds to the active site and is positioned for optimal interaction with the zinc-bound OH$^-$; (3) The hydroxide ion attacks the carbonyl of CO$_2$, producing HCO$_3^-$; (4) The release of HCO$_3^-$ regenerates the enzyme.

| CA class | Lig       |
|----------|-----------|
| α CA     | His, His, His |
| β CA     | His, Cys, Cys |
| γ CA     | His, His, His |
| δ CA     | His, His, His |
| ζ CA     | His, Cys, Cys |
| η CA     | His, His, Gly |
### Table 1. Metals as physiologically-relevant cofactors of CA.

| CA Families | Metals as Physiologically-Relevant CA Cofactors | Ref. |
|-------------|-----------------------------------------------|------|
| α-CA        | Zn$^{2+}$                                      | [21] |
| β-CA        | Zn$^{2+}$                                      | [7]  |
| γ-CA        | Fe$^{2+}$; Zn$^{2+}$                           | [9,22] |
| δ-CA        | Zn$^{2+}$                                      | [10] |
| ζ-CA        | Cd$^{2+}$; Zn$^{2+}$                           | [12] |
| η-CA        | Zn$^{2+}$                                      | [2]  |

Apart from zinc, other metals have been found to be physiologically-relevant cofactors of some CAs.

#### 2.1. Metals as Physiologically-Relevant Cofactors of CA

Zn$^{2+}$ is one of the most widely-used metallic elements as enzyme cofactor in nature, and its presence in all of the CA families is a successful confirmation of its peculiar properties. The reason for its success lies in the filled $d$ orbital (d10). Unlike other first-row transition elements (e.g., Sc$^{2+}$, Ti$^{2+}$, V$^{2+}$, Cr$^{2+}$, Mn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$ and Cu$^{2+}$), Zn$^{2+}$ is not involved in redox reactions, but rather, it acts as a Lewis acid accepting a pair of electrons [18]. This makes zinc a good metal cofactor for biochemical reactions requiring a redox-stable ion to function as a Lewis acid-type catalyst [19], such as proteolysis and carbon dioxide hydration. Zinc complexes have low thermodynamic stabilities, as well as variable geometries, which in turn account for low activation barriers. This makes zinc a versatile and suitable as an active site metal [20]. Zinc is in the +2 state, and it is positioned in a cleft in the center of the CA molecule (Figure 2). It is coordinated by the three key amino acid residues (see above). The fourth coordination site is occupied by a water molecule. The role of Zn$^{2+}$ in the CA catalytic mechanism is to promote the deprotonation of H$_2$O with the production of the nucleophilic OH$^-$, which in turn can attack the carbonyl group of CO$_2$ to convert it into HCO$_3^-$.

Fe$^{2+}$ has been demonstrated to be a physiological metal cofactor of γ-CAs [22,23]. The γ-CA class is among the most ancient, with homologs widespread in Archaea and Bacteria [24]. CAM, the prototypic γ-class CA from the anaerobic Archaea species *Methanosarcina thermophila*, has been demonstrated to contain zinc in the active site when overproduced in *Escherichia coli* and purified in aerobic experimental conditions [22]. On the other hand, when the enzyme is purified anaerobically, it has been demonstrated to contain Fe$^{2+}$ in the active site (Fe-CAM) and to have a three-fold increased activity. These contrasting results are explained by the fact that in aerobic conditions, Fe$^{3+}$ is oxidized and is rapidly substituted by Zn$^{2+}$ present in the reaction buffers not treated with chelating agents. The stability of complexed Zn$^{2+}$ is much greater than that for Fe$^{2+}$ ligated with the nitrogen atoms of histidine residues coordinating the active-site metal in CAM. Thus, aerobic purification results in the loss of Fe$^{3+}$ and substitution with Zn$^{2+}$. These findings clearly suggest Fe$^{2+}$ as the physiologically-relevant metal [22–24] in the active site of the CAM enzyme. Considering that Fe$^{2+}$ is available in oxygen-free environments, the finding of iron as a physiological metal cofactor in CAM from an anaerobic species suggests the possibility that iron could act as a metal cofactor in γ-class CA enzymes and possibly in other classes in anaerobic organisms [23]. Moreover, evidence for the role of Fe$^{2+}$ in CA catalytic activity has been found also in the α-class. In fact, in duck erythrocytes, the addition of Fe$^{2+}$ in the reaction medium increases CA activity [25].

Cd$^{2+}$ has been demonstrated to be naturally used as a catalytic metal in the cadmium-carbonic anhydrase (CDCA1), a ζ-CA isolated from the marine diatom *Thalassiosira weissflogii* [26–28]. Its homolog gene has been found in a number of diatom species, as well as natural assemblages [12]. CDCA1 provides the first evidence of the biological role of cadmium, which is usually considered a toxic element associated with environmental pollution [29]. CDCA1 is considered a “cambialistic” enzyme because it can use either Zn or Cd for the catalytic reaction [12]. The ability to use Cd in CDCA allows the diatom *T. weissflogii* to support the needs of fast growth also in Zn$^{2+}$ limiting conditions, and this has ecologically relevant implications. In fact, in the oceans, which are known to be poor...
in metals, this ability might have given a competitive advantage to diatoms with respect to other species, contributing to the radiation of diatoms during the Cenozoic Era and to the parallel decrease in atmospheric CO$_2$ [12]. In the ocean, Cd shows a nutrient-like concentration profile, with a low concentration at the surface, because of phytoplankton uptake, and an increased concentration at depth due to remineralization of sinking organic matter. This Cd profile is thought to be explained by the use of this metal by CDCA.

Co$^{2+}$ has been demonstrated to in vivo substitute Zn$^{2+}$ in the other genetically-distinct CA form (TWCA1) (δ-CA) isolated from the diatom T. weissflogii [30]. Although the affinity of TWCA1 for Co$^{2+}$ is lower than for Zn$^{2+}$ and the Co-substituted enzyme is less active than the Zn-form [30], it has been suggested that the Co$^{2+}$ substitution can alleviate the Zn$^{2+}$ limitation in diatoms in open oceans, as also suggested for Cd$^{2+}$ [26,30].

$\text{Figure 2.}$ Human CAII: in detail, the metal binding site with the zinc ion as a sphere, the direct ligand histidines, H94, H96, H119, and the water molecule. Modified from Mahon et al [31] and from Dutta and Goodsell [32].

2.2. Metals as Displacers of the Native Metal Cofactor

In addition to the role of some metals as CA physiologically-relevant metal cofactors, many divalent metal ions, such as Co$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, Cd$^{2+}$ and Hg$^{2+}$, have been demonstrated to easily bind to the three-histidine moiety within the CA active site in in vitro experimental conditions [33–35]. Most of this information comes from transmetallation experiments on the α-CAII. The CO$_2$ hydration catalytic activity of metal-substituted human CAIIs, relative to wild-type CAII activity, is about 7% for the Mn$^{2+}$-substituted CA, about 2% for the Cd$^{2+}$-substituted and about 0% for the Cu$^{2+}$-substituted and Hg$^{2+}$-substituted ones, respectively [36,37]. Only Co$^{2+}$ is able to produce a catalytically-functioning enzyme with a wild-type catalytic efficiency ($K_{\text{cat}}/K_{\text{m}} = 8.7 \times 10^7$ M$^{-1}$·s$^{-1}$ for Zn$^{2+}$ vs. $8.8 \times 10^7$ M$^{-1}$·s$^{-1}$ for Co$^{2+}$) [34] because only cobalt besides zinc has a tetrahedral coordination at a pH around eight. In particular, Co-CA represents an ideal metallovariant for studying the CA catalytic site. In contrast to Zn$^{2+}$, lacking a spectroscopic signature, the paramagnetic Co$^{2+}$ is accessible to spectroscopic analysis, providing information about the environment of the metal ions in the CA active site [38].

2.3. Metal Binding to Other Sites in the CA Protein

In addition to the ability of some metals to bind the three key amino acid residues of the CA active site, metals are also able to bind CA protein in other sites elsewhere in the molecule thanks to their affinity for thiol and histidyl groups. Mercuric ions have been demonstrated by X-ray
crystallography [39] to bind to the His-64 ring (the so-called proton shuttle) and also to Cys-206 of CAII [40]. Copper ion is known to bind to the imidazole of His-64 in CAII, preventing its function in the H⁺ transfer from the zinc-bound H₂O to buffer molecules outside the active site [41]. In addition, Cu²⁺ is also able to bind CA in another binding site [42] nearby the N-terminus of the proteins, presumably in a four-coordinate tetragonal Cu²⁺ site with two histidine ligands and an N-terminal amine [43]. Because of the presence of this high affinity site, CA seems to be a member of a growing number of proteins and peptides that have been found to have an N-terminal Cu²⁺-binding site, including serum albumin and amyloid β-peptide complexes [43]. It is unknown why CA has this N-terminal Cu²⁺ site that does not show any catalytic activity. It is possible that this site could function as a sequestering site of adventitious Cu²⁺. In this respect, an intriguing hypothesis suggests that this N-terminal site can be effective in defending the native zinc(II)-(His)₃ active site of CA, as well as other metalloproteins, from Cu²⁺ displacement, and in turn, inactivation [43].

3. Metals and CA Activity Inhibition

The ability of some transition metals either to displace Zn²⁺ in the active site or to bind histidine and cysteine residues in sites other than the active site accounts for the inhibitory effect of some metals on CA activity, reported by several authors in a number of tissues in a variety of animal species. Table 2 summarizes the results obtained in in vitro studies.

In the teleost fish Ictalurus punctatus, Cd²⁺, Cu²⁺, Ag⁺ and Zn²⁺ were demonstrated to inhibit erythrocyte CA activity [44]. In the euryhaline teleost Anguilla anguilla, a significant in vitro tissue-specific inhibition of branchial and intestinal CA activity by Cd²⁺ was found [45,46]. In particular, in the intestine, a cytosolic and a membrane-bound CA were detected, with the cytosolic isoform more sensitive to the metal than the membrane-bound one. In the European seabass (Dicentrarchus labrax), Al³⁺, Cu⁺², Pb⁺², Co⁺³, Ag⁺¹, Zn⁺² and Hg⁺² were found to be competitive inhibitors of liver CA activity [47]. In the liver of sea bream (Sparus aurata) Ag⁺, Ni⁺², Cd²⁺ and Cu²⁺ were shown to be noncompetitive inhibitors of CA activity [48].

In rainbow trout (Oncorhynchus mykiss), Co⁺², Cu²⁺, Zn²⁺, Ag⁺ and Cd²⁺ inhibited brain CA activity. Ag⁺ and Cd²⁺ were competitive inhibitors, Cu²⁺ was noncompetitive inhibitor, while Zn²⁺ was uncompetitive inhibitor [49].

In the estuarine crab Chasmagnathus granulate, Cd²⁺, Cu²⁺ and Zn²⁺ inhibited branchial CA activity both in vitro and in vivo [50]. In other euryhaline crabs, Callinectes sapidus and Carcinus maenas, a significant in vitro inhibition of gill CA by Ag⁺, Cd³⁺, Cu²⁺ and Zn²⁺ was demonstrated, with strong differences between the gill CA of the two species, Callinectes sapidus CA being more sensitive than Carcinus maenas CA [51]. In humans, the cytosolic HCAI and II were in vitro inhibited by Pb⁺², Co⁺² and Hg⁺². Pb⁺² was noncompetitive inhibitor for HCAI and competitive for HCAII, Co⁺² was competitive for HCAI and noncompetitive for HCAII, and Hg⁺² was uncompetitive for both isoforms [52].

In the sturgeon Acipenser gueldenstaedti, Ag⁺, Zn⁺², Cu²⁺ and Co²⁺ were weak inhibitors of the erythrocyte CA activity [53]. In sheep kidney, Pb⁺², Co⁺², Hg⁺², Cd²⁺, Zn⁺², Se⁺², Cu²⁺ and Al³⁺ competitively inhibited CA activity in the low molar/millimolar range [54]. In the Turkish native chicken, Al³⁺, Hg⁺², Cu²⁺, Pb⁺² and Cd²⁺ were competitive inhibitors of erythrocyte CA activity. Pb⁺² exhibited the strongest inhibitory action; Cd²⁺ and Hg⁺² were moderate inhibitors, while Al³⁺ and Cu²⁺ were weaker inhibitors [55].

Ki or IC₅₀ values for in vitro metal inhibition of CA activity range from micromolar to millimolar depending on species, tissue and isoform. Although in some cases the Ki or IC₅₀ values are in the submicromolar or millimolar range, suggesting a weak inhibition, they may be significant for infield inhibition due to the bioaccumulation process occurring for most metals in many exposed organisms under prolonged exposure [56].
Table 2. *In vitro* inhibition of CA activity by trace metals. IC$_{50}$ and $K_i$ values are expressed as mM concentrations.

| Species                  | Tissue       | Cd$^{2+}$ (mM) | Cu$^{2+}$ | Hg$^{2+}$ | Zn$^{2+}$ | Co$^{2+}$ | Pb$^{2+}$ | Ag$^{+}$ | Ref. |
|--------------------------|--------------|----------------|-----------|-----------|-----------|-----------|----------|---------|------|
| *Callinectes sapidus*    | gills        | $K_i 0.1 \times 10^{-3}$ | $K_i 3.6 \times 10^{-3}$ | $K_i 2-6 \times 10^{-3}$ |          |          |          |          | [51] |
| *Carcinus maenas*        | gills        | $K_i$ 0.6–2.5 | $K_i$ 0.6–2.5 |          |          |          |          |          | [51] |
| *Anguilla anguilla*      | gills        | $IC_{50} 9.9 \times 10^{-3}$ |          |          |          |          |          |          | [46] |
| *Anguilla anguilla*      | intestine    | $IC_{50} 36.4 \times 10^{-3}$ |          |          |          |          |          |          | [46] |
| *Sparus aurata*          | liver        | $K_i 17.7$ (non-competitive) | $K_i 36.2$ (non-competitive) |          |          |          |          | $K_i 0.02$ (non-competitive) | [48] |
| *Ictalurus punctatus*    | erythrocyte  | $IC_{50} 0.9$ | $IC_{50} 0.065$ | $IC_{50} 0.7$ |          |          | $IC_{50} 0.035$ |          | [44] |
| *Oncorhynchus mykiss*    | brain        | $K_i 94.2 \times 10^{-3}$ (non-competitive) | $K_i 27.6 \times 10^{-3}$ (non-competitive) | $K_i 1.20$ | $K_i 0.035$ |          |          |          | [49] |
| *Acipenser gueldenstaedti* | erythrocyte  | $IC_{50} 5.2$ | $IC_{50} 2.8$ |          |          |          | $IC_{50} 1.7$ |          | [53] |
| *Dicentrarchus labrax*   | liver        | $K_i 0.76$ (competitive) | $K_i 0.72$ (competitive) | $K_i 0.53$ (competitive) | $K_i 0.24$ (competitive) |          |          |          | [47] |
| *Gallus*                 | erythrocyte  | $K_i 2.78$ (competitive) | $K_i 1.26$ (competitive) |          |          |          | $K_i 0.97$ (competitive) |          | [55] |
| *Ovis aries*             | kidney       | $K_i 1.04$ (competitive) | $K_i 4.70$ (competitive) |          | $K_i 0.96$ (competitive) |          |          |          | [54] |
| *Homo sapiens*           | Erythrocyte, CA I | $K_i 3.22$ (uncompetitive) | $K_i 3.22$ (uncompetitive) | $K_i 1.45$ (competitive) | $K_i 1$ (non-competitive) |          |          |          | [52] |
| *Homo sapiens*           | Erythrocyte, CA II | $K_i 0.312$ (uncompetitive) | $K_i 0.312$ (uncompetitive) | $K_i 1.7$ (non-competitive) | $K_i 0.056$ (competitive) |          |          |          | [52] |
The inhibitory effect of metals on CA activity demonstrated in in vitro studies has been confirmed by works carried out in in vivo exposure conditions. In the mantle of the filter-feeding mussel *Mytilus galloprovincialis*, CA was significantly inhibited by exposure to 1.78 μM Cd$^{2+}$ for 15 days [57]. Considering the role of CA in carbonate salt deposition, the inhibitory effect of Cd$^{2+}$ on mantle CA activity could explain the significant decrease in shell growth previously observed in *M. galloprovincialis* exposed to heavy metals. [58]. In the marine anemone *Aiptasia pallida*, a decrease of CA activity was observed after waterborne exposure to metal mixtures (Cu$^{2+}$, Zn$^{2+}$, Ni$^{2+}$ and Cd$^{2+}$) with a significant CA activity reduction following treatment with 100 μg/L for three days [59]. CA activity in the gills of the freshwater bivalve *Anodonta anatina* was significantly inhibited by the exposure to 0.35 μM Cu$^{2+}$ for 15 days [60]. A CA activity decrease with increasing exposure concentrations of Cu$^{2+}$ was observed in two species of scleractinian corals, such as *Acropora cervicornis* and *Montastraea faveolata*. Significant effects were detected in *A. cervicornis* exposed to 10 and 20 μg/L Cu$^{2+}$ and in *M. faveolata* exposed to 20 μg/L Cu$^{2+}$ for five weeks [61], respectively. A significant decrease in CA activity was recorded also in the anemones *Condylactis gigantea* and *Stichodactyla helianthus* exposed to Cu$^{2+}$, Ni$^{2+}$, Pb$^{2+}$ and V$^{2+}$ for 48 h [62].

From all of the available studies, a great variability in the sensitivity of CA activity to in vitro and in vivo metal exposure was observed among species, tissues and metals. This great variability can be explained by the complexity and multifaceted aspects of the binding of metals with CA isoforms, producing a number of different inhibitory responses to different metals in different tissues and species. It is possible to hypothesize that structural differences in CA isoforms could generate different metal-binding affinities and in turn different inhibitory responses.

4. Metals and CA Protein Expression

The inhibition exerted by some metals on CA-specific isoforms, as a result of the displacement of the native metal cofactor or the binding to other sites in the CA molecule, did not explain all of the biological effects of metals on CA.

It is known in plants and phytoplankton that biosynthesis of CA may be regulated by some environmental factors, including environmental trace metal concentrations [26,63]. Concerning animals, less information is available on this aspect. Some works indicate CA protein expression to be influenced by Zn$^{2+}$ availability. For example, in humans, patients with CAVI deficiency show stimulation of CAVI synthesis/secretion by Zn$^{2+}$ treatment [64], presumably through metal-induced upregulation of the CAIV gene. In rats, zinc deficiency induced a decrease in CAII protein expression in the submandibular gland [65].

In the fish *Cyprinodon variegatus*, CAII gene expression in gills and intestine was influenced by Cu$^{2+}$ exposure in a concentration-dependent manner, with a two-fold upregulation in the gills and a three-fold upregulation in the intestine following nine days of exposure to 100 mg/L Cu$^{2+}$ [66]. The effect of Cu$^{2+}$ on CA expression appears more complex if we consider that the metal in the same fish is also able to interfere with the regulation of CA expression by osmotic stress, disrupting the osmotic stress induction of the intestinal CAII. These results outline that CA plays a role in the combined effects of copper and osmotic stress on ion homeostasis and show how this enzyme represents a factor linking the physiological responses of the organism to multiple stresses.

In mussels, Caricato et al. [67] demonstrated that cadmium exposure (1.785 μM CdCl$_2$ for 14 days) enhances CA protein expression in digestive gland, as assessed by Western blotting analysis, CA activity measurement and immunofluorescence analysis in both laboratory and field experiments [67,68]. In basal conditions, digestive gland cells show a high CA specific activity, which can be functional to the well-developed lysosomal compartment of these cells. In fact CA, catalyzing the H$^+$ production from metabolic CO$_2$, can provide the H$^+$ necessary for the lysosome acidification. Therefore, the authors hypothesized that the observed metal-induced CA increased expression in mussel digestive gland is functionally related to the activation of the lysosomal system known to occur in pollutant-exposed organisms [69] and well described in mussel digestive gland [70].
The metal induction of CA expression arouses the question of how to interpret the different opposite effects that some metals can exert on CA, such as inhibition of the activity and upregulation of the gene expression. In mussel digestive gland, the IC$_{50}$ value for Cd$^{2+}$ inhibition of CA activity in vitro was in the millimolar range, while CA induction was observed following in vivo exposure to 1.785 $\mu$M CdCl$_2$ for two weeks [67]. In this case, it is possible to argue that the effect of Cd$^{2+}$ on CA is dose-dependent with upregulation of CA expression at low concentrations and inhibition of the CA activity at higher concentrations. Moreover, the type of effect of metals on CA, induction vs. inhibition, is tissue specific. In fact, in the case of mussels, the same organisms exposed to 1.785 $\mu$M CdCl$_2$ for two weeks showed mantle CA significantly inhibited [57], while digestive gland CA significantly increased [67].

Although future work is needed to clarify this intriguing aspect and to deepen the research on the regulation of CA expression by metals, these results enrich the panorama of information about the effects of metals on CA, pointing to the importance of the concentration of exposure, the type of exposure and the metal and tissue specificity on the observed effects.

5. CA and Trace Metals: Applicative Insights and Perspectives

As emerged from the above described studies, the relationships between metals and CA are complex and multifaceted, metals being cofactors of CA, but also inhibitors of CA activity and modulators of CA expression. In this field, new applicative perspectives have been recently developed in the biotechnological and environmental fields, allowing translating new advances in basic science on the interaction between CA and metals into practice and novel applications.

5.1. CA Metallovariants

The ability of many divalent metal ions, such as Co$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, Cd$^{2+}$ and Hg$^{2+}$, to easily bind to the three-histidine moiety within the CA active site in transmetallation experiments generated a number of CA metallovariants.

The interest in CA metallovariants goes beyond the study of the metal-protein interaction in the catalytic site structure. It allows probing the changes certain metals make to the function of the enzyme, including new catalytic reactions. This has been observed particularly in CA enzymes containing Co$^{2+}$ and Mn$^{2+}$ metal centers [71]. The natural function of CA is to catalyze the reversible hydration of CO$_2$ to HCO$_3^-$, but it is also known for its ability to catalyze the hydrolysis of esters with moderate enantioselectivity. Replacing zinc with manganese in the active site produced the manganese-substituted carbonic anhydrase (CA[Mn]) with peroxidase activity [72]. In the presence of bicarbonate and hydrogen peroxide, CA[Mn] catalyzed the efficient oxidation of O-dianisidine with $K_{cat}/K_m$ comparable to that for horseradish peroxidase. CA[Mn] also catalyzed the moderately enantioselective epoxidation of olefins to epoxides. This enantioselectivity of CA[Mn] is similar to that observed in natural heme-based peroxidases, with the advantage that CA[Mn] avoids the formation of aldehyde side products [71].

Therefore, the ability of some transition metals to displace the native Zn$^{2+}$ in the catalytic site discloses a CA catalytic promiscuity that has begun to be recognized as a valuable research and synthesis tool in potential biotechnological applications, leading to improvements in existing catalysts and providing novel synthesis pathways currently not available.

5.2. CA-Based Biosensors for Metal Ions

Pollution by trace metals is a world-wide problem, which raises concerns about potential effects on human health and the environment [73]. Mining and smelting, industrial and urban waste, wastewater discharges and shipping activity are the major anthropogenic sources of metals in the aquatic environment. Many sensitive and selective analytical methods for the detection of metal ions at low concentrations in environmental samples have been developed. In this field, great is the interest for simple, rapid and inexpensive alternatives to the classical analytical methods, able to provide a
continuous analysis of metal concentration in situ and in real time. This interest is expressed by the development of biosensors for trace metal detection. Some of them are fluorescence-based biosensors for the determination of free metals in solution [74–76], such as Cu$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Cd$^{2+}$ and Ni$^{2+}$ at concentrations down the picomolar range [76,77]. They utilize the affinity of the apoCA (in general variants of human CAII) for metals [76,78].

Therefore, the high affinity of CA for metals has driven the development of CA-based biosensing that has been shown to be a viable approach for determining certain divalent cations in environmental media, serving also as an archetype for other fluorescence-based biosensors [79].

5.3. CA-Based Ecotoxicological Biomarkers and Bioassays for Metal Pollution Assessment

In the last few decades, the use of effect-based methodologies, such as ecotoxicological bioassays and biomarkers, has received growing interest in environmental monitoring and assessment. Ecotoxicity bioassays are widely used for the assessment of environmental media quality, because they provide an integrated measurement of the various complex effects exerted by contaminants present in an environmental sample on the test biological system [80,81]. On the other hand, molecular and cellular biomarkers measured in exposed organism in the field can be helpful for detecting the occurrence of pollutant-induced stress syndrome in living organisms and gaining insight regarding the mechanisms causing the observed effects of chemicals [82,83]. Bioassays and biomarkers provide useful information about the degree of exposure to pollutants and the resulting effects on the organisms. In this regard, the use of bioassay/biomarkers provides early warning information useful for improving the processes of environmental risk assessment [84].

Recently, new perspectives on the potential use of CA as pollution biomarkers in environmental biomonitoring rose from the sensitivity of CA activity and expression to trace metals in several species. Therefore, CA measurements have been included in multi-biomarker approaches on bioindicator organisms in field studies [85]. For example CA activity inhibition measurement in the bioindicator fish Pimelodus maculatus has been included in the multi-marker biomonitoring of three water reservoirs along the Paraiba do Sul River in an industrialized area of Brazil [86]. In corals, CA activity inhibition by metal exposure has been related to inhibition of coral growth through alteration of the calcification process, and it has been suggested as a potential biomarker of exposure to metal pollution [61].

The applicability of digestive gland CA activity and expression in a multimarker approach has been also demonstrated in the bioindicator organism Mytilus galloprovincialis [67,87] in two studies addressing the ecotoxicological biomonitoring and assessment of a coastal marine area in Southern Italy.

Branchial CA activity measured in the filter-feeding species Crassostrea rhizophorae was shown to be very responsive to coastal contamination in a recent study carried out in three human-impacted Brazilian estuaries [88]. Data were consistent with the usefulness of branchial CA in this species as a supporting biomarker for inexpensive and rapid analysis.

All of the available studies demonstrate the applicability of metal-induced CA alterations as pollution biomarkers. However, the successful use of CA as a biomarker in environmental biomonitoring requires a thorough knowledge of the specific metal-induced CA response in the specific bioindicator species used, because of the high species-specific and tissue-specific effects of metals on CA isoforms.

The in vitro inhibition of CA activity by metals has been applied by Lionetto et al. [57,89,90] to the development of an in vitro bioassay sensitive to the synergic effects of metals in a mixture and useful for the toxicity assessment of environmental aqueous samples [90]. This patented method has been recently applied to the monitoring of harbor sediments [68] and of reclaimed waste waters [91], showing a high agreement with standardized in vivo bioassays.

All of the studies carried out to date on the in vitro and in vivo sensitivity of CA to trace metals contribute to developing effect-based methodologies, such as CA-based pollution biomarkers and
bioassays. They allow translating new advances in basic science into practice and novel applications in environmental biomonitoring (Figure 3).

![Diagram](image)

**Figure 3.** From *in vivo* and *in vivo* assessment of the sensitivity of CA to trace metals to environmental monitoring.

6. Conclusions

Although CA has been widely investigated in several aspects of its structure and functions, its complex relationships with metals have been only partially investigated to date, and some questions still remain to be clarified, such as, for example, the metal regulation of CA expression and its underlying mechanisms. This is an intriguing aspect of the research on this metalloenzyme that could raise new perspectives in the understanding of CA function and regulation.

The responses of CA activity and expression to metal exposure in animals need to be further characterized, and this could improve the potentiality of this enzyme for biotechnological and environmental applications.

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