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Manganese inactivation of renal betaine aldehyde dehydrogenase from swine

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ABSTRACT

Manganese is an essential micronutrient for mammals, however high manganese concentrations cause adverse health effects. Swine renal betaine aldehyde dehydrogenase catalyzes the synthesis of glycine betaine, which plays an important role in renal cells osmoregulation. *In vitro* inactivation of BADH was observed by incubating the purified enzyme in the presence of 1 mM $MnCl_2$ under physiological and low ionic strength conditions. Enzyme inactivation followed first order kinetics in a monophasic process with an inactivation constant of 0.126 ± 0.011 min^{-1} and 0.137 ± 0.017 at physiological and low ionic strength, respectively. Enzyme inactivation was not prevented by physiological ionic strength, nor by the substrates NAD^+ and betaine aldehyde at saturated concentrations. The enzyme was reactivated with DTT and GSH. Native-PAGE of the inactivated enzyme showed no change in the tetrameric conformation. Intrinsic protein fluorescence studies demonstrated an increased exposure of the tryptophan residues to the aqueous solvent when the enzyme was incubated with Mn^{2+}. These results suggest that BADH inactivation by Mn^{2+} may result from the oxidation of cysteines, which induces changes in the tertiary structure of the enzyme.

Keywords: betaine aldehyde dehydrogenase; fuel pollution; glycine betaine; manganese; renal osmoregulation.

INTRODUCTION

Manganese is an essential micronutrient for mammals and is involved in metabolic pathways as a cofactor for several enzymes (Barceloux, 1999). High manganese concentrations cause adverse health effects. Exposure to manganese through inhalation has been observed in industrial workers and in urban areas with high traffic density (Iregren, 1999; Joselow et al., 1978; Salehi et al., 2006). Iregren *et al.* found that exposure to air concentrations of 1 mg of Mn/m^3 can lead to adverse respiratory, neurological, and reproductive effects. Under normal conditions higher manganese concentrations are localized in the lungs, brain, kidneys, liver, pancreas, bone, and blood (Bush et al., 1995; Rahil-Khazen et al., 2002; Erdogan et al., 2004). The majority of the studies on the toxic effects of manganese, known as manganism, have focused on the central nervous system because manganese may cause neurological symptoms similar to those of Parkinson's

disease (Salehi et al., 2006). Other sources of manganese intoxication are from organometallic fungicides and fertilizers (Guven et al., 1998; Huang and Lin, 2004). Guven *et al*. found manganese accumulation in the kidneys in both fetal and female rats during pregnancy and a variety of histological effects in renal tissue when female Wistar rats were exposed orally to the fertilizers Propineb and Maneb (Guven et al., 1998). The toxicological effects of fertilizers have also been observed in humans as reported by Huang and Lin where acute renal failure was associated with the ingestion of manganese-containing fertilizer (Huang and Lin, 2004).

During the physiological mechanism that concentrates urine, the renal medulla cell must synthesize and accumulate osmolytes to achieve osmoregulation (Burg, 1995). The main renal osmolytes are glycerophosphorylcholine, glycine betaine, sorbitol, and mannitol (Bagnasco et al., 1986). Glycine betaine is synthesized in two choline oxidation steps. The first is catalyzed by choline dehydrogenase (Grossman et al., 1989) and the second by betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8) (Figueroa-Soto et. al., 1999). Swine renal BADH has been purified (Guzman-Partida and Valenzuela-Soto, 1998), kinetically characterized (Figueroa-Soto and Valenzuela-Soto, 2000), and the required cations studied (Valenzuela-Soto et al., 2003; Valenzuela-Soto et al., 2005). Renal BADH needs physiological ionic strength induced by monovalent cations to maintain its stability (Valenzuela-Soto et al., 2003). The functional conformation of BADH is a 52-kilodalton subunit homo-tetramer; the tetramer is dissociated to inactive dimers and monomers in the absence of physiological ionic strength (Valenzuela-Soto et al., 2003). These correspond to the environment surrounding the enzyme during the concentrating mechanism of urine (García-Perez and Burg, 1991). In contrast, K^+ enhances the stability of BADH against thermal denaturation, which cannot be achieved by Mg^{2+} at the same ionic strength and suggests that the monovalent cation has a specific effect (Valenzuela-Soto et al, 2005).

BADH has been classified as aldehyde dehydrogenase (ALDH) class 9; thus, kidney BADH is an ALDH9 (Yoshida et al., 1998). Mono and divalent cations regulate yeast, and human liver cytosolic (ALDH1) and mitochondrial (ALDH2) aldehyde dehydrogenase (Vallari. and Pietruszko, 1984b; Dickinson and Hywood, 1987; Dickinson, 2003). It is known that ALDH2 is activated by Mg^{2+}, whereas ALDH1 is inhibited by Mg^{2+} (Vallari and Pietruszko, 1984a; Vallari and Pietruszko, 1984b; Ni et al., 1997). The addition of Mg^{2+} appears to decrease the rate of NADH release for ALDH1 (Perez-Miller and Hurley, 2003).

Although high Mn^{2+} concentrations can be found in the kidneys, little is known about the potentially adverse renal effect of this metal. Ponnapakkam *et al*. found that Sprague-Dawley male rats treated with high manganese dose developed severe renal disease (Ponnapakkam et al., 2003). Wistar Albino rats treated with gentamicin and manganese chloride (20 mg kg^{-1} per day) showed severe tubular necrosis (Atessahin et al., 2003). For rats Arregui *et al*. found that intrarenal administration of $Mn_2(CO)_{10}$ increased water and sodium excretion (Arregui et al., 2004). This finding suggests defective rat kidney osmoregulation. Impaired renal osmoregulation has a significant impact on kidney physiology and therefore health. Recently, mammals have been exposed to increased Mn^{2+} concentrations from fuel emissions and chemical products, such as fertilizers and fungicides. In this study, we evaluated the

effect of Mn^{2+} on the activity and stability of BADH since this enzyme is directly related to renal osmoregulation.

MATERIALS AND METHODS

BADH Purification and Activity Assay

BADH was purified from porcine kidney and its activity was assayed at 30 °C as previously reported (Guzman-Partida and Valenzuela-Soto, 1999). The pure enzyme was stored at 20 °C in a 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.2 M KCl and 10% glycerol. The enzyme (0.15 mg/mL) was dialyzed overnight at 4 °C against 10 mM HEPES-KOH buffer, pH 7.0, 1mM EDTA, 10 mM β-mercaptoethanol (buffer A), or against buffer A plus 150 mM KCl (buffer B).

Effect of Mn^{2+} Concentration on BADH Activity

$MnCl_2$ concentrations of 0 to 20 mM were added to the BADH activity buffer, the dialyzed enzyme was added and the activity measured immediately afterwards.

Kinetics of Inactivation by Mn^{2+}. Dialyzed BADH (0.15 mg/mL) was incubated at 1 mM $MnCl_2$ and its remaining activity determined by standard assay at the times indicated. The kinetics of reactivation was studied in enzyme samples partially inactivated during 60 min of incubation at 1 mM $MnCl_2$ plus 5 mM DTT, (Dithiothreitol) or 10 mM glutathione (GSH). The recovery of activity was followed for an additional 120 min. Enzyme inactivation and reactivation time courses were analyzed by nonlinear fit of the experimental data to single exponential decay and growth equations, respectively.

Determination of Protein Concentration

Protein concentration was determined with Bradford's method (Bradford, 1976) using the Bio-Rad protein assay reagent with bovine serum albumin as the standard.

Electrophoresis. Samples of dialyzed BADH incubated at room temperature with 0 and 1 mM $MnCl_2$ for 60 min were analyzed in 4-15% nondenaturing PAGE (Laemmli, 1976) and stained with the silver technique (Wray et al., 1981).

Fluorescence Spectroscopy

Fluorescence spectra were recorded with a PTI fluorescence spectrophotometer (PTI-840) in a quartz cell with a 5nm path length, using an excitation wavelength of 296 nm and emission wavelength of 300-450 nm. BADH in 10 mM HEPES pH 7.0 was incubated in the presence of 1 mM $MnCl_2$ for 30 min before the spectra were recorded. Protein concentration was 0.1 mg/mL for all experiments, and the measurements were carried out at 25 °C. Fluorescence spectral centers of mass (intensity-weighted average emission wavelengths, λ_{av}) were calculated according to the following equation:

$$\lambda_{av} = \Sigma \lambda I(\lambda) / \Sigma I(\lambda)$$

where λ is the emission wavelength and $I(\lambda)$ represents the fluorescence intensity at wavelength λ.

Kinetic Analysis

Activity data were analyzed by nonlinear regression using the program Microcal Origin (OriginLab Corporation).

RESULTS AND DISCUSSION

Porcine kidney BADH activity was measured at increasing concentrations of $MnCl_2$ at low (10 mM HEPES) and physiological (0.15 M KCl) ionic strength conditions; in both cases, the enzyme was inhibited. BADH activity decreased 50 % and 70 % at 10 mM Mn^{2+} at low and physiological ionic strength, respectively (Fig. 1). Physiological ionic strength seems to protect against Mn^{2+} inactivation.

Previously, it was found that the renal BADH is slightly protected from temperature denaturation by K^+ (Valenzuela-Soto et al., 2005), and this is in accordance with this new result.

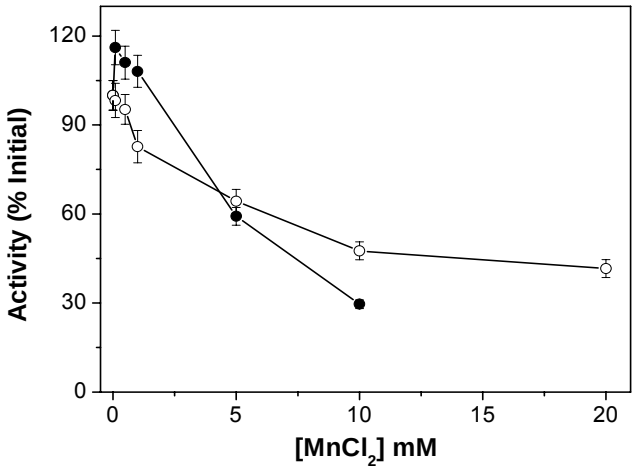

Figure 1: Effect of $MnCl_2$ concentration on porcine kidney BADH activity. The enzyme (0.1 mg/mL) was assayed at 0-20 mM $MnCl_2$ in the presence (●) and absence of KCl (○). Each point is the mean ± SEM of 3 independent experiments.

Atessahin et al., did not find changes in glutathione peroxidase and catalase activities in rat kidney treated with low (2 mg kg^{-1} per day) or high (20 mg kg^{-1} per day) $MnCl_2$ dose (Atessahin et al., 2003).

The effect of Mn^{2+} on kidney BADH stability was studied in the presence and absence of 0.15 M KCl. Inactivation of the enzyme at physiological ionic strength incubated with 1 mM Mn^{2+} followed first order kinetics in a monophasic process with an inactivation constant (*Ki*) of 0.126 ± 0.015 min^{-1} (Fig. 2). The kinetics of the inactivation of BADH at low ionic strength was similar; *Ki* = 0.137 ± 0.015 min^{-1} (Fig. 2).

To our knowledge, this is the first report regarding the effect of manganese on mammal BADH activity and stability. Several studies have demonstrated the outcome of Mg^{2+} and/or Ca^{2+} on the activity of ALDHs (Vallari and Pietruszko, 1984a; Ni et al., 1997; Dickinson, 2003; Ho and Weiner, 2005).

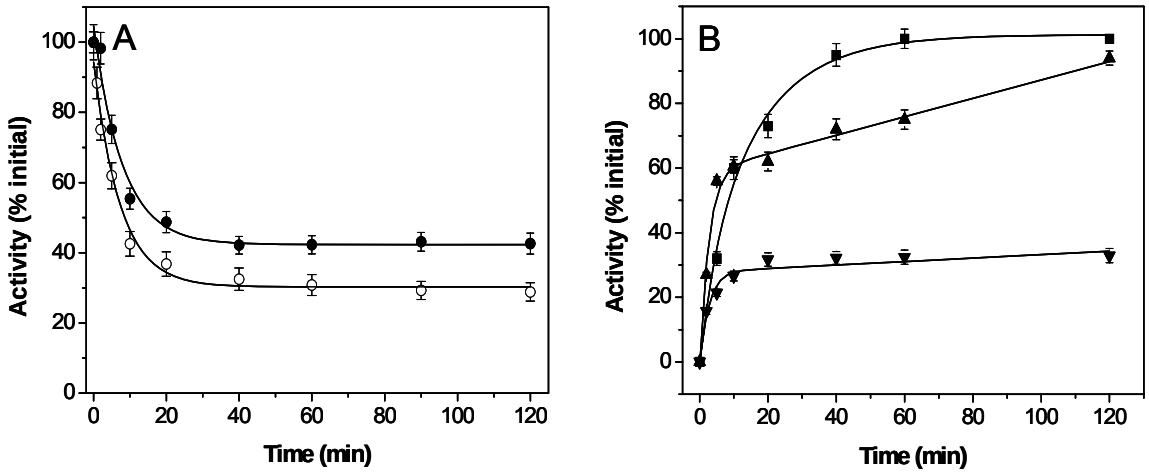

Figure 2: (A) Effect of 1 mM $MnCl_2$ on the time-course of porcine kidney BADH inactivation in the (A) presence (●) and absence (○) of 0.15 M KCl. BADH (0.1 mg/mL) was incubated at 25 °C in buffer A plus 1 mM $MnCl_2$. (B) Reactivation by 5 mM DTT (■), 10 mM GSH (▲) and thionein (▼). Remaining activity was determined at the time intervals indicated. Each point is the mean ± SEM of 3 independent experiments. The line is the result of the best fit of the experimental data to a single exponential decay equation.

It is known that Mg^{2+} activates ALDH2, but inhibits ALDH1 (Vallari and Pietruszko, 1984a and b; Ni et al., 1997). Perez-Miller *et al.* found that nicotinamide isomerization during turnover plays an important role in ALDH2 catalysis (Perez-Miller and Hurley, 2003). Their data also suggest that the addition of Mg^{2+} ions accelerates the nicotinamide isomerization step during turnover, thus explaining the activation of ALDH2 and the inhibition of ALDH1 by Mg^{2+} (Perez-Miller and Hurley, 2003). Muñoz-Clares and Mújica-Jiménez found a burst of NADH production in the pre-steady-state kinetics of amaranth BADH indicating that the rate-limiting step occurs after the hydride transfer (Muñoz-Clares and Mujica-Jimenez, 2001). It is worth noting that amaranth and renal BADH share similar characteristics including their kinetic mechanisms (Figueroa-Soto and Valenzuela-Soto, 2000). Therefore, the inhibition of kidney BADH by Mn^{2+} could be related to the slow speed in the isomerization step similar to that reported for Mg^{2+} in ALDH1 (Perez-Miller and Hurley, 2003).

Treatment of the inactive enzyme with DTT completely restored enzymatic activity (Fig. 2B). Reactivation was fast, with 100% of activity recovered in 1 h. Similar results were obtained with GSH; however, with this compound reactivation was slower, with 94% of activity recovered at 2 h (Fig. 2B). Metallothioneins are proteins which can bind several metal atoms and they are abundant in mammal kidneys (Vasak, 2005). Therefore, Mn-thionein is an ideal candidate for transporting and supplying Mn^{2+} for renal BADH inactivation. With the goal of testing this hypothesis, fully

inactivated BADH was incubated with 14 μM thionein for 2 h. The enzyme recovered 32% of its activity (Fig. 2B).

The reactivation of BADH by DTT and GSH suggests that BADH inactivation *in vivo* could be prevented by the physiological reductant GSH. In addition, the reactivation data correlates with a possible oxidation of cysteines, specifically with the site active cysteine, which binds the substrate betaine aldehyde. Thioneins are the biological chelating agents (Vasak, 2005). Their effect on BADH inactivation indicates that Mn^{2+} binding is reversible. However, we do not know if thionein can remove manganese from the catalytic cysteine.

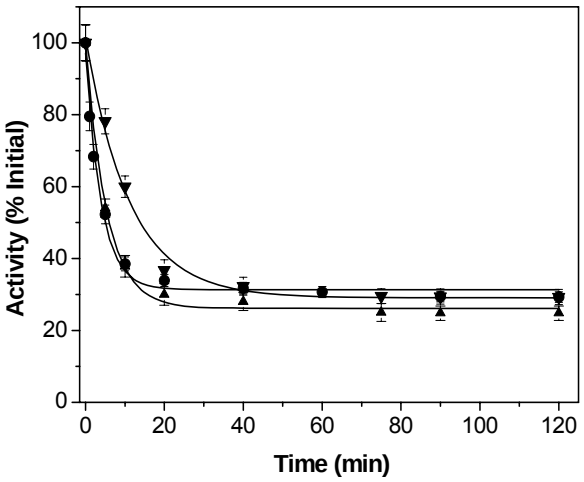

Figure 3: Substrate effect on renal BADH inactivation by $MnCl_2$. The enzyme (0.1 mg/mL) was incubated at 25 °C in buffer A at 1 mM $MnCl_2$ (●), or 1 mM NAD^+ (▼), or 0.5 mM BA (▲). Remaining activity was determined at the time intervals indicated. Each point is the mean ± SEM of 3 independent experiments, and the line is the best fit of the experimental data to a single exponential decay equation.

The coenzyme and substrate role played by Mn^{2+} in the inactivation of BADH was analyzed by adding 1 mM NAD^+ or 0.5 mM BA to the incubation medium. Enzyme inactivation was not prevented by NAD^+ nor by the substrate BA under low (data not shown) or physiological ionic strength (Fig. 3). Therefore, *in vivo* the continued activity of BADH depends on GSH when Mn^{2+} concentrations are high.

To determine if the loss of enzyme activity during incubation time was related to changes in the aggregation state, a native-PAGE was run. Incubating the enzyme for 30 min with $MnCl_2$ in the absence of K^+ does not change its migration profile (tetrameric conformation; Fig. 4). Renal BADH requires ionic strength to maintain its tetrameric conformation indicated by the gel as an abundant protein band at 232 kDa (tetramer) and another one (less abundant) at 125 kDa (dimer) with or without Mn^{2+} as previously reported (Valenzuela-Soto et al., 2003). This result suggests that Mn^{2+} is inactivating the enzyme because it is acting

at the level of a catalytic residue and or tertiary structure.

Since the tetrameric conformation of the enzyme did not change, intrinsic tryptophan fluorescence studies were carried out to determine whether the enzyme's tertiary structure was affected by Mn^{2+}. The fluorescence spectra of the enzyme in the presence and absence of K^+ showed a decrease in the maximum and also a red shift in fluorescence emission (Fig. 5A). The addition of 1 mM Mn^{2+} to the enzyme in the presence and absence of K^+ did not modify the fluorescence spectra (Fig. 5A).

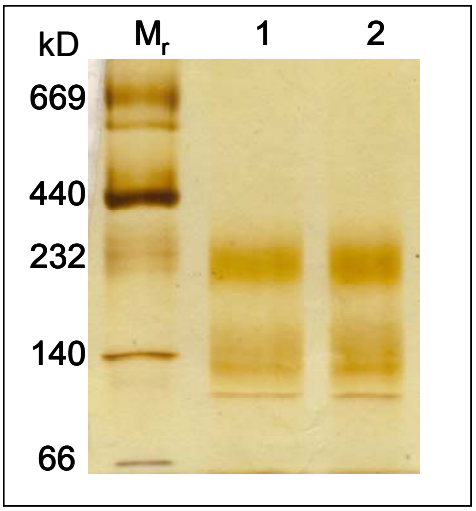

Figure 4: Native-PAGE of kidney BADH. The enzyme without KCl was incubated for 30 min lane 1, with 1 mM $MnCl_2$, and lane 2, without divalent cation.

However, the fluorescence spectral centers of mass calculated for the enzyme plus 1 mM Mn^{2+} in the absence of K^+ was 344 nm whereas it was 356 nm for the BADH with K^+. This indicates a tryptophan spatial change in the protein owing to the presence of K^+. The change in enzyme maximum and red shift of fluorescence emission depends on incubation time (Fig. 5B). For the enzyme incubated for 30 min with 1 mM Mn^{2+} in absence of K^+, the fluorescence spectral center of mass was 7 nm different from the enzyme at the start of incubation. Similar results were found for the enzyme in the presence of K^+.

As demonstrated for other proteins and other divalent cations (Johnson et al., 1986; Zhang et al., 2001; Broder and Miller, 2003), Mn^{2+} increased the exposure of the BADH tryptophan residues to the aqueous solvent, which suggests that Mn^{2+} inactivation of kidney BADH could be produced by changes in its tertiary structure. This change in protein tertiary structure decreases the BADH activity.

In conclusion, the results of this study indicate that mammal renal BADH is a target of manganese toxicity, and at least one adverse effect could be a decrease in glycine betaine synthesis. This osmolyte plays a key role in osmoregulation and in

counteracting the adverse effects of urea in renal medulla cells (Burg, 1995; Bagnasco et. al., 1986). A decrease in glycine betaine synthesis and therefore in its concentration, would negatively affect the normal renal osmoregulation mechanism and induce a deficient protection of proteins from the deleterious effect of the urea. The renal damage demonstrated to rat kidneys could be related with a deficient enzymes activities, like showed in this work (Atessahin et al., 2003; Ponnapakkam et al., 2003).

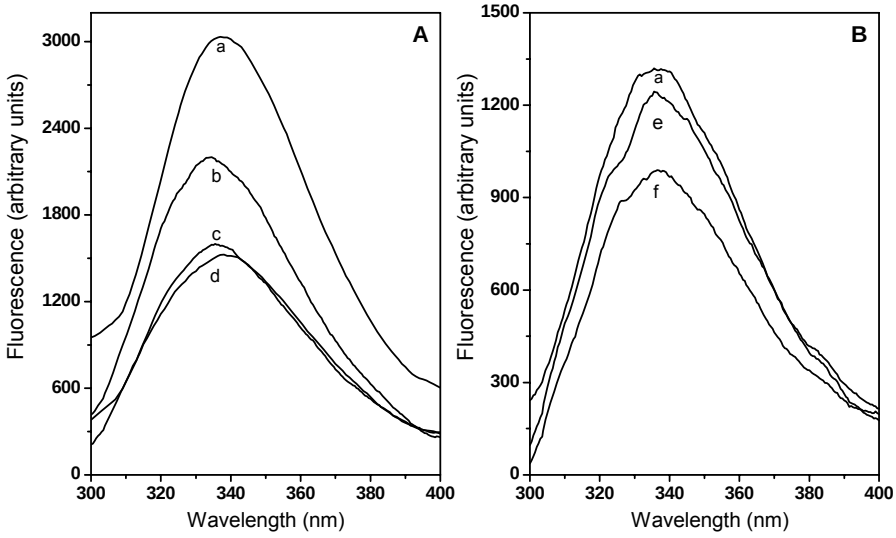

Figure 5: Kidney BADH fluorescence spectra. (A) Effect of Mn^{2+} on fluorescence emission of: a) BADH in buffer A; b) BADH in buffer A plus 0.15 M KCl; c) BADH in buffer A plus 1 mM $MnCl_2$; d) BADH in buffer A plus 0.15 M KCl and 1 mM $MnCl_2$. (B) Effect of incubation time on the fluorescence spectra of the enzyme with $MnCl_2$. e) BADH in buffer A plus 1 mM $MnCl_2$; f) BADH incubated for 30 min at room temperature.

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REFERENCES

Arregui B, López B, García Salom M, Valero F, Navarro C, Fenoy FJ, Acute renal hemodynamic effects of dimanganese decacarbonyl and cobalt protoporphyrin, *Kidney Int*., 2004; 65:564-574.

Atessahin A., Karahan I., Yilmaz S., Ceribasi A.O., Princci I., The effect of manganese chloride on gentamicin-induced nephrotoxicity in rats, *Pharmacol Res*, 2003; 48:637-642.

Bagnasco S, Balaban R, Fales HM, Yang Y-M, Burg MB, Predominant osmotically active solutes in rat and rabbit renal medullas, *J. Biol. Chem*. 1986; 261:5872-5877.

Barceloux DG, Manganese, *J. Toxicol Clin Toxicol*, 1999; 37:293-307.

Bradford MM, A rapid and sensitive method for the quantitation of micrograms of protein utilizing the principle of protein-binding, *Anal. Biochem*. 1976; 72:248-254.

Broder DH, Miller CG, DapE can function as an Aspartyl Peptidase in the presence of Mn^{2+}, *J. Bacteriol*., 2003; 181:4748-4754.

Burg MB, Molecular basis of osmotic regulation, *Am. J. Physiol.,* 1995; 268:F983-F996.

Bush BJ, Moyer TP, Batts KP, Parisi JE, Essential and toxic element concentrations in fresh and formalin-fixed human autopsy tissues. *Clin. Chem*., 1995; 41:284-294.

Dickinson FM, Conformational changes and activation of yeast aldehyde dehydrogenase by various agents, *Chem-Biol Interact,* 2003; 143-144:169-174.

Dickinson FM, Hywood GW, The role of the metal ion in the mechanism of the K^+ activated aldehyde dehydrogenase of *Saccharomyces cerevisiae*, *Biochem. J*., 1987; 247:377-384.

Erdogan S, Celik S, Erdogan Z, Seasonal and locational effects on serum, milk, liver and kidney chromium, manganese, copper, zinc and iron concentrations of dairy cows, *Biol. Trace Elem. Res*., 2004; 98:51-61.

Figueroa-Soto CG, López-Cervantes G, Valenzuela-Soto EM, Immunolocalization of betaine aldehyde dehydrogenase in porcine kidney, *Biochem. Biophys. Res. Commun*., 1999; 258:732-736.

Figueroa-Soto CG, Valenzuela-Soto EM, Kinetic study of porcine kidney betaine aldehyde dehydrogenase, *Biochem. Biophys. Res. Commun*., 2000; 269:596-603.

García-Perez A, Burg MB, Renal medullary organic osmolytes, *Physiol. Rev.*, 1991; 71:1081-1115.

Grossman EB, Hebert SC, Renal inner medullary choline dehydrogenase activity: characterization and modulation, *Am. J. Physiol. (Renal Fluid Electrolyte Physiol.)*, 1989; 256:F107-F112.

Guven K, Deveci E, Akba O, Onen A, de Pomerai D, The accumulation and histological effects of organometallic fungicides propineb and maneb on the kidneys of fetus and female rats during pregnancy, *Toxicol. Lett*., 1998; 99:91-98.

Guzman-Partida AM, Valenzuela-Soto EM, Porcine kidney betaine aldehyde dehydrogenase: purification and properties, *Comp. Biochem. Physiol*. 1998; 119(Part B): 485-491.

Ho KK, Weiner H, Isolation and characterization of an aldehyde dehydrogenase encoded by the *aldB* gene of *Escherichia coli*, *J. Bacteriol*., 2005; 187:1067-1073.

Huang WH, Lin JL, Acute renal failure following ingestion of manganese-containing fertilizer, *Toxicol. Clin. Toxicol*., 2004; 42:305-307.

Iregren A, Manganese neurotoxicity in industrial exposures: proofs of effects, critical exposure level, and sensitive tests, *Neurotoxicology*, 1999; 20:315-324.

Johnson EA, Levine RL, Lin ECC, Inactivation of glycerol dehydrogenase of *Klebsiella pneumoniae* and the role of divalent cations, *J. Bacteriol*., 1986; 164:479-483.

Joselow MM, Tobias E, Koehler R, Colemang S, Bogden J, Gause D, Manganese pollution in the city environment and its relationship to traffic density, *Am. J. Public Health*, 1978; 68:557-560.

Laemmli UK, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4, *Nature,* 1976; 277:680-685.

Muñoz-Clares RA, Mujica-Jimenez C, Complexes of NADH with betaine aldehyde dehydrogenase from leaves of the plant

Amaranthus hypochondriacus L, *Chem-Biol. Interact*, 2001; 130-132:71-80.

Ni L, Sheikh S, Weiner H, Involvement of glutamate 399 and lysine 192 in the mechanism of human liver mitochondrial aldehyde dehydrogenase, *J. Biol. Chem*., 1997; 272:18823-18826.

Perez-Miller SJ, Hurley TD, Coenzyme isomerization is integral to catalysis in aldehyde dehydrogenase, *Biochemistry,* 2003; 42:7100-7109.

Ponnapakkam T., Iszard M., Henry-Sam G., Effects of oral administration of manganese on the kidneys and urinary bladder of Sprague-Dawley rats, *Int J Toxicol*, 2003; 22:227-232.

Rahil-Khazen R, Bolann BJ, Myking A, Ulvik RJ, Multi-element analysis of trace element levels in human autopsy tissues by using inductively coupled atomic emission spectrometry technique (ICP-AES). *J. Trace Elem. Med. Biol.*, 2002; 16:15-25.

Salehi F, Krewski D, Mergler D, Normandin L, Kennedy G, Philippe S, Zayed J, Bioaccumulation and locomotor effects of manganese phosphate/sulfate mixture in Sprague-Dawley rats following subchronic (90 days) inhalation exposure, *Toxicol. Appl. Pharmacol*., 2003; 191:264-271.

Valenzuela-Soto EM, Ayala-Castro HG, Muñoz-Clares RA, Effects of monovalent and divalent cations on the thermostability of porcine kidney betaine aldehyde dehydrogenase, in: H. Weiner, E. Maser (Eds.) *Enzymology and Molecular Biology of Carbonyl Metabolism 12*, Purdue University Press, Purdue, 2005;12:104-110.

Valenzuela-Soto EM, Velasco-Garcia R, Mujica-Jimenez C, Gaviria-Gonzalez L, Muñoz-Clares RA, Monovalent cation requirements for the stability of betaine aldehyde dehydrogenase from *Pseudomonas aeruginosa*, porcine kidney and amaranth leaves, *Chem-Biol Interact*., 2003; 143-144:139-148.

Vallari RC, Pietruszko R, Interaction of Mg^{2+} with human liver aldehyde dehydrogenase. II. Mechanism and site of interaction, *J. Biol. Chem*. 1984; 259:4927-4933(b).

Vallari RC, Pietruszko R, Interaction of Mg^{2+} with human liver aldehyde dehydrogenase. I. Species difference in the mitochondrial isozyme, *J. Biol. Chem*. 1984; 259:4922-4926(a).

Vasak M, Advances in metallothionein structure and functions, *J. Trace Elem. Med. Biol*, 2005; 19:13-17.

Wray W, Boulikas T, Wray VP, Hancock R., Silver staining of proteins in polyacrilamide gels, *Anal. Biochem.,* 1981; 118:197-203.

Yoshida A, Rzhetsky A, Hsu LC, Chang C, Human aldehyde dehydrogenase gene family, *Eur. J. Biochem*., 1998; 251:549-547.

Zhang Y, Wang P, Brew K, Specificity and mechanism of metal ion activation in UDP-galactose: b-galactoside-a-1,3-galactosyltransferase, *J. Biol. Chem*., 2001; 276:11567-11574.