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CARBONIC ANHYDRASE IN COMPLEX RELATION WITH METALS AND ITS APPLICATION AS POLLUTION BIOMARKER.

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ABSTRACT

Carbonic anhydrase constitute few unrelated classes of metallo-enzymes mainly responsible for the conversion of carbon dioxide to bicarbonate and hydrogen ionic moieties by reversible hydration. The resulting molecules are important metabolic pathway molecules. This enzyme plays important physiological functions in archaea, bacteria and in mammals. Metals interact with carbonic anhydrase by acting as cofactors for the carbonic anhydrase active site and sometimes as inhibitors of the enzyme. Metal ions can displace the native cofactor molecules from enzyme moiety and the expression level of carbonic anhydrase protein is affected by the metal ion concentrations in the surrounding. Complex relations between carbonic anhydrase and metal ions provide the application of CA as metallo-variants and pollution biomarkers. Carbonic anhydrase as pollution biomarkers are being applied for bio-monitoring in bioassays and in biosensors. Electrometric method is one of the major techniques extensively applied in bio-monitoring. Carbonic anhydrases are introduced in bio-indicator organisms when utilized as a pollution biomarker.

KEYWORDS: Metallo-enzyme, reversible hydration, pollution biomarker, bio-monitoring.

INTRODUCTION

Carbonic anhydrases (EC 4.2.1.1) are the metallo-enzymes encoded by different gene families. There are five unrelated families (represented as α-, β-, γ-, δ, ζ) of Carbonic anhydrase enzyme. Alpha, beta and delta class carbonic anhydrases[1] involve Zn^{2+} ions, gamma class enzymes incorporate Fe^{2+} and zeta class enzyme incorporate Cd^{2+} as well as Zn^{2+} ions in the active domain (site) of enzyme.16 alpha- carbonic anhydrase isoforms have been isolated in mammals, where they take part in essential physiological functions. The beta-delta-carbonic anhydrase representatives are widely found in plants, diatoms, eubacteria and archaea. The main mechanism of carbonic anhydrase involves the reversible hydration of CO_2 to bi-carbonate and H+ ion.[2] All the five distinct families have the ability to perform the same reaction of carbon dioxide hydration to bicarbonate but still each family has its own specific the amino acid sequence and 3D tertiary structure.[3] The alpha carbonic anhydrase has acquired the ability to catalyze a number of different hydrolysis processes.[4] The catalytic principle followed while catalysis by the alpha carbonic anhydrases involves the active sites of enzymes containing Zn^{2+} ions which are co-ordinated by three residues of histidine and a water or OH moiety. The latter includes dynamic species, playing role as a potent nucleophilic element. In case of beta and gamma carbonic anhydrase, the zinc hydroxide mechanism is also convincing. Carbonic anhydrase catalysis in inhibited by certain inhibiting elements including metal making complex with anions and sulfonamides along with their isosteres (sulfamates, sulfamides etc.). Many other significant physiological and physio-pathological roles are performed by carbonic anhydrase isozymes in members of the whole phylogenetic tree. They play key roles related to respiration, carbon dioxide or bicarbonate, pH and carbon dioxide homeostasis, delivery of CO_2 and HCO_3^- between respiratory surfaces and body tissues etc[5], electrolyte secretion and reactions for biosynthesis like urea genesis and carbon dioxide fixing (in algae and plants). Carbonic anhydrases occurrence in several tissues in addition to various iso-forms is an indication to design many inhibitors or activators with biomedical applications.

Prokaryotic carbonic anhydrases

Carbonic anhydrases are important group of enzymes in prokaryotic domains of Archaea and Bacteria. Carbonic anhydrases has been purified from five species only. Neisseria sicca was the first specie used to obtain carbonic anhydrase. All the three classes of enzyme encoding genes are found in prokaryotes with genes dominating for beta and gamma classes. Mammalian isozymes of carbonic anhydrase belong to alpha class while domain bacteria has only nine alpha isoforms and none of alpha class members found in domain archaea.

Beta class carbonic anhydrases have been found in both archaea and bacterial members. The only carbonic anhydrase belonging to gamma class is obtained from *Methanosacrina thermophila*.[6] Prokaryotes have multiple genes encoding for the same enzyme and this illustrates the importance of carbonic anhydrase in prokaryotic physiology. Some major functions performed by carbonic anhydrase in prokaryotes include cyanobacterial carbon dioxide fixation[7], cyanate degradation and endurance of intracellular pathogens in host. Various bacterial metabolic pathways involve carbon dioxide and or bicarbonates and these ions are regulated by carbonic anhydrase activities.[1]

METALS INTERACTION WITH THE CATALYTIC SITE OF CARBONIC ANHYDRASE

All carbonic anhydrase iso-enzymes catalyze the synthesis of HCO3 and H+ from carbon dioxide by a metal-hydroxide [$Lig^3M^{2+}(OH)^-$] mechanism of hydration and the metal involved in this mechanism is Zn^{2+} for all classes. There are six classes of carbonic anhydrase and each with its own cofactors; as shown in the following table.

Table 1: Various metal ions acting as cofactors for different families of Carbonic anhydrase

CA Families	Metals as cofactors
α-CA	Zn^{2+}
β-CA	Zn^{2+}
γ-CA	Fe^{2+}, Zn^{2+}
δ-CA	Zn^{2+}
ζ-CA	Cd^{2+}, Zn^{2+}
$Cd2^+$	Zn^{2+}

In case of the carbonic anhydrase enzyme, Zn^{2+} is an extensively used metallic element as the cofactor, and its occurrence in all the carbonic CA families is a conquering verification of its unusual characteristics. Its filled *d* orbital (d10) is the reason to Zn^{2+} success. Zn^{2+} doesn't play a significant role in redox reactions as transition elements of the first periodic row, rather, it behaves as a Lewis acid as it accept an electron pair. [8] That property makes zinc a fine metal enzyme cofactor for biochemical reactions that want a redox-stable ion to act as Lewis acid-kind of catalyst such as carbon dioxide hydration and proteolysis. The complexes of Zinc have variable geometries and low thermodynamic stabilities that in return are responsible for low activation barriers. That makes Zn a suitable and versatile metal for active site. Zinc is located in a cleft in center of the CA molecule and is in the +2 oxidation state. Zinc is coordinated by the 3 key amino acid residues the fourth coordination site is occupied by a molecule of water. The essential catalytic step involves the interaction between CO_2 and the OH bound to the Zn^{2+} ion, giving a HCO_3^- ion that is then displaced from the metal by H_2O. In the α-, γ- and δ-CA classes, Lig3 is symbolized by three key amino acid molecules, which are 3 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.[9] Function of the Zinc in CA catalytic mechanism is to promote deprotonation of water with nucleophilic OH^- production that can attack the C=O group of CO_2 to convert it into bicarbonate, then a molecule later displaces the HCO_3^- at metal.[10] Following is the human CAII depicted in figure 1, affinity site for metal showing Zn ion as ball, the ligand Histidine, HIS119, HIS 94, HIS 96, and a water molecule.

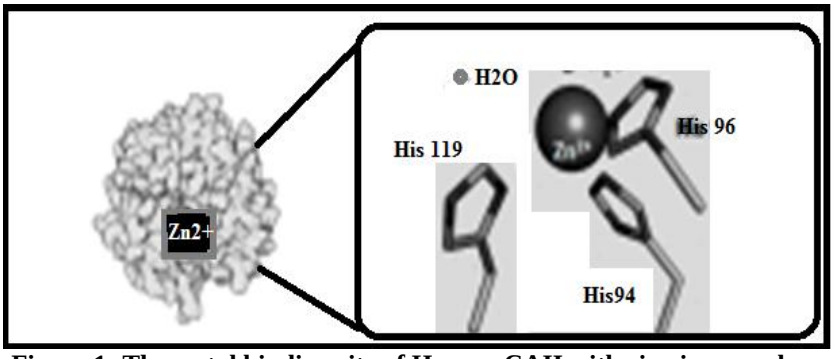

Figure 1: The metal binding site of Human CAII with zinc ion as sphere

CA as metallovariants

Due to the capability of various divalent ions like CO^{2+},Ni^{2+}, Mn^{2+},Cu^{2+},Cd^{2+} and Hg^{2+} to feasibly bind to 3 histidine moieties within CA active centre in trans-metallation experiments generate a variety of CA metallovariants. In CA metallovariants the interest goes far away the study of protein-metal contact in the catalytic structure. This is reported particularly in CA enzymes containing the Co^{2+} and Mn^{2+} metal centers.[11] Function of CA enzyme is catalysis of reversible hydration of carbon dioxide, but it also has the potential to catalyze the hydrolysis of esters. Manganese-substituted enzyme (CA[Mn]) is produced by substituting Zn with Mn in active site that has peroxidase

activity. [12] CA[Mn] also catalyzed the moderately enantio-selective epoxidation of olefins to epoxides.[11] Therefore, the CA catalytic promiscuity disclose when some of transition metals relocate the resident Zn^{2+} in the catalytic site that began to be recognized as a synthesis tool and helped in prospective biotechnological applications, resulting in improvement in obtainable catalysts and provided that novel synthesis pathways are that currently not available.

Metals can displace natural metallic cofactor

Since physiologically relevant metals play a key role in the enzyme cofactors but there comes some problems as is seen in vitro experimental conditions, that there are many divalent metal ions, such as Co^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+}, are demonstrated that can easily bind to the three-histidine moiety within the CA active site.[13] From trans-metallation experiments on the α-CAII this information has been collected. The % of the metals that are substituted in CO_2 hydration catalysis of human CAII comparative to wild-type CAII activity, is 2% for the Cd^{2+}-substituted, is almost\ 7% for Mn^{2+}-substituted CA, and roundabout 0% for Cu^{2+}-substituted and Hg^{2+}substituted metals, respectively**.** Only Co^{2+} is able that can produce a functioning enzyme with a wild-type catalytic effectiveness. In this regard, Co-CA shows the best metallo-variant to study the CA catalytic site.[14]

Metals and carbonic anhydrase protein expression

Due to the dislocation of the resident metallic cofactor or binding to other sites in the CA protein, the inhibition by a few metals on CA specific iso-forms, are not the entire range of the biological effects of metals on CA. Synthesis of CA, in plants and phyto-plankton, is synchronized by few environmental factors, that include trace metal concentrations in environment.[15] As is concerned of Kingdom Animal, less data exists on that side. Few experiments indicate CA protein expression to be prejudiced by accessibility of Zn^{2+}.[16] For example, In human beings, CAVI deficiency patients experience stimulated production of CAVI secretion on curing with Zn^{2+}[16] presumably by induced up regulation of the CAIV gene. In Rat's mandibular gland, deficiency of zinc induced a decline in expression of CAII protein.[17]

INHIBITORY EFFECTS OF METALS ON CARBONIC ANHYDRASES

The metals are able to bind the three key amino acid residues of the CA active site, but there are a number of metals that are able to bind CA protein at other sites. Instead of binding to the three key enzymes elsewhere in the molecule, metals bind to other sites due to their affinity for thiol and histidyl groups. For example, Mercuric ions have been shown by X-ray crystallography to bind to the His-64 ring and also to Cys-206 of CAII.[18] Some transition metals have the potential to displace Zn^{2+} in the active site or that can bind cysteine and histidine residues at such sites other than the active site, and it exerts the inhibitory effect of some metals on CA activity that is reported in a variety of tissues of animal species by several authors. In this regard various examples have been reported like in one of the experiments the teleost fish (*Ictalurus punctatus)*, Ag^{+}, Zn^{2+}, Cd^{2+}, and Cu^{2+},were inhibitory to RBC's CA activity.[19] Metal's inhibitory effect on CA activity shown by the study under in vitro conditions has been verified by working in in vivo conditions. It is probable to put forward that differences in CA iso-forms structures can produce variant affinities for meal binding and thus inhibitory responses which are also different.[20]

CA ACTING AS POLLUTION BIOMARKER

In the biotechnological and in environmental fields, new applicative perspectives have been recently developed, allowing translating new advances in basic science on the interaction between metals and CA into novel and practical applications. Biomarkers are the variations caused by pollutants at cell level or in biochemical elements in living beings on exposure to environmental contaminants. Biomarkers make two classes including type 1 related to exposure and 2nd to effects.[21] Biomarkers related to exposure (class 1) are the alterations at cell level which are reversible, providing timely signals on exposure to micro-pollutants. They have specificity for a single class of pollutants. Biomarkers of class 2 (effect) provide estimation of physiological special effects on living beings. These biomarkers have direct relation with the undesirable health affect risks. As the disturbances caused by pollutants are expressed late at population or community levels so the detection of damage at molecular or cellular level would be a sensitive early sign of warning.[22] The properties essential for a biomarker include its sensitivity, and whether the response is easily measurable. Carbonic anhydrase is an enzyme of importance in both human and wildlife. So the abnormalities in CA activity would be a risk to survival of organisms. This suggests the significance of CA enzyme as a biomarker for environment. Various a biotic (temperature, pH etc) and biotic (sex, ability to produce etc) affect the biological responses and must be interpreted carefully for biomarker applications.[23] Variety of biological responses is demonstrated on exposure to different types of pollutants occurring at the same time in environment. Therefore a number of biomarkers are required for effective bio-monitoring programs to understand the complex stress syndrome initiated by pollutants in organisms. Multi-biomarker strategies on bio-indicator living beings for successful evaluation and monitoring are applied. Assessment of CA gives the evaluation of pollutant induced stress syndrome as CA is involved in many physiological functions such as homeostasis and osmotic regulation etc. For instance, hindrance in the activity of CA by heavy metals results in slowing down of calcification process and retardation of coral escalation.

BIO-MONITORING

Chemical contamination of environment is a major concern of today due to increased human activities.

Heavy metal ions, products based on oil, pesticides, plastic goods and fertilizers affect the aquatic and terrestrial environment in negative way.[24] This tempts the development of advance technologies for the detection, management and elimination of pollutants from environment.[25] Monitoring of environment by biological methodologies is one of the modern and efficient techniques. Figure 2 depicts the applications of CA related to environmental monitoring.

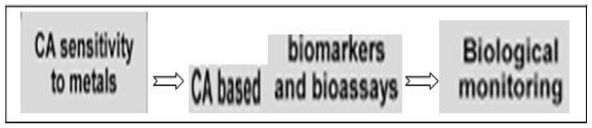

Figure 2: CA as pollution biomarker.

More than single kinds of pollutants present in environment interact simultaneously to cause complex and extensive changes characteristics of pollutants like toxicity and bioavailability. One of the biologically based techniques for environmental monitoring is CA inhibition, which is the biochemical shift indicating environmental health rapidly and sensitively. Different types of CA from various tissue types in a number of species are susceptible to various kinds of pollutants (38). A biological response as a result of toxic pollutants must have the standard specifications to be used as a biomarker for monitoring environment.

Major pollutants being monitored

Organic chemical pollutants

Pesticides have toxic effects directly on man and normal biota of living organisms. CA obtained by purification from erythrocytes of *Acipenser gueldenstaedtii* is inhibited by 2,4-dichlorophenol, dithiocarbamates, parathion and carbaryl which is a pesticide.[26] Dicarbamates are actually the low molecular weight inhibitors of carbonic anhydrase. Herbicides and fungicides have also been reported as inhibitory elements of human CA from erythrocytes.[27] Deltamehtrin is the most effective pesticide inhibitor (in vivo) of enzyme and this effect has been confirmed by various studies.[29] CA II are highly sensitive to carbamate pesticide carbaryl and polychlorinated biphenyl arochlor and show 34.4% inhibition of enzymatic function even at the concentration of 10 nanograms per liter. CA feedback to organic pollutants is analyzed for mankind and environment health in bio-monitoring.[28]

Heavy metal pollution

Heavy metal pollutants are one of the major toxicological contaminants of environment causing threat to living organisms. They have the ability to evade bodies of organisms via water, air or absorption through skin.[20] Some of the leading causes of heavy metal pollution involve waste disposal and fuel combustion. Heavy metals act as co-factors for catalysis by carbonic anhydrase but they also inhibit CA activity in vitro.[19] The inhibitory effect has been reported for both the vertebrate and invertebrates. CA (cytosolic isoform) activity found in gills of eel specie *Anguilla anguilla* is sensitive to heavy metals more than the isoforms bound to membrane found in its intestine.[40] The difference of 10-30 minutes has been observed for the inhibition of above two isoforms by cadmium ion. Reason behind this time gap is the duration of time needed by the cadmium to displace metal ion (Zn) linked to the enzyme resulting in Cadmium substituted CA which is inactive. Cadmium being in same periodic table group, owns same oxidation state and same size in ionic form as that of Zinc makes it capable to replace zinc in biological systems. Membrane bound CA isoforms shows delayed inhibition due to hard access to catalytic site of enzyme. Effect on CA activity with respect to metallic ion concentration has been graphically represented in figure 3.

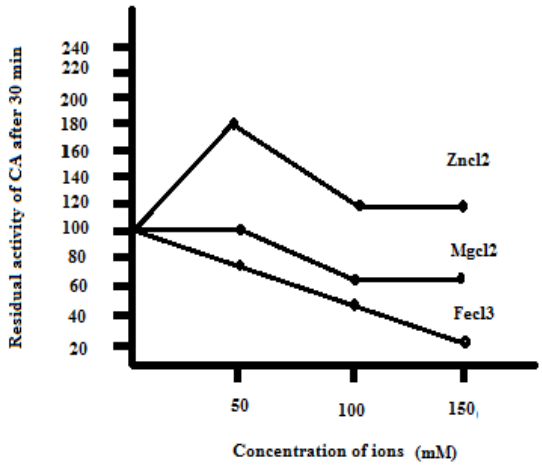

Figure 3: Graphical representation of effect of metallic ion concentration on CA activity.

In pollution monitoring program the CA function was greatly retarded by in vivo and in vitro exposure to cadmium ions. CA have a significant role in calcification process, therefore the heavy metal pollution results in hang-up of shell growth process in mussels. Cadmium exerts inhibitory effects on CA in mantle but the same metal ions enhance the CA activity in digestive glands and expression. But the increased CA function in digestive gland is less sensitive to Cd ion exposure so only the enhanced concentration of these metallic ions would exert the positive effect. CA inhibition by heavy metals follows the rule of high specie specificity. Difference in binding attraction between enzyme and pesticide are responsible for variation in susceptibility of CA in different species to these inhibitors.

Electrometric method in bio-monitoring

Functioning ability of CA is evaluated using various methods. Electrometric method[30] is one of the most widely used methods for this purpose. This technique follows the principle of evaluation of rate at which pH declines in reaction media having CA and respective substrate (carbon dioxide). Electrometric technique is beneficial as it is easy to manipulate and applies cheap apparatus. It provides precise quantitative results.[31] Due to these elements CA activity evaluation is possible in

field biomarker applications involving sentinel organisms. Biomarkers response in a dose dependent manner to the existence of heavy metals. Dose-response sensitive activity of CA in response to different pollutants like heavy metals has been observed during in vivo and in vitro studies. These studies provided the indication of using alterations in CA functioning as pollution biomarker. Alterations in CA response are tissue and specie specific so the knowledge about precise bio-indicator species expressing pollutants initiated variation in CA activity is necessary.

OTHER WAYS TO APPLY CA AS POLLUTION BIOMARKER

Biomarkers of different sources or types have variations in responses due to natural differences (e.g temperature and weight etc). One of the essential issues regarding bio-monitoring by biomarkers is that response of biomarker must depict the link to hazardous effects at organism level like reproduction, growth and many more.

Biomarkers applied in bio-indicator organisms

M. galloprovincials

Another study on CA expression in multi-marker technique in bio-indicator living being *(M. galloprovincialis*) was performed[32] to assess the eco-toxicological risk in marine coastal area on exposure to urban and industrial impact. Mussels on exposure to anthropogenic affected site for a month showed considerable increase in digestive gland CA expression and function more than those not exposed to such conditions. This retort paralleled the metalothionein induction in same animals. This study was a clear indication that heavy metal environment results in the increase of CA expression in mussel digestive glands. Its digestive gland has well established endolysosomal system.[33] CA catalyses the hydrogen ion production by carbon dioxide, so it provides proton for lysosomal acidification. Up regulation of CA on heavy metal contact activates the lysosomal system in digestive gland. Evaluation of digestive gland CA expression compliments the data provided by lysosomal system in a mussel.

Rainbow trout (*Oncorhynchus mykiss*)

Impact from the kidney of rainbow trout has also been studied. Contamination of water and soil is a major threat in the era of technology. Heavy metals in addition to being contaminants have significant contribution in enzyme activity and metabolism because of bio-accumulative and non-biodegradable characteristics of metals. Toxicity due to heavy metals results in abnormal metallothioneins protein formations, damage to renal and destruction of bone structure in wildlife and human beings. It has been studied that heavy metals affect CA from the kidney of rainbow trout. Using esterase method the hindering effects of Co, Zn, Cu, Cd and Ag on function ability of CA have been studied in vitro. Cobalt had the maximum potential for CA inhibition while rest of metal were in the order of inhibition like given ahead; Zinc> Copper> Cadmium> Silver.[34]

Water quality assessment

Bio-indicator catfish (*Pimelodus maculatus*) was used for this experiment. Brachial CA was reduced during dry season whereas the renal CA functioning was retarded during rainy season. This depicted that the osmoregulatory and acid-base regulatory phenomenon when disturbed result in alteration of CA activity. And the degree of alteration helps to evaluate whether the water is fit for consumption or not.[35]

Carbonic anhydrase based environmental assay

Carbonic anhydrase (metalloenzyme) is found in plants, animals and bacterial kingdoms for various physiological roles. CA activity is sensitive to contact with cadmium in teleosts and dichlorodiphenyl-dichloroethane in birds. In vitro enzymatic assay applying this enzyme is used for environment monitoring. Water quality was checked by calculating the active CA units byrate of proton formation in reaction solution (having carbon dioxide as the substrate) against blank having acetazolamide which is a precise CA retarder. Proton deviation was done at 0 degree Celsius. CA activity was inhibited in the assay for heavy metals. Thus CA in vitro bioassay provides a rapid tool to assess the toxicity of samples from environment.[28] Environment bio-monitoring involves bioassays (in vivo and in vitro) and biomarkers. Cell extracts or cell cultures are used to detect the existence of pollutants using in vitro bioassays. These assays aid in the measurement of inhibition of enzyme, binding to receptor and alterations in genome expression. Evaluation of biomarkers in living organisms exposed the polluted environment aid in the detection of stress syndromes caused by pollution.

Bio-sensing of metal ions based on Carbonic anhydrase

Metal extraction from mines, industrial and urban wastes as well as transportation via shipping routes have been proved to be some major causes of metal pollution in the surrounding. Attraction of CA towards the metal ions is the basis of CA based bio-sensing.[36] Bio sensors based on the fluorescence phenomenon evaluates the amount of free metal ions in solution[38] applying CA. Some metal ions like Zn, Cd and Cu are detected even in the concentrations of Pico-moles[37] by anisotropy, and changes in fluorescence release. These biosensors utilize the affection of apoCA for metal ions.[39] Bio sensors with improved sensitivity, kinetics and stability have been launched.

CONCLUSIONS

CA is a potent enzyme as it is liable for important physiological roles in prokaryotes as well as in eukaryotes. Complex interactions between CA and metal ions have been studied widely but advance research in some useful aspects like regulation of CA expression by metals can result in new beneficial perspectives. CA

being sensitive to organic and heavy metal pollution is serving in bio-monitoring as multipurpose pollution biomarker for the evaluation of pollution induced stress at cellular and biochemical level.

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