

# Structural and Activity Changes in Renal Betaine Aldehyde Dehydrogenase Caused by Oxidants

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## 1. Introduction

Oxidative stress has been implicated in a variety of diseases such as glomerulonephritis and tubulointerstitial nephritis, renal insufficiency, proteinuria, Alzheimer's and Parkinson's disease, diabetes and hypertension, as well as contributing to the pathogenesis of ischemia reperfusion injury in the kidney (Banday & Lokhandwala, 2011; Martin & Goeddeke-Merickel, 2005; Touyz, 2004; Vaziri, 2004). One of the most important functions of kidney is the regulation of liquid volume. Broad shifts in osmolality, high urea concentrations and low oxygen tension are required by the urine concentrating mechanism to produce concentrated urine (Kwon et al., 2009; Burg & Ferraris, 2008; Neuhofer & Beck, 2006). There is evidence indicating that the osmotic stress and low oxygen tension produced in medullary cells generates an increase in the concentration of reactive oxygen species (ROS), which triggers damage to kidney cells.

The source of ROS in kidney is (1) the hyperosmolality of the environment where the medulla cells are found, (2) NADH oxidase activity and (3) the mitochondrial respiratory chain (Banday & Lokhandwala, 2011; Harper et al., 2004; Mori & Crowley, 2003; Zou et al., 2001). The most abundant ROS in renal cells are the superoxide radical ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ). Hydrogen peroxide is produced during the incomplete reduction of oxygen to  $H_2O$ , and it is biologically important because it is reduced to the highly reactive hydroxyl radical (Rhee et al., 2003). The hydroxyl radical has been implicated in endothelial dysfunction (Coyle et al. 2006). The amount of ROS produced is in balance with the quantity eliminated by the antioxidants. However, when this balance is upset the possibility of cellular damage by ROS increases (Haliwell, 2007; Lushchak 2011).

Metal ions such as iron, cadmium and copper have been found to play a role in several human diseases owing to their capacity to generate oxidative stress (Bonda et al., 2011;

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Butterworth, 2010; Jomova & Valko, 2011; Jomova et al., 2010). Copper generates ROS (hydroxyl  $\bullet\text{OH}$  and superoxide anion radical  $\text{O}_2\bullet^-$ ) by two mechanisms: Fenton and/or Haber-Weiss chemistry (Jomova & Valko, 2011; Prousek, 2007) and by decreasing glutathione levels (Speisky et al., 2009). Glutathione has several functions including acting as a powerful cell antioxidant and as a copper chelating agent; therefore decreased glutathione levels can enhance oxidative stress (Mattie & Freedman, 2004; Steinebach & Wolterbeek, 1994).

Copper ions display a high affinity to thiol groups in proteins and can directly bind to them causing enzyme inactivation or altered protein conformation (Giles et al., 2003; Letelier et al., 2005; Prudent & Girault, 2009; Zhang et al., 2010). For example, the enzyme glutathione S-transferase (GST) from rat liver, crab and shrimp is inhibited by copper (Elumalai et al., 2002; Letelier et al., 2006; Salazar-Medina et al., 2010), and proteins such as LDL and HDL are also oxidized by copper (Burkitt, 2001). The thiol group exhibits a variety of oxidation states which allow for nucleophilic attack, electron transfer, hydride transfer and oxygen atom transfer (Giles et al., 2003). The binding between copper and the thiol group can oxidize the sulphur, producing sulfenic and sulfinic acids or a disulfide bridge between vicinal cysteines (Giles et al., 2003).

Identifying the proteins that are susceptible to oxidative damage could lead to a better understanding of the complexity of the alterations caused by oxidative stress, and allow us to counteract the injury caused by ROS. A decrease in the activity of the enzymes involved in osmolyte synthesis and stress response are associated with renal injury, and as such could generate other diseases in the organism. Further study of the regulation and biochemical characteristics of those enzymes is necessary in order to identify and understand the effects of ROS in kidney and their relationship to the osmolyte system. One of those enzymes is betaine aldehyde dehydrogenase (BADH EC 1.2.1.8), which catalyzes the oxidation of betaine aldehyde to synthesize glycine betaine.

Glycine betaine (GB) is one of the major non-perturbing osmolytes that are actively accumulated by plant, bacteria and mammalian cells under hypertonic conditions, and this process is important in the regulation of cell volume (Chen & Murata, 2011; Burg & Ferraris, 2008; Burg et al., 2007). Glycine betaine has also been found to suppress increases in oxidative stress in old rats (Go et al. 2005), prevent isoprenaline-induced myocardial infarction due to its antioxidant properties (Ganesan et al. 2009), prevent tauro lithocholate 3-sulfate-induced oxidative stress in rat hepatocytes (Graf et al. 2002), activate chaperone-mediated disaggregation—suggesting that GB has a specific interaction of activation with ClpB and/or DnK (Umenishi et al., 2005), and to promote aquaporine PI (AQPI) expression under severe hypertonic conditions in kidney (Diamant et al., 2001). Glycine betaine has been described as an osmoprotectant against deleterious effect of urea (Burg et al. 1996).

Betaine aldehyde dehydrogenase (BADH, EC 1.2.1.8) belongs to the superfamily of aldehyde dehydrogenases (ALDH9) (Julián-Sánchez et al., 2007; Vasiliou et al., 1999) and has been found in many prokaryotic and eukaryotic organisms. The enzyme has been purified from and characterized for a number of these sources: plants (Arakawa et al., 1987; Figueroa-Soto & Valenzuela-Soto, 2001; Livingstone et al., 2003; Oishi & Ebina, 2005; Pan, 1988; Valenzuela-Soto & Muñoz-Clares, 1994; Weretylnik & Hanson, 1989), microorganisms (Boch et al., 1997; Falkenberg & Strom, 1990; Mori et al., 1992; Mori et al., 1980; Nagasawa et al., 1976; Velasco-García et al., 1999) and animals (Chern & Pietruszko, 1999; Guzman-Partida & Valenzuela-Soto, 1998; Hjelmquist et al., 2003; Kurys et al., 1989; Rothschild & Guzman-Barron, 1954).

Studies of steady-state kinetics have shown that BADH from swine kidney and amaranth plants follows an iso bi-bi ordered mechanism (Figuroa-Soto & Valenzuela-Soto, 2000; Valenzuela-Soto & Muñoz-Clares, 1993), that of *Pseudomonas aeruginosa* follows a random steady-state, with a much preferred route in which the nucleotide first links to the enzyme (Velasco-Garcia et al., 1999), and a ping-pong mechanism for the enzymes from the fungus *Cylindrocarpon didymum* (Mori et al., 1980), and the bacterium *Escherichia coli* (Falkenberg & Strom, 1990). In an iso bi-bi ordered mechanism, NAD<sup>+</sup> is the first substrate to combine with the enzyme and NADH is the last product released.

The enzyme betaine aldehyde dehydrogenase from swine kidney (skBADH) requires renal physiological ionic strength to maintain its tetrameric conformation; at low ionic strength the enzyme dissociates, forming dimers which are inactive and very stable (Valenzuela-Soto et al., 2003). Additionally, the enzyme requires the physiological ionic strength provided by a monovalent cation for maximum thermostability (Valenzuela-Soto et al., 2005).

SkBADH, like the majority of aldehyde dehydrogenases, has a catalytic cysteine (Cys 288) (González-Segura et al., 2002; Muñoz-Clares et al., 2003), and also has a vicinal cysteine (Cys 289). The chemical mechanism of BADH involves the following steps: 1) There is a nucleophilic attack by the thiolate group of the catalytic cysteine on the carbon of carbonyl group of the aldehyde, forming the thiohemiacetal intermediate; 2) The transfer of the hydride to the C-4 position of the nicotinamide portion of NAD(P)<sup>+</sup>, thus reducing the nucleotide; 3) A water molecule acting as a nucleophile breaks the thioester bond, thus forming the acid product of the reaction (Muñoz-Clares & Valenzuela-Soto, 2008; Muñoz-Clares et al., 2010).

In previous studies, we found that in swine kidney BADH the thiolate from catalytic cysteine can be oxidized by hydrogen peroxide, forming a disulfide bond between catalytic and vicinal cysteine (Rosas-Rodríguez & Valenzuela-Soto, 2011). With the aim of better understanding how ROS affect the stability and activity of the enzymes involved in osmolyte synthesis, in this study we examined the impact of hydrogen peroxide and copper on the activity, structure and stability of renal BADH. Physiological conditions such as pH and ionic strength were analyzed and contrasted with the conditions that are optimal for enzyme activity.

## 2. Materials and methods

### 2.1 Chemicals

Betaine aldehyde, DTT, EDTA, glycine betaine, GSH, HEPES, 2-mercaptoethanol, NAD<sup>+</sup> (sodium salt), KCl and CuCl<sub>2</sub> were obtained from Sigma-Aldrich SA de CV, México. All other chemicals and solvents used in this study were of analytical grade.

### 2.2 Enzyme purification and activity assay

BADH was purified from porcine kidney tissue and its activity was assayed at 30 °C as previously reported (Guzman-Partida & Valenzuela-Soto, 1998) with a modification in the affinity chromatographic step, where an N-6-Hexyl-AMP-sepharose matrix was used. The pure enzyme was stored at -20 °C in a 10 mM potassium phosphate buffer, pH 6.8, 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.2 M KCl and 10% glycerol. The standard assay system

contained 0.1 M Hepes-KOH, pH 8.0, 0.5 mM betaine aldehyde (BA) and 1.0 mM NAD<sup>+</sup> in a final volume of 0.4 mL. SkBADH activity was assayed spectrophotometrically measuring NAD<sup>+</sup> reduction by the increase in extinction at 340 nm using an Ultrospec 4000 (Pharmacia) spectrophotometer.

Prior to treatments skBADH (0.15 mg/mL) was dialyzed overnight at 4 °C against 10 mM Hepes-KOH buffer, pH 7.0, 1 mM EDTA, 10 mM 2-mercaptoethanol (buffer A, low ionic strength), and against buffer A plus 150 mM KCl (buffer B, physiological ionic strength).

### 2.3 Effect of hydrogen peroxide in enzyme activity

SkBADH activity in the presence of hydrogen peroxide was evaluated at distinct pH values and ionic strengths. The enzyme was assayed in the presence of 0.1 mM of hydrogen peroxide in 10 mM Hepes buffer, pH 6.8, 7.0, 7.2, 7.4 and 8.0 and enzyme activity was measured. The effect of ionic strength was evaluated by incubating the enzyme at each pH tested with 0.15 M KCl for 60 minutes prior to the enzyme activity assay.

### 2.4 Kinetics of inactivation by copper

The enzyme was dialyzed with 10 mM Hepes buffer, pH 7 or pH 8.0, 10% glycerol (v/v), and 1 mM 2-mercaptoethanol. The enzyme was incubated for 120 min with 10 μM CuCl<sub>2</sub> in the presence or absence of 0.15 M KCl, an aliquot was taken at different times and residual enzyme activity was measured by the standard assay. Rate constants for inactivation were calculated by fitting the data to a single exponential decay equation (Eq. 1) using OriginPro 8.0 (OriginLab, Northampton, MA, USA).

$$A_t = A_\infty + A_0 e^{-k_{\text{obs}} \cdot t} \quad (1)$$

Where  $A_t$ ,  $A_0$  and  $A_\infty$  are enzyme activity at time  $t$ , zero, and infinite, respectively, expressed as a percentage of the initial activity;  $k_{\text{obs}}$  is the observed pseudo-first order constant in monophasic inactivation.

### 2.5 Enzyme reactivation kinetics

The reactivation of inactivated enzyme was carried out with 5 mM dithiothreitol (DTT), 10 mM glutathione (GSH) or 10 mM 2-mercaptoethanol. An aliquot was taken at different times and enzyme activity was measured using the standard assay. Activity data were fitted to a double exponential growth equation (Eq. 2) using OriginPro 8.0.

$$A_t = A_1 (1 - e^{-k_{\text{obs}} \cdot t}) + A_2 (1 - e^{-k_{\text{obs}2} \cdot t}) \quad (2)$$

Where  $A_t$ ,  $A_0$  and  $A_\infty$  are enzyme activity at time  $t$ , zero, and infinite, respectively, expressed as a percentage of the initial activity;  $k_{\text{obs}}$  is the observed pseudo-first order constant in monophasic inactivation.

### 2.6 Enzyme fluorescence assay

Intrinsic tryptophan fluorescence was used to estimate skBADH fluorescence quenching by enzyme substrates (BA and NAD<sup>+</sup>), products (GB and NADH) and inhibitors (H<sub>2</sub>O<sub>2</sub> and copper) at the concentrations given in each figure legend. Another set of experiments was

carried out in which the enzyme was incubated for 120 min in the presence of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  or 10  $\mu\text{M}$   $\text{CuCl}_2$ . Fluorescence spectra were recorded at an excitation wavelength of 296 nm and emission wavelength of 300-400 nm (5 nm bandwidth) using a QM-2003 fluorometer (Photon Technology International) with a 75-W xenon lamp as the light source. The spectra were corrected by subtracting the spectrum obtained for the solvent under identical conditions. Three readings of emission data were accumulated. The assay was done using 2  $\mu\text{M}$  of enzyme monomer in 100 mM Hepes-KOH buffer at pH 7.0 and then at pH 8.0. Fluorescence spectral centers of mass (intensity-weighted average emission wavelengths,  $\lambda_{\text{av}}$ ) were calculated according to the following equation:

$$\lambda_{\text{av}} = \frac{\sum I(\lambda)}{\sum I(\lambda)} \quad (3)$$

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

## 2.7 Enzyme structural models

The structural model of the skBADH tetramer was constructed using MOE v2009.10 software (Chemical Computing Group). The homology model was built using the cDNA-deduced swine kidney BADH amino acid sequence. The crystallographic structures from cod liver (1A4S, DOI:10.2210/pdb1a4s/pdb) and *Escherichia coli* BADH (1WNB, DOI:10.2210/pdb1wnb/pdb) were used as templates to generate the final homology model.

The hydrogen peroxide and copper interaction models with the skBADH active site were built using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. The model was designed based on previously described experimental evidence of the alteration of skBADH active site (Rosas-Rodríguez & Valenzuela-Soto, 2011).

## 3. Results

### 3.1 Effect of pH and hydrogen peroxide on enzyme activity

To test the influence of pH on skBADH oxidation provoked by hydrogen peroxide, enzyme activity was assayed from pH 6.8 to 8.0. Enzyme activity was higher at pH 8.0 in the absence or presence of hydrogen peroxide (Fig. 1). SkBADH activity decreased 50% in the presence of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  at all pH values tested relative to the enzyme assays done with no hydrogen peroxide (Fig. 1A). Chemical modification studies carried out with BADH from *Pseudomonas aeruginosa* indicated that the catalytic cysteine residue exists as thiolate at pH values of 5.5 to 9.0 (González-Segura et al., 2002). Therefore,  $\text{H}_2\text{O}_2$  is able to oxidize the catalytic cysteine in the skBADH at the pH values assayed in this work.

Physiological ionic strength obtained using 0.15 M KCl was tested to analyze its influence on skBADH oxidation by 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . Enzyme activity decreased 50% when hydrogen peroxide was included in the activity assay medium (Fig. 1B). Physiologic ionic strength was not able to maintain enzyme activity in the presence of hydrogen peroxide at any of the pH values tested (Fig. 1B).

Previous studies indicated that enzyme exposure to  $\text{Na}^+$  or  $\text{K}^+$  ions maintains more than 50% of skBADH activity and that physiological ionic strength is necessary to maintain the tetrameric structure of skBADH (Guzman-Partida & Valenzuela-Soto, 1998; Valenzuela-

Soto et al., 2003). SkBADH is inhibited in the presence of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , a concentration that may be found in medulla cells during concentrated urine formation, and which requires high osmolarity and low oxygen tension (Burg & Ferraris, 2008; Neuhofer & Beck, 2006). In addition, these peroxide concentrations may be found in the medulla cells of hypertensive (Touyz & Briones, 2011; Thengchaisri & Kuo, 2003) and diabetic patients (Coughlan et al., 2009; Forbes et al., 2008), i.e. pathologies related to high ROS concentrations.

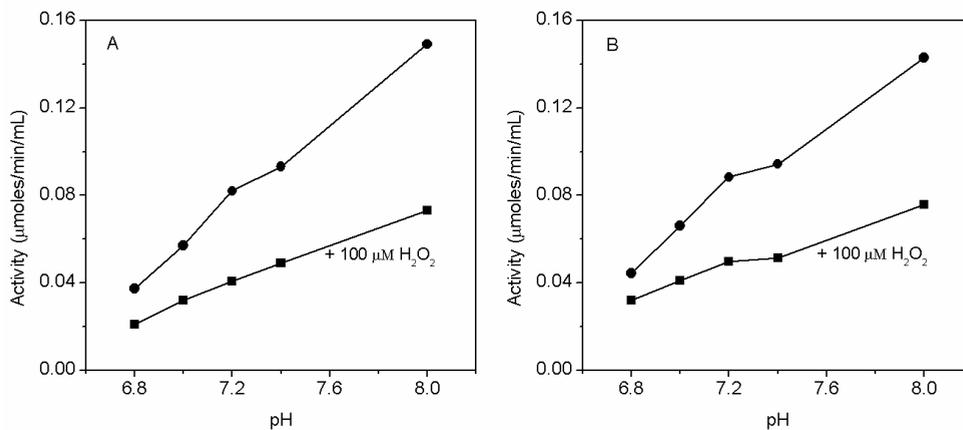


Fig. 1. Effect of pH and ionic strength on skBADH inactivation caused by hydrogen peroxide. Enzyme activity measured at: (A) low ionic strength; (B) physiological ionic strength (0.15 M KCl).

### 3.2 The effect of hydrogen peroxide on enzyme fluorescence

A methodical evaluation of the effect of hydrogen peroxide on enzyme tertiary structure was performed by incubating the enzyme in the presence of 0-0.5 mM  $\text{H}_2\text{O}_2$ . SkBADH maximum emission was detected at 333 nm (Fig. 2). Enzyme fluorescence was quenched by all hydrogen peroxide concentrations tested (Fig. 2), however fluorescence spectral centers of mass did not change and no blue or red shift was found.

A hydrogen peroxide concentration of 100  $\mu\text{M}$  was chosen to analyze the effect of incubation time on enzyme quenching, because this concentration can be found in kidney. When the enzyme was incubated for 120 min in the presence of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , the fluorescence of skBADH was quenched in a time dependent manner (Fig. 3). The presence of hydrogen peroxide decreased the skBADH maximum emission by more than 50% at 120 min of incubation. Changes in enzyme emission fit a single decay equation with an R-square of 0.9893 (Fig. 3 Inset). It is noteworthy that skBADH fluorescence quenching was only detected in the presence of peroxide and the shape of the fluorescence spectra did not change. The fluorescence spectral centers of mass did not change either.

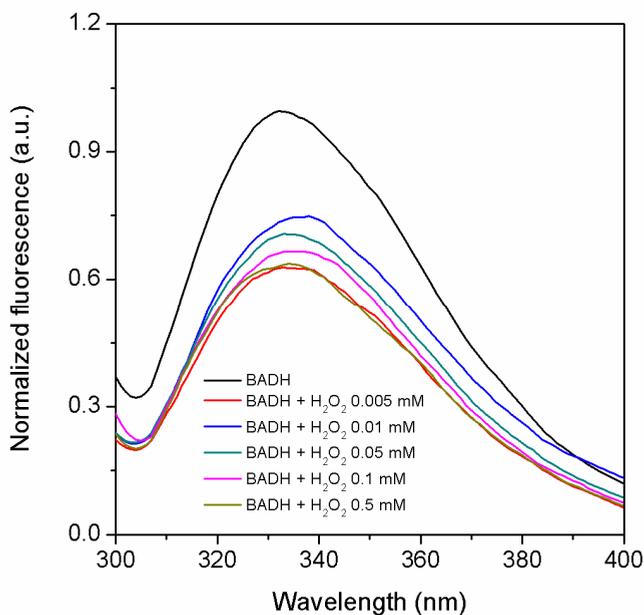


Fig. 2. Fluorescence spectra for skBADH in the presence of hydrogen peroxide.

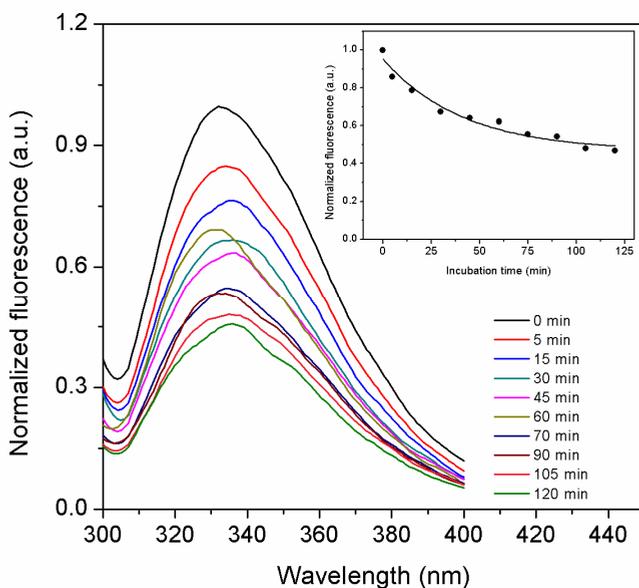


Fig. 3. Fluorescence spectra for skBADH incubated with 100 μM H<sub>2</sub>O<sub>2</sub>. Inset: SkBADH time dependent quenching.

In addition, with respect to the maximum emission wavelength, no blue/red shift could be detected which suggests the direct alteration of the enzyme site rather than a change in protein folding, and which also rules out any enzyme denaturation.

### 3.3 The effect of ligands on enzyme quenching provoked by hydrogen peroxide

The skBADH fluorescence spectra at both pH 7.0 and pH 8.0 showed that the maximum emission and the wavelength at which the maxima occurred were equal (Fig. 4). Fluorescence spectral centers of mass changed between 1 and 2 nm. At pH 7.0, hydrogen peroxide increased the enzyme's maximum emission but did not cause a blue or red shift (Fig. 4A). The presence of BA produced a red shift indicating a change in solvent exposition of tryptophans, caused by hydrogen peroxide and BA mix (Fig. 4A). However, the enzyme product GB had no effect on the quenching provoked by peroxide. At pH 8.0, as before, skBADH fluorescence was quenched by hydrogen peroxide without any blue or red shift (Fig. 4B). BA and GB did not play a role in the enzyme fluorescence quenching induced by hydrogen peroxide (Fig. 4B).

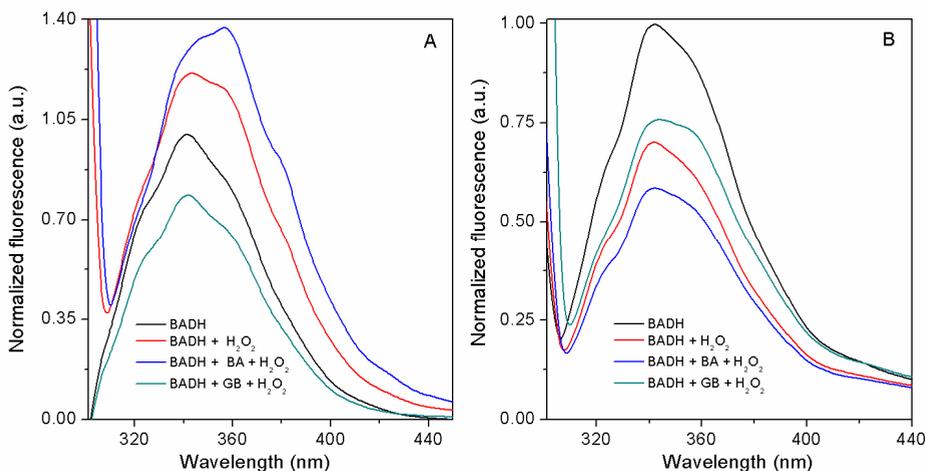


Fig. 4. Fluorescence spectra for skBADH incubated at pH 7.0 (A) and pH 8.0 (B) in the absence and presence of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , betaine aldehyde (BA) or glycine betaine (GB).

### 3.4 The effect of copper on enzyme activity

SkBADH incubated with 10  $\mu\text{M}$   $\text{CuCl}_2$  at pH 7.0 and 8.0 was inactivated in a time dependent manner (Fig. 5). In assays performed at pH 8.0, enzyme activity decreased 80%, however when KCl was included in the incubation assay, enzyme activity decreased 45% (Fig. 5A). Data analysis revealed single exponential decay kinetics with  $k_{\text{obs}} = 0.0112 \pm 0.005 \text{ min}^{-1}$  for the enzyme incubated with KCl and  $k_{\text{obs}} = 0.022 \pm 0.002 \text{ min}^{-1}$  for the enzyme incubated without KCl (Fig. 5A).

The enzyme incubated with 10  $\mu\text{M}$   $\text{CuCl}_2$  at pH 7.0 had a different inactivation pattern: skBADH lost 48% of its activity in absence of KCl, whereas in its presence enzyme

inactivation reached 59% (Fig. 5B). The inactivation process follows single exponential decay kinetics with  $k_{\text{obs}} = 0.0412 \pm 0.0057 \text{ min}^{-1}$  for the enzyme incubated with KCl and  $k_{\text{obs}} = 0.0147 \pm 0.0048 \text{ min}^{-1}$  for the enzyme incubated without KCl (Fig. 5B). Under renal pH and ionic strength conditions skBADH is inactivated by copper concentrations that can occur in kidney.

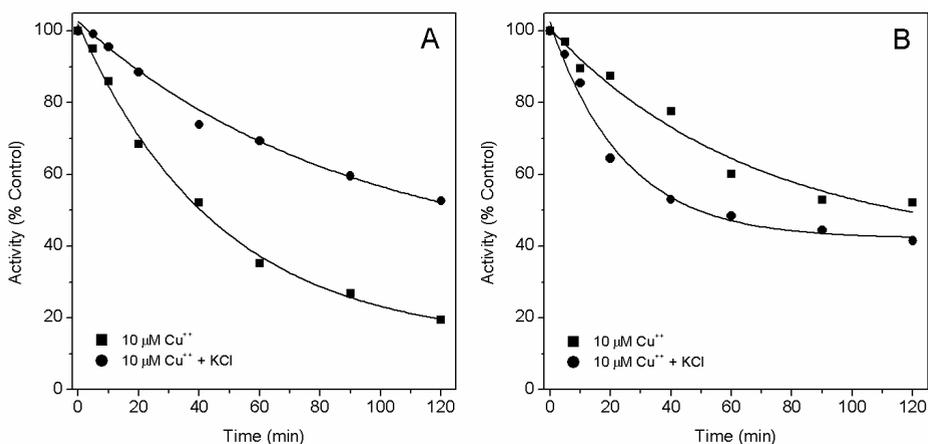


Fig. 5. Time courses of the inactivation of skBADH by copper. (A) Enzyme incubated in Hepes-KOH buffer at pH 8.0; (B) Enzyme incubated in Hepes-KOH buffer at pH 7.0. The lines are the best fit of the inactivation data to a single exponential decay equation.

### 3.5 Enzyme reactivation kinetics

The fully inactivated enzyme was incubated in the presence of 10 mM DTT, 10 mM GSH or 10 mM 2-mercaptoethanol. SkBADH incubated with DTT at pH 8.0 or pH 7.0 recovered 93% and 96% of its activity, respectively (Fig. 6). When the reducing agent used was 2-mercaptoethanol, under conditions of pH 8.0 or pH 7.0 the enzyme recovered 86% and 84% of its activity (Fig. 6). With GSH at pH 8.0, skBADH recovered 90% of its activity (Fig. 6B), while at pH 7.0 the enzyme recovered 30% of its activity (Fig. 6A). Enzyme recovery activity was faster at pH 8.0 than at pH 7.0, regardless of the reducing agent tested (Fig. 6). SkBADH incubated with reducing agents in the presence of 0.15 M KCl behaved very similarly to the curves in Figure 6 (data not shown). GSH was not able to recover enzyme activity when there was potassium in the assay medium. No significant skBADH activity changes were obtained after long incubation periods.

The failure of GSH to reactivate skBADH and other BADHs inactivated by thiol-specific reagents had been reported (Rosas-Rodríguez & Valenzuela-Soto, 2011; Velasco-García et al., 2003; Vallari & Pietruszko, 1982); however, the reason for this failure is not yet clear and may be related to the larger size of GSH, which prevents it from accessing the active site.

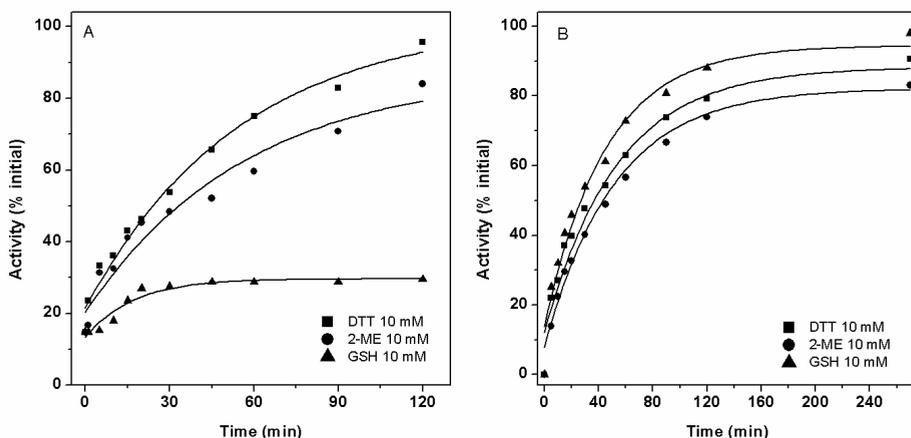


Fig. 6. Reactivation kinetics for skBADH inactivated by copper. Enzyme inactivated by 50  $\mu\text{M}$   $\text{CuCl}_2$  to a residual activity of 15% over a 150 min reaction. Enzyme incubated: at pH 7.0 (A) or pH 8.0 (B). The lines are the best fit of the inactivation data to a single exponential decay equation.

### 3.6 The effect of copper on enzyme fluorescence

The skBADH fluorescence spectra were obtained from enzyme incubation assays with 10  $\mu\text{M}$   $\text{CuCl}_2$  at pH 8.0 or pH 7.0. Enzyme fluorescence was quenched by copper at both pH values (Fig. 7 and 8). Enzyme maximum emission changed with respect to the time of incubation at both pH values. However, incubation time did not have any effect on the wavelength at which maximum emission occurred.

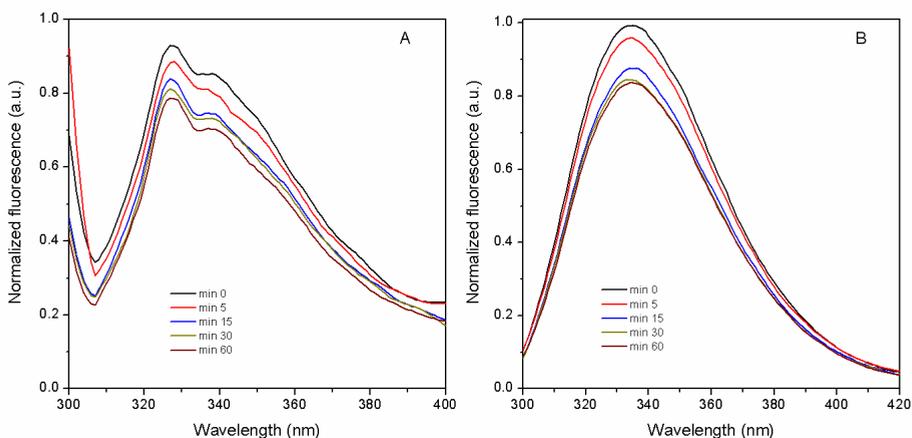


Fig. 7. The effect of copper and incubation time on skBADH fluorescence spectra. Enzyme incubated at pH 7.0 with 10  $\mu\text{M}$   $\text{CuCl}_2$  (A), plus 0.15 M KCl (B).

The drop in the enzyme's maximum emission was similar at both pH values, though the wavelength at which that maximum occurred was different at pH 7.0 and pH 8.0. At pH 7.0 the maximum emission wavelength for the enzyme incubated only with copper was 327 nm (Fig. 7A). Copper caused a blue shift in the enzyme, implying that the tryptophans were less exposed to the solvent. The presence of 0.15 M KCl reversed the blue shift (Fig 7B). The skBADH fluorescence spectra at pH 8.0 with 10  $\mu$ M CuCl<sub>2</sub> did not exhibit a blue or red shift (Fig. 8A) and the presence of KCl did not cause any changes (Fig. 8B).

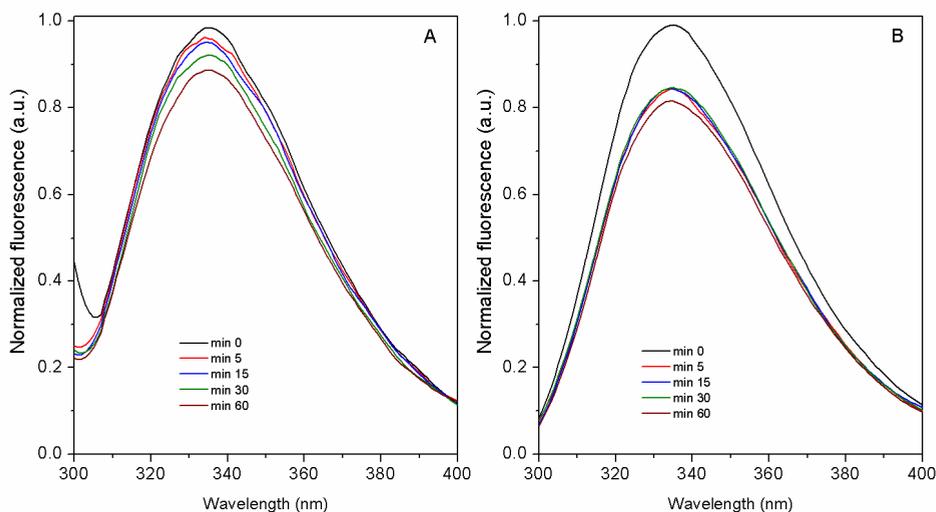


Fig. 8. The effect of copper and incubation time on skBADH fluorescence spectra. Enzyme incubated at pH 8.0 with 10  $\mu$ M CuCl<sub>2</sub> (A), plus 0.15 M KCl (B).

#### 4. Discussion

SkBADH has four tryptophan residues per subunit, which means there are sixteen residues in the enzyme's active conformation (tetramer), making Trp fluorescence a useful technique for structure-function studies. Alvarez et al. (2010) report that the presence of reactive oxygen species could impact the fluorescent properties of a specific protein (Cyan Fluorescent Protein), due to chemical modifications that cause changes in the chromophore pocket (Alvarez et al., 2010). These authors suggest that the alterations could be related to the torsion of amino acid residues, which leads to a quenching process.

The quenching of skBADH fluorescence by hydrogen peroxide is consistent with data reported by Tsourkas et al. (2005) who detected a 50% decrease in fluorescent emission for Red Fluorescent Protein (DsRed) and 95% for Enhanced Yellow Fluorescent Protein (EYFP) in the presence of 50  $\mu$ M hydrogen peroxide (Tsourkas et al., 2005). The authors attributed the quenching process to intramolecular cross-linking between two cysteines which puts stress on the protein structure (Tsourkas et al., 2005).

A previous study reported that skBADH is inactivated by H<sub>2</sub>O<sub>2</sub> in an oxygen independent modification process (Rosas-Rodríguez & Valenzuela-Soto, 2011). Our data for enzyme fluorescence quenching by peroxide indicates that the protein's tertiary structure was not changed (Fig. 2). Together, the inactivation and fluorescence data support the idea that enzyme inactivation is caused by a disulfide bond between vicinal cysteines (C288-C289) at the active site, as previously postulated (Rosas-Rodríguez & Valenzuela-Soto, 2011).

Based on the mechanism of the hydrogen peroxide and cysteine reaction (Luo & Anderson, 2008), we propose a structural model showing the interaction of peroxide with the skBADH active site that leads to enzyme inactivation (Fig. 9). The structural model of skBADH was compared with that of methanol dehydrogenase (pdb 1g72) to look for type VIII folding, because that kind of folding has been found in proteins forming disulfide bridges between vicinal cysteines (Carugo et al., 2003). Both models were very similar, thus we think that skBADH exhibits the type VIII  $\beta$ -turn folding that allows for disulfide bridge formation.

Copper had the strongest inactivation effect on skBADH under physiological pH and ionic strength conditions (Fig. 5). Because the potassium concentrations in kidney medulla cells are those tested in this study, skBADH is a target for copper oxidation which leads to a decrease in glycine betaine synthesis and accumulation. In addition to its function as an osmolyte, glycine betaine functions as an osmoprotector (Rosas-Rodríguez et al., 2010); the insufficient osmoprotection and osmoregulation of renal cells may be related to some kidney pathologies.

The highest levels of BADH activity and protein occur in human liver, the adrenal gland, and kidney (Izaguirre et al, 1991). Besides of the role played by BADH in kidney, mammalian BADH catalyzes the oxidation of different amino aldehydes in addition to betaine aldehyde, so this enzyme is thought to be involved in: (i) the biosynthesis of GB, which may function as a methyl donor for methionine synthesis in liver (duVigneaud, 1946, Muntz, 1950), (ii) polyamine catabolism (Ambroziak & Pietruszko, 1991), (iii) the synthesis of the inhibitory neurotransmitter  $\gamma$ -aminobutyrate (GABA) particularly in adrenal glands where putrescine is a source of  $\gamma$ -aminobutyric acid (Lin et al., 1996), and (iv) carnitine biosynthesis (Vaz et al., 2000). It is possible that all BADHs might be susceptible to copper inactivation, but it is important to conduct studies to test this idea.

SkBADH was inactivated by 10  $\mu$ M CuCl<sub>2</sub> as a consequence of its thiol groups being oxidized (Fig. 5). The oxidation process was reversed by thiol reducing agents, DTT and 2-ME and GSH (at pH 8.0) (Fig. 6B), whereas at pH 7.0 DTT and 2-ME were able to restore enzyme activity which is consistent with the generation of a disulfide bond in the enzyme (Fig. 6A). A disulfide bond could be formed between Cys 288 and Cys 289 at the active site (Fig. 10). GSH was not able to restore enzyme activity at pH 7.0, which suggests that GSH cannot prevent skBADH inactivation *in vivo*.

Inactivation by copper due to thiol oxidation has been observed for other enzymes such as rat and human aldose reductase, where inactivation results from disulfide bridge formation (Cecconi et al., 2002). Similarly, Hadizadeh et al. (2009) observed that the enzyme xanthine oxidase is inactivated by copper in a concentration and time dependent manner (Hadizadeh et al. 2009).

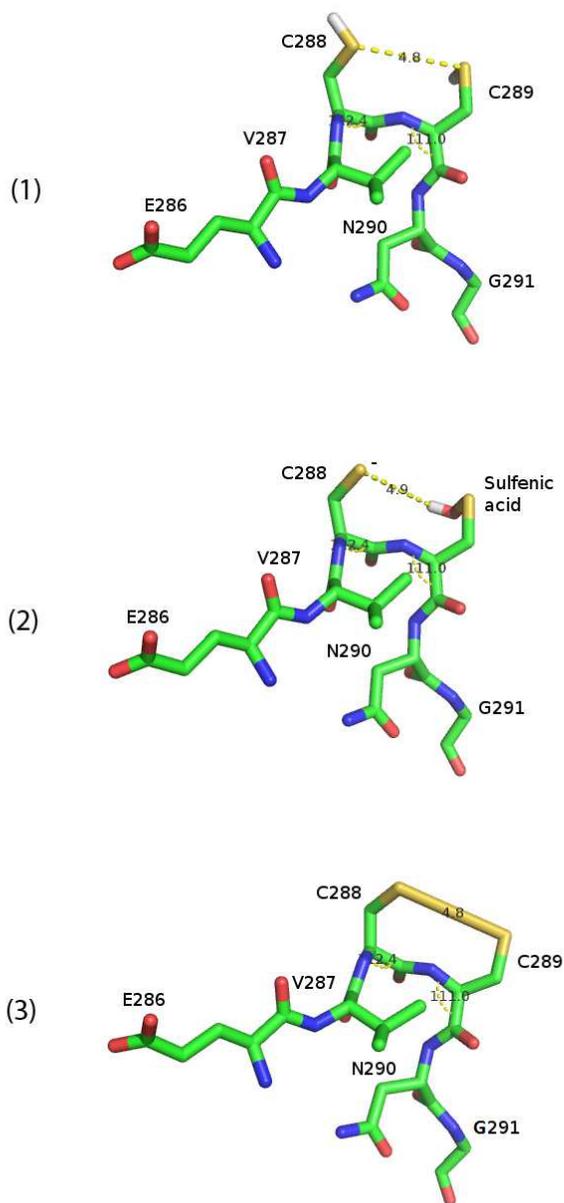


Fig. 9. Proposed mechanism for the reaction of the skBADH active site and  $H_2O_2$ . Hydrogen peroxide reacts with the thiol group (CSH) of the catalytic cysteine (C288) or the vicinal cysteine (C289) (1) leading to sulfenic acid formation (RSOH) (2), the proximity of a second thiolate group allows the intermediate sulfenic acid to combine with the thiolate group and form a disulfide bond (RSSR) between cysteines (3).

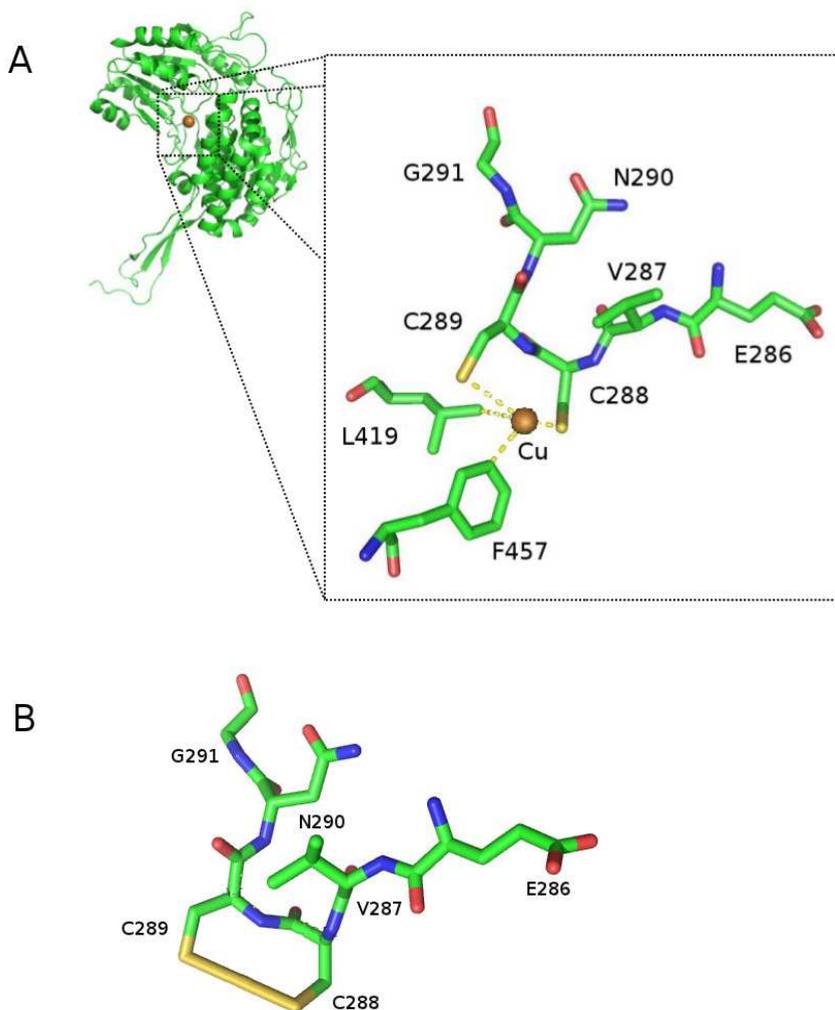


Fig. 10. Proposed mechanism for the reaction of the skBADH active site and copper. Copper reacts with the thiol group (CSH) of the catalytic cysteine (C288) or the vicinal cysteine (C289) (A), leading to the formation of a disulfide bond (RSSR) between cysteines (B).

SkBADH fluorescence spectra with 10  $\mu\text{M}$   $\text{CuCl}_2$  at pH 7.0 and pH 8.0 exhibited differences in the wavelengths at which the maximum emission occurred (Fig. 7 and 8), demonstrating that copper is changing the tryptophans' access to the solvent. In addition, potassium seems to be playing an interesting role in exposing the tryptophans in the enzyme to the solvent. Previous studies have demonstrated that skBADH requires a monovalent cation to maintain its tetrameric conformation (Valenzuela-Soto et al., 2003). SkBADH crystallographic studies are needed to see whether there is a site on the enzyme for potassium or for a different monovalent cation, such as that found in *P. aeruginosa* BADH (González-Segura et al., 2009).

Further studies are underway to determine if skBADH inactivation is dependent on copper concentration, to identify the time course of disulfide generation, and to detect whether there are differences in inactivation when Cu(I) or Cu(II) is used in the treatments.

## 5. Conclusion

SkBADH was oxidized by hydrogen peroxide and by copper at concentrations that likely reflect physiologically relevant conditions (Atmane et al. 2003; Caselli et al. 1998; Kazi et al., 2008; Meng et al. 2002; Pravodh et al. 2011) in kidney, and the targets of oxidation were thiol groups, mainly those of the active site. Interestingly, the thiol groups were not oxidized to sulfenic or sulfinic acid, but rather a disulfide bond was formed between catalytic cysteine (Cys288) and a vicinal cysteine (C289). Potassium, under physiological conditions, increased the oxidation of the enzyme by copper, possibly because it maintains the enzyme active tetramer or enables the oxidation of the cysteine residues. Since GB synthesis is catalyzed by BADH and it is crucial for the kidney cells to be able to withstand osmotic stress conditions, this type of inhibition can have serious physiological implications. In addition, BADH is an enzyme that is also found in the liver and brain where its inactivation by oxidative stress is associated with other pathologies. In summary, identifying the specific modifications caused by ROS on key enzymes contributes to a greater understanding of the changes that occur during oxidative stress and provides insight into how oxidative stress can be prevented. Further work is underway to determine the mechanisms by which renal BADH can be protected from the oxidative stress that occurs in renal medulla cells.

## 6. Perspectives

Hydrogen peroxide plays a role as an oxidant and as a secondary messenger, however the molecular basis of these roles and the concentration of H<sub>2</sub>O<sub>2</sub> in cells has not been established. In addition, copper can interact with proteins, causing oxidation, or can generate oxidative stress. In light of this it is important to pursue this line of research, also because hydrogen peroxide and copper have been associated with some human pathologies that are characterized by some interaction between hydrogen peroxide and copper and enzymes and proteins. This opens up new areas for developing strategies to contend with some of the diseases directly related to oxidative stress.

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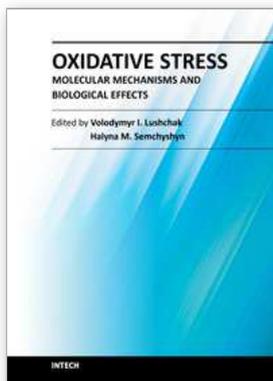
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## **Oxidative Stress - Molecular Mechanisms and Biological Effects**

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Since the discovery of free radicals in biological systems researchers have been highly interested in their interaction with biological molecules. Denoted in 1980, and due to fruitful results and ideas, oxidative stress is now appreciated by both basic and applied scientists as an enhanced steady state level of reactive oxygen species with wide range of biological effects. This book covers a wide range of aspects and issues related to the field of oxidative stress. The association between generation and elimination of reactive species and effects of oxidative stress are also addressed, as well as summaries of recent works on the signaling role of reactive species in eukaryotic organisms. The readers will gain an overview of our current understanding of homeostasis of reactive species and cellular processes they are involved in, as well as useful resources for further reading.

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