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ALAD (δ-aminolevulinic Acid Dehydratase) as Biosensor for Pb Contamination

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1. Introduction

Heavy metals are defined as those having a specific density of more than 5 g/cm^3. The main threats to human health are associated with exposure to lead, cadmium, mercury and arsenic (arsenic is a metalloid, but is usually classified as a heavy metal) (Järup 2003). Heavy metals have been used in many different areas for thousands of years. Lead has been used for at least 5000 years, early applications including building materials, pigments for glazing ceramics, and pipes for transporting water. In ancient Rome, lead acetate was used to sweeten old wine, and some Romans might have consumed as much as a gram of lead a day. Mercury was allegedly used by the Romans as a salve to alleviate teething pain in infants, and was later (from the 1300s to the late 1800s) employed as a remedy for syphilis.

Although adverse health effects of heavy metals have been known for a long time, exposure to heavy metals continues and is even increasing in some areas. Since the middle of the 19th century, production of heavy metals increased steeply for more than 100 years, with concomitant emissions to the environment. Emissions of heavy metals to the environment occur *via* a wide range of processes and pathways, including to the air, waters, and soil (Järup 2003).

Lead, mercury, cadmium and arsenic form a significant potential threat to human health, both occupational and environmental (Hu 2000). Over the past few decades, heavy metal contamination of aquatic system has attracted the attention of a number of researchers in all over the world. Many industrial and agricultural processes have contributed to the contamination of fresh water systems thereby causing adverse effects on aquatic biota and human health (Wang 2002, Dautremepuits 2004). The fact that heavy metals cannot be destroyed through biological degradation and have the ability to accumulate in the environment make these toxicants deleterious to the aquatic environment and consequently to humans who depend on aquatic products as sources of food. Heavy metals do mainly accumulate in the tissues of aquatic animals and can be of public health concern to human beings (Kalay 1999, Ashraf 2005).

In fact, there are several heavy metals known to be carcinogens, including arsenic, chromium and nickel. Many of the toxic effects of metals, including carcinogenicity, can be modified by concurrent exposure to other metals. The emphasis now is on testing the effects of mixtures of chemicals to simulate actual environmental conditions. Researches have already demonstrated links between lead and polychlorinated biphenyl exposure and

neurodevelopmental effects. It is now frightened that neurobehavioural and neurodevelopmental deterioration may take place in the next generations (Tang et al. 1999, Winneke et al. 2002, Stein et al. 2002, Yang et al. 2003).

Reports which link autoimmune diseases to environmental factors have appeared in recent literatures (Molina & Ehrenfeld 2003, Dooley & Hogan 2003). In many exposed populations, some individuals are extra sensitive while some extra tolerant. Especially, children are more susceptible to mercury and lead exposures (Needleman et al. 1990). In order to comprehend the basis of these special high or low risk groups, regarding comparative anatomy, physiology, metabolism and genetics, more in depth studies in toxicodynamics and toxicokinetics are needed. It is assumed that state-of-the-art technology where occupational safety, product safety, environmental quality maintenance and accident prevention are in-built under good manufacturing practices. As a result of these studies, conventional problems in occupational toxicology are expected to decrease. Exposure to chemicals already loaded in the environment will still be responsible for delayed effects and indirect exposure. Molecular epidemiology, armed with designed molecular probes and noninvasive diagnostics will become a leading component in risk assessment and health management in future.

2. Lead and lead pollution

Lead (Pb) is one of the most widely used metals in industries and almost in all over the world exposure to Pb continues to be a common problem. Batteries, paints and pigments, plastic, ceramic, secondary foundries and welding are the most important occupational settings. The general population may get exposed to Pb due to food and water contamination, and air pollution caused by industrial emission and fuel containing Pb compounds.

Due to environmental ubiquity and persistence of Pb, its accumulation in organisms and biomass throughout the trophic chain, imply a continuous exposure. This metal can cause mortality in cases of acute poisoning or can indirectly affect the populations by altering reproductive success, behavior, immune response, and physiology in cases of chronic exposure (Mazliah et al. 1989, Burger 1995, Burger & Gochfeld 2000b, Fair & Ricklefs 2002).

Sometimes wild birds might be exposed to very high metal levels, for example, at waste disposal sites or through the ingestion of lead-shot pellets. Such acute poisonings are easily diagnosed, although longer-term effects are difficult to assess. Lead in blood is a good indicator of newly exposure, while chronic exposure can be estimated when concentrations in accumulator tissue(s) are available. Although studies on dead animals provide useful information, ethical, legal, and scientific reasons indicate the need for other types of more easily available samples (feathers, eggs, excrements, regurgitated food, etc), which enable us to estimate exposure conditions (Burger & Gochfeld 2000a, Dauwe et al. 2000). In recent years the usefulness of feathers as a biomarker of heavy metal exposure has been investigated. Results were very satisfying to monitore mercury and lead levels, while contradictory results have been obtained for cadmium (Furness 1993).

In spite of significant reductions in use, most notably in paint production and as a fuel additive, Pb continues to enter the environment primarily by anthropogenic resources, retaining its status as a priority pollutant (USEPA 2006).

As the focus has turned towards remediation concerning to prevent the human exposure, much is still needed in the way of determining appropriate measures to monitor and protect

the aquatic environment. Usually, water quality criteria (WQC) continue to rely principally on water hardness (i.e. Ca^{2+}) despite growing evidence that other chemical parameters [e.g. pH, salinity and dissolved organic carbon (DOC)], which may vary greatly on a local basis, also strongly influence Pb toxicity (Macdonald et al. 2002, Grosell et al. 2006).

Efforts to improve WQC for metals have resulted in several toxicity models which designed to encircle the influences of all major water chemistry parameters. The most widely accepted model, the biotic ligand model (BLM), is currently used by the USEPA to set WQC for copper. In core, the BLM is resposible for site-specific water conditions considering the competitive effects from other cations and complexity with organic/inorganic agents that prevent the metal from interacting with the site of toxic action (Paquin et al. 2002).

There has been no demonstrated biological need for Pb. Therefore, its uptake and toxicity is likely mediated through imitating of other cations (Ballatori 2002). The most reasonable candidate is Ca^{2+}. There are strong evidences that Pb acts as a Ca^{2+} antagonist (Busselberg et al. 1991, Rogers & Wood 2004). However, the identification of a specific ligand for Pb remains elusive. As in mammals, the principal effects of chronic Pb exposure to fish are presumably hematological (Hodson et al. 1978), neurological (Davies et al. 1976) and renal (Patel et al. 2006) defects. Some studies have also examined reproduction and behavioral effects (Holcombe et al. 1976, Weber 1993).

These metals and other toxicants are commonly present as mixtures in the environment. Genomic approaches are well suited to locate such problems by filling in where more conventional methods prove insufficient to precise key environmental stressors or elucidate the contributions and additive effects from multiple toxicants. Additionally, microarrays give opportunities not only for establishing the molecular basis of toxicity, but potential for gaining insights into modes of action and higher order effects. Thus, defining toxicant-specific mechanisms that link signature gene transcript profiles to chronic effects would greatly help in monitoring and diagnosing water quality and also prioritizing higher ranked tests in ecological risk assessment. The significance of genomics in this regard was recently referred by the USEPA (Dix et al. 2006).

3. Lead toxicity and δ-ALAD as biosensor for lead toxicity

Pb is a natural component of ecosystems with no known biological role and is highly toxic. Its toxicity originates from its ability to mimic biologically important metals and to produce membrane damage through lipid peroxidation. Most Pb poisoning symptoms are thought to occur by interfering with an essential enzyme, δ-aminolevulinic acid dehydratase (ALAD), the activity of which is markedly inhibited by Pb. This is in total agreement with almost all studies and confirms the toxic effects of Pb for the taxa including bacteria, fishes, amphibians, reptiles, birds, mammals and humanbeings.

A biosensor is a device for the detection of an analyte that combines a biological component with a physicochemical detector component (http://en.wikipedia.org/wiki/Biosensor). It consists of 3 parts:

- The *sensitive biological element* biological material (eg. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.), a biologically derived material or biomimic] the sensitive elements can be created by biological engineering.
- the *transducer* or the *detector element* (works in a physicochemical way; optical, piezoelectric, electrochemical, etc.) that transforms the signal resulting from the

interaction of the analyte with the biological element into another signal (i.e., transducers) that can be more easily measured and quantified;

• Associated electronics or signal processors that are primarily responsible for the display of the results in a user-friendly way (Cavalcanti 2008).

An enzyme, an analytical device, can be used as a biosensor. This combines an enzyme with a transducer to produce a signal proportional to target analyte concentration. This signal can result from a change in proton concentration, release or uptake of gases, such as ammonia or oxygen, light emission, absorption or reflectance, heat emission, and so forth, brought about by the reaction catalyzed by the enzyme used. The transducer converts this signal into a measurable response, such as current, potential, temperature change, or absorption of light through electrochemical, thermal, or optical means. This signal can be further amplified, processed, or stored for later analysis. Because of their specificity and catalytic properties, enzymes have found widespread use as sensing elements in biosensors. Since the development of the first enzyme-based sensor by Clark & Lyons (1962), who immobilized glucose oxidase on an oxygen-sensing electrode to measure glucose, there has been an impressive proliferation of applications involving a wide variety of substrates. A variety of the enzymes belonging to classes of oxido-reductases, hydrolases, and lyases have been integrated with different transducers to construct biosensors for applications in health care, veterinary medicine, food industry, environmental monitoring, and defense (Guilbault 1984).

Enzyme biosensors have been widely used in clinical and food analysis, where analytes represent natural substrates of the enzymes employed. At difference, in environmental analysis, pollutants (e.g. pesticides, heavy metals, etc.) are generally detected as monitoring the inhibition of enzymatic activity caused by those toxic materials. The reduced specificity of inhibition phenomenon makes only possible the determination of such parameters, i.e. total concentration of the substances belonging to a certain class. On the other hand, it is expected that biosensor detects only contaminants, which are actually harmful to life.

Enzyme biosensor has got some advantages and same disadvantages. Their advantages are: being more specific than cell based sensors; faster responds due to shorter diffusion a path (no cell walls). Disadvantages: being more expensive to produce; and enzymes are often unstable. Additonally, many enzymes need cofactors for the detection of substances (http://www.rpi.edu/dept/chem-eng/Biotech Environ/BIOSEN/enzbio.htm).

Biosensors for heavy metals have been mainly developed in environmental analysis in water (Evtugyn et al. 1999). As far as enzyme biosensors for heavy metal determination are concerned, a certain number of papers have appeared, reporting the use of different enzymes and biosensor configurations/transducers (Ciucu et al. 2001, Compagnone et al. 1995 and Donlan et al. 1989b, Starodub et al. 1999, Vel Krawczyc et al. 2000, Pirvutoiu et al. 2001, and Dzyadevych et al. 2003). In several cases the inhibition of enzymes by heavy metals is reversible, even if for rapid restoration of enzymatic activity the use of strong ligands, like EDTA, is required.

Most of the heavy metals bind to the sulfhydryl groups, thus inhibiting enzymic activity, disrupting cellular transport and causing changes in protein functions. The toxicity of heavy metals includes the blocking of functional groups of important molecules, e.g. enzymes, polynucleotides, transport systems for essential nutrients and ions, and substitution of essential ions from cellular sites.

During the last decades there was an increasing interest to investigate other sublethal endpoints, especially in relation to those biochemical responses that may be considered as
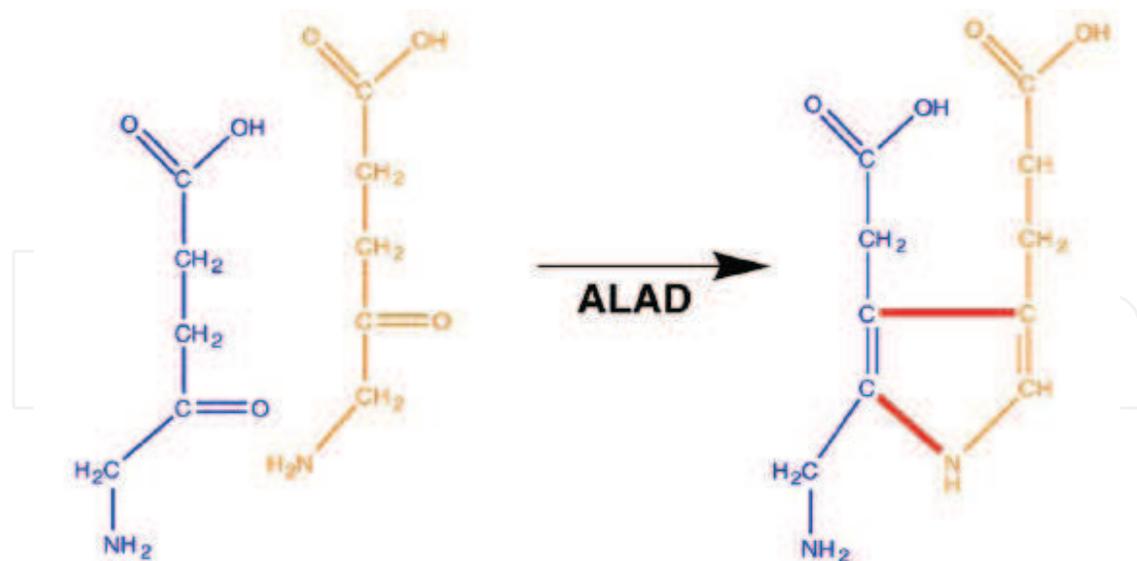
early biosensors of contamination (Huggett et al. 1992) Among them, the inhibition of the enzyme δ-aminolevulinic acid dehydratase (ALAD, E.C. 4.2.1.24) is recognized as a useful biomarker of Pb exposure and effect, both in humans and other animal species (Rand 1995, Timbrell 2000).

Endogenous metals are essential components of many enzyme systems, for instance, δ-aminolevulinate dehydratase (δ-ALAD or called PBGS, EC 4.2.1.24) is a metalloenzyme requiring zinc ions for activity (Jaffe et al. 1995). δ-ALAD catalyses the asymmetric condensation of two aminolevulinic acid (ALA) molecules to form porphobilinogen (PBG) in heme biosynthesis (Gibson et al. 1955) (Figure 1-2) pathway. The pyrrole is common precursor of the tetrapyrrole pigments such as heme, chlorophyll, and cobalamin, corrins, and its biosynthesis pathways are similar in all organisms (Senior et al. 1996, Shoolingin-Jordan 2003). PBGS is very highly conserved in sequence and structure but contains a remarkable phylogenetic variation in metal ion usage for catalytic and allosteric functions

Fig. 1. The heme biosynthetic pathway. Mitochondrial enzymes are depicted in green and cytosolic enzymes in red (Richard et al., 2006).

Fig. 2. Synthesis of porphobilinogen (PBG). Two molecules of ALA (blue and orange) are condensed to form PBG, a monopyrrole, by the cytosolic enzyme aminolevulinic acid dehydratase (ALAD) (Richard et al. 2006).

(Jaffe 2000, 2003). As of 2003, approximately one-half of the ~130 PBGS sequences available contained the binding determinants for a catalytic zinc ion, and about one-half did not (Jaffe 2003). On the other hand, approximately 90% of the known PBGS sequences contain the binding determinants for allosteric magnesium. The only known PBGS sequences that lack the binding determinants for both the catalytic zinc and the allosteric magnesium are in the bacterial genus *Rhodobacter* (Jaffe 2003).

δ-ALAD is a sulfhydryl containing enzyme (Gibson et al. 1955, Barnard et al. 1977) and numerous metals such as lead (Rodrigues et al. 1989, 1996, and Goering 1993), mercury (Rocha et al., 1993, 1995), and other compounds that oxidize sulfhydryl groups modified its activity (Emanuelli et al. 1996, Barbosa et al. 1998, Flora et al. 1998). Therefore, δ-ALAD is inhibited by substances that compete with zinc and/or that oxidize the –SH groups (Farina et al. 2002, Nogueira et al. 2003a-b, Santos et al. 2004) and is linked to situations associated with oxidative stress (Folmer et al. 2002, Pande et al. 2001, Pande & Flora 2002, Tandon et al. 2002, Soares et al. 2003). In addition, human exposure to Pb^{2+} causes an important inhibition of blood δ-ALAD (Meredith et al. 1979, Fujita et al. 1981, Pappas et al. 1995, Polo et al. 1995, Pires et al. 2002) and is associated with an intense anemia accompanied by an increase in urinary δ-ALA excretion (Oskarsson 1989). Therefore, δ-ALAD activity is used as one of the most reliable indicators of Pb^{2+} intoxication in humans and other animals (Meredith et al. 1979, Pappas et al. 1995).

ALADs have been purifed from a wide variety of sources, including bovine liver (Gibson et al. 1955), human erythrocytes (Anderson & Desnick 1979), *Rhodopseudomonas capsulatus*, *Rhodobacter sphaeroides*, (Nandi & Shemin 1973; Nandi et al. 1968), *Escherichia coli* (Spencer & Jordan 1993) and spinach, *Spinacia oleracea* (Liedgens et al. 1983) during the time. Although the fundamental catalytic properties of all ALADs are similar, differences in enzyme primary structure, metal ion requirement and thiol sensitivity have been observed between the various purified enzymes. Metal dependency allows ALADs to be divided into two main categories, the Zn^{2+} -dependent and the Mg^{2+} -dependent dehydratases. The Zn^{2+} - dependent enzymes include the ALADs from mammalian sources, which have 'pH optima'

of between 6.3 and 7.1 and have been shown to require Zn^{2+} for maximal catalytic activity (Shemin 1972, Cheh & Neilands 1976). The yeast and *E. coli* enzymes can also be included in this class, requiring Zn^{+2} for activity but with more alkaline pH optima than the animal counterparts: 9.8 for the yeast and 8.5 for the enzyme from *E. coli* (Borralho et al. 1990). The animal, yeast and *E. coli* ALADs have a homo-octameric structure and have thiol groups that are extremely sensitive to oxidation. The oxidation of the thiol groups has been shown to be accompanied by a decrease in catalytic activity and a stoichiometric loss of bound metal ions (Tsukamoto et al. 1979), thereby demonstrating that the cysteine residues are required for Zn^{2+} binding. It has been established that ALADs from this class contain both catalytic and non-catalytic Zn^{+2} (Dent et al. 1990). Techniques such as EXAFS predict that the non-catalytic Zn^{2+} has a tetrahedral co-ordination of at least two and often four cysteine residues. The catalytic Zn^{2+} can be bound in either a tetrahedral or pentaco-ordinate fashion with cysteine, histidine and often water as ligands (Jaffe 1993). The Mg^{2+}-dependent class of dehydratases includes the plant ALADs, which have been reported to have alkaline pH optima of c.a. 8.0-8.5 (Liedgens et al. 1983), but again these values were determined by measurement of an average rate of reaction as described above. They have an absolute dependence on Mg^{2+} as well as subtle differences in their primary structure, especially in the putative metal-binding domains. In addition, some of the plant enzymes seem to be homohexameric and to be less sensitive to oxidation than their animal counterparts; consequently a minor role has been postulated for their thiol groups (Liedgens et al. 1983). This may be due to the fact that the cysteine residues are not involved in metal chelation in the Mg^{2+}-dependent enzymes and their oxidation therefore does not lead to the loss of metal ions. In an attempt to show conclusive differences and/or similarities in the ALADs, a detailed study of the properties of the ALADs from *E. coli*, yeast and pea was conducted. Evidences were presented supporting this hypothesis that the variances in metal binding between the enzymes are a reflection of significant biochemical differences that affect substrate recognition and binding and can therefore be used in the design of specific inhibitors (Senior et al. 1996). It was also revealed that the yeast enzyme, previously assumed to be only Zn^{2+}-binding, is similar to the *E. coli* ALAD in that Mg^{2+} can be substituted at the catalytic site to restore enzyme activity although there is no stimulation of activity. Finally, the crystallization of the yeast ALAD is reported, which will permit the determination of the structure of the enzyme by X-ray diffraction methods.

The yeast ALAD has been overexpressed, purified and found to be a Zn^{2+}-dependent. Mg^{2+}-binding enzyme that is similar in behaviour to, but not identical with, the ALAD from *E. coli*.

Comparative studies with the ALADs from three different sources have given an insight into some of the features required for molecular recognition, demonstrating that there are real differences both between and within the different classes of ALADs. These are sufficient to enable the selective inhibition of the enzymes. It will not be possible to rationalize all of the inhibition results collected until the three-dimensional structure of the yeast enzyme has been solved and substantial progress has been made towards this goal with the reported crystallization of the yeast enzyme.

More specific biochemical screening methods are being used by toxicologists such as, protein kinase variants, nitric oxide, interleukin 4 (IL4) and auto-antibodies in plumbism apart from gross changes such as stippling of erythrocytes or inhibition of ALAD (Nag et al. 1996).

Not only δ-ALAD is inhibited but also a number of other enzymes in heme biosynthesis pathway, including coproporphyrinogen oxidase and ferrochelatase are affected by lead.

Inhibitions of ALAD are most profound, and the degree of erythrocyte ALAD inhibition has been used clinically to estimate the degree of Pb poisoning in humans. ALA has neurotoxic activity and may contribute to Pb-induced neurotoxicity (Sithisarankul et al. 1997). At the molecular level, Pb displaces a zinc ion at the metal binding site, not the active site, producing inhibition through a change in the enzyme quaternary structure. ALAD is the second enzyme in the heme biosynthetic pathway, which is cytosolic and non-limiting in heme synthesis in healthy cells. Heme plays important roles in oxygen transport, electron transport systems, detoxification, and transcriptional regulations.

Porphobilinogen is the pyrrole precursor utilized by all living systems for the biosynthesis of tetrapyrroles, including hemes and chlorophylls (Jordan 1991). The ALAD polymorphism has not been established, but it is clear that geographic and strainspecific factors define the distribution of the two recognized ALAD alleles (Fleming et al. 1998). It has also been shown that organisms bred in environments containing high levels of Pb are endowed with multiple copies of the ALAD gene (Bishop et al. 1998).

4. Result

Determination of the blood Pb level alone cannot indicate the toxicity of Pb, since each individual has different degrees of tolerance of Pb (Marcus 1985). As known, atomic absorption spectrophotometry (AAS) or ICP is required for the determination of Pb, and both are expensive. These instruments are available only in specialist laboratories, and can be operated only by a well-trained technician. For these resons same Pb affected enzymes have been employed as biosensors in monitoring Pb toxicity. Among these, ALAD is most popular one, and its results showed good combination with the blood Pb level determined by atomic absorption spectrophotometry or ICP. There are some mathematical equations deviced by several authors to give the Pb concentration by looking at the activity of ALAD (Ogunseitan 1999, Ogunseitan et al. 2000, Korcan et al. 2007, Ciğerci et al. 2008, Konuk et al. 2008).

The expression of ALAD activity gives us a clear indication of the severity of the effect of Pb pollution along the pollution gradient. That is why it is an important biosensor for Pb contamination and pollution. Further studies should be focused on the determination of molecular basis of its effect on ALADs in different organisms and these studies should be strengthened by immobilization studies.

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Intelligent and Biosensors

Edited by Vernon S. Somerset

The use of intelligent sensors have revolutionized the way in which we gather data from the world around us, how we extract useful information from that data, and the manner in which we use the newly obtained information for various operations and decision making. This book is an attempt to highlight the current research in the field of Intelligent and Biosensors, thereby describing state-of-the-art techniques in the field and emerging new technologies, also showcasing some examples and applications.

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