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**Mitochondrial Respiratory Chain and Thioredoxin Reductase Regulate Intermembrane Cu,Zn-Superoxide Dismutase Activity: Implications for Mitochondrial Energy Metabolism and Apoptosis**

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## ABSTRACT

Intermembrane space Cu,Zn-superoxide dismutase (IMS Cu,Zn-SOD) is inactive in isolated intact rat liver mitochondria and is activated following oxidative modification of its critical thiol groups. The present study aimed to identify biochemical pathways implicated in the regulation of IMS Cu,Zn-SOD activity and to assess the impact of its functional state on key mitochondrial events. Exogenous H<sub>2</sub>O<sub>2</sub> (5 μM) activated Cu,Zn-SOD in intact mitochondria. However, neither H<sub>2</sub>O<sub>2</sub> alone nor H<sub>2</sub>O<sub>2</sub> in the presence of mitochondrial peroxiredoxin III activated Cu,Zn-SOD, which was purified from mitochondria and subsequently reduced by DTT to an inactive state. The reduced enzyme was activated following incubation with superoxide generating system, xanthine and xanthine oxidase. In intact mitochondria, the extent and duration of Cu,Zn-SOD activation was inversely correlated with mitochondrial superoxide production. Presence of thioredoxin reductase-1 (TxrR-1) was demonstrated in the mitochondrial IMS by western blotting. Inhibitors of TxrR-1, CDNB or auranofin, prolonged duration of H<sub>2</sub>O<sub>2</sub>-induced Cu,Zn-SOD activity in intact mitochondria. TxrR-1 inactivated Cu,Zn-SOD purified from mitochondria in an active oxidized state. Activation of IMS Cu,Zn-SOD by exogenous H<sub>2</sub>O<sub>2</sub> delayed CaCl<sub>2</sub>-induced loss of trans-membrane potential, decreased cytochrome c release, and markedly prevented superoxide-induced loss of aconitase activity in intact mitochondria respiring at state-3. These findings suggest that IMS Cu,Zn-SOD activity is reversibly regulated by H<sub>2</sub>O<sub>2</sub>, superoxide, and TxrR-1 and that the active enzyme is implicated in protecting vital mitochondrial functions.

## INTRODUCTION

Recent studies on the subcellular distribution of superoxide dismutase (SOD) demonstrated that Cu,Zn-SOD is localized in not only cytosol [1] but also in the mitochondrial intermembrane space (IMS) of rat liver [2, 3] and yeast [4]. Comparative kinetic studies, however, revealed that Cu,Zn-SOD is inactive in isolated intact rat liver mitochondria, unless the outer membrane is disrupted by digitonin [5, 6, 7, 8]. Presence of alkylating agents during disruption of the outer membrane prevents activation of the enzyme whereas exposure of intact mitochondria to low concentrations of H<sub>2</sub>O<sub>2</sub> (5 μM) activates Cu,Zn-SOD in the IMS [8]. These findings suggest that intra-subunit disulfide bond necessary for the active state of Cu,Zn-SOD has not been formed in mitochondria [8].

The biochemical pathways catalyzing reversible redox activation of Cu,Zn-SOD are not defined in mitochondria. Activation of Cu,Zn-SOD following exposure of mitochondria to low levels of H<sub>2</sub>O<sub>2</sub> suggest involvement of an enzyme-mediated thiol oxidation rather than direct oxidation of the enzyme by H<sub>2</sub>O<sub>2</sub>. Potential candidates to catalyse such a reaction are mitochondrial peroxiredoxins (III and/or V), a family of peroxidases that reduce H<sub>2</sub>O<sub>2</sub> to water through reducing equivalents provided by thiol-containing proteins [9-10]. Thioredoxin reductase (TxrR) and glutathione reductase are major mitochondrial systems catalyzing reduction of protein disulfide bonds through reducing equivalents provided by thioredoxin (Txr) and glutathione (GSH) [11,12]; hence, they may be implicated in reduction, and as a corollary inactivation of IMS Cu,Zn-SOD.

The role of IMS Cu,Zn-SOD in mitochondrial functions is not known. However, its localization in the IMS suggests that the enzyme has a functional relationship with the occurrence of superoxide in this compartment. Several mitochondrial sites are primary targets of regulation by fluctuating levels of superoxide and/or H<sub>2</sub>O<sub>2</sub>. Enzymes containing 4Fe-4S clusters, such as aconitase and fumarase, are target of inactivation by superoxide [13, 14]. Superoxide and/or H<sub>2</sub>O<sub>2</sub> also mediate the opening of permeability transition pore (PTP) leading to the loss of mitochondrial transmembrane potential ( $\Psi_m$ ), cytochrome c release, and apoptosis [15, 16]. Therefore, IMS Cu,Zn-SOD activity may potentially play an important role in key mitochondrial events (e.g. energy metabolism, transemembrane potential, and cytochrome c release) to maintain the integrity of mitochondria. Hence, the aims of this study were to elucidate the biochemical pathways regulating IMS Cu,Zn-SOD activity, and to establish a functional link between its activity and key mitochondrial events.

## EXPERIMENTAL

### Chemicals.

*p*-hydroxyphenylacetate, Bovine Cu,Zn-superoxide, fatty acid free bovine serum albumin (BSA), 3'-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT), digitonin, iodoacetamide (IAM), xanthine oxidase, catalase, reduced glutathion, Txr, TxrR-1, antimycin, horseradish peroxidase, NADP<sup>+</sup>, isocitrate dehydrogenase, rotenone, and rhodamine 123 were from Sigma Chemical Co. (St. Louis, MO, USA).

### Isolation of liver mitochondria.

Liver mitochondria were isolated from adult male Wistar rats by differential centrifugation as described previously [17]. Rat livers were excised, chopped into fine pieces, washed with 0.25 M sucrose and homogenized in isolation buffer consisted of 210 mM mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.4, and 0.05% BSA. The homogenate was centrifuged at 800g for 8 min, the pellet was removed, and the centrifugation process was repeated. The supernatant was centrifuged at 8,000g for 10 min, the pellet was washed with the isolation buffer, and the centrifugation was repeated. The pellet containing mixture of organelles were further fractionated by centrifugation at 8,500 g for 10 min in Percoll gradient consisted of three layers of 18%, 30%, and 60% (w/v) Percoll in sucrose-Tris buffer (0.25 M sucrose, 1 mM EDTA, and 50 mM Tris), pH 7.4. Mitochondria were collected from the interface of 30% and 60% Percoll and washed with the sucrose-Tris buffer. Mitochondrial proteins were determined by Bradford assay [18].

### **Isolation of mitochondrial IMS contents.**

Mitochondrial outer membrane was selectively disrupted by treating mitochondria (40 mg/ml) with 0.11 mg of digitonin per mg of protein [19]. The IMS contents were then freed of mitoplasts by centrifugation at 10,000g for 15 min.

### **Measurement of SOD activity.**

SOD activity was assayed by generating  $O_2^{\cdot-}$  with the xanthine/xanthine oxidase system in the presence of sulphonated tetrazolium salt, XTT [20]. Cyanide at 5.0 mM was used to selectively inhibit Cu,Zn-SOD activity.

### **Measurement of $H_2O_2$ .**

Fluorescence measurements were performed using Perkin-Elmer LS-5 spectrofluorometer.  $H_2O_2$  formation was measured by monitoring horseradish peroxidase-catalysed  $H_2O_2$ -dependent oxidation of *p*-hydroxyphenylacetate ( $\lambda_{ex} = 320$  nm;  $\lambda_{em} = 400$  nm) [21].

### **Temporal kinetics of IMS Cu,Zn-SOD activation by $H_2O_2$ .**

Mitochondria (2 mg/ml) were incubated with 5  $\mu$ M  $H_2O_2$  for 5, 10, 20, 30 minutes at 37°C, washed with sucrose-Tris buffer, and then incubated with digitonin in the presence of iodoacetamide (IAM) for 1 hour at room temperature. Cu,Zn-SOD activity was assayed in the mitochondrial IMS preparations.

### **Preparation of the oxidized and reduced IMS Cu,Zn-SOD.**

Rat liver IMS Cu,Zn-SOD was purified in the oxidized state as described previously [2]. The reduced enzyme was prepared following incubation of the purified enzyme with 4 mM dithiotreitol (DTT) at 37°C overnight. DTT was then removed by

dialysis. The activity of the oxidized or reduced Cu,Zn SOD was measured following incubation with various agents for indicated times at 37°C.

#### **Measurement of IMS Cu,Zn-SOD activation by mitochondrial superoxide.**

Mitochondria were incubated with various concentrations of antimycin ranging from 0.01 to 0.1 µg/mg of mitochondria respiring at state 4 in the presence of 1 mM *p*-hydroxyphenylacetate. H<sub>2</sub>O<sub>2</sub> formation was measured by monitoring horseradish peroxidase-catalyzed (5 U/ml) H<sub>2</sub>O<sub>2</sub>-dependent oxidation of *p*-hydroxyphenylacetate. Mitochondria were also incubated with 0.01-0.1 µg/mg of antimycin for indicated times at 37°C, washed with sucrose-Tris buffer, and incubated with digitonin in the presence of iodoacetamide (IAM) for 1 hour at room temperature. Cu,Zn-SOD activity was assayed in the mitochondrial IMS preparations.

#### **Detection of TxrR-1 in mitochondrial IMS.**

Mitochondrial IMS and cytosolic fractions were resolved on polyacrylamide gels and immunoblotted onto nitrocellulose membranes using anti-TxrR-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Cytosolic contamination was assessed by using anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO, USA.).

#### **Measurement of aconitase activity.**

Mitochondria were incubated in the presence or absence of 5 µM H<sub>2</sub>O<sub>2</sub> for 5 minutes at 37°C. The state 3 respiration was then initiated by addition of 2 mM ADP, 4 mM MgCl<sub>2</sub>, 5 mM KH<sub>2</sub>PO<sub>4</sub>, and 10 mM succinate. Aliquots were taken at indicated times to determine mitochondrial aconitase activity. Aconitase activity was assayed at room temperature by following formation of NADPH at 340 nm for 10 minutes in a reaction mixture containing 50 mM Tris HCl, pH 7.4, 30 mM sodium citrate, 0.6 mM



MnCl<sub>2</sub>, 0.2 mM NADP<sup>+</sup> and 1 U/ml of isocitrate dehydrogenase. One nmole NADPH formed per min was corresponded to 1 mU of aconitase [22].

### **Measurement of mitochondrial membrane potential and cytochrome c release from mitochondria.**

Mitochondria (1 mg/ml) were incubated in a buffer containing 0.250 mM sucrose, 10 mM Hepes/KOH, pH 7.4, 0.1% fatty-acid free bovine serum albumin, 6 mM succinate, 1 µg/ml rotenone, and 1 µM Rhodamine 123 for 5 min at 25°C in order to equilibrate mitochondria with the cationic dye. Mitochondria were incubated with 5 µM H<sub>2</sub>O<sub>2</sub> for 3 minutes at 25°C. CaCl<sub>2</sub> (12 or 25 µM) was then added to induce dissipation of membrane potential. Membrane potential was assessed fluorimetrically by measuring the membrane potential dependent uptake of Rhodamine 123 (1 µM) with excitation at 505 nm and recording at 534 nm. [23]. Pellets and supernatants were treated with SDS sample buffer and boiled, and aliquots were resolved on polyacrylamide gels and immunoblotted onto nitrocellulose membranes using anti-cytochrome c antibodies (BD Bioscience, San Jose, CA, USA.).

### **Data presentation**

Data were reported as mean ± SD of at least three independent experiments performed in triplicate. Analysis of variance and Duncan's Multiple Range Test was used to analyse the difference among group means. A *P* value of < 0.05 was considered a statistically significant difference.

## RESULTS

### **Superoxide and H<sub>2</sub>O<sub>2</sub> promote activation of IMS Cu,Zn-SOD.**

In order to determine the biochemical processes involved in the activation of IMS Cu,Zn-SOD, a series of experiments were conducted with intact mitochondria and the purified inactive enzyme. Low micromolar concentrations (5  $\mu$ M) of exogenous H<sub>2</sub>O<sub>2</sub> activated IMS Cu,Zn-SOD at a rate of 2 units/mg mitochondrial protein/min (Fig. 1A). The enzyme activity reached to its maximum level, 10 units/mg mitochondrial protein, after 5 minutes of mitochondria exposure to H<sub>2</sub>O<sub>2</sub>, decreased sharply at 10 minutes, and ultimately returned to the basal levels at 30 minutes (Fig. 1A). The possibility of direct interaction between IMS Cu,Zn-SOD and H<sub>2</sub>O<sub>2</sub> was assessed by exploiting Cu,Zn-SOD, which was purified from mitochondria and subsequently reduced by DTT to inactive state. The specific activity of the purified IMS Cu,Zn-SOD was determined as 3200  $\pm$  270 units/mg enzyme/min following rupture of the outer membrane with digitonin alone but prior to reduction with DTT. Neither H<sub>2</sub>O<sub>2</sub> alone nor H<sub>2</sub>O<sub>2</sub> in the presence of mitochondrial peroxiredoxin III activated the reduced Cu,Zn-SOD (Fig. 1B). However, the enzyme was markedly activated following incubation with superoxide generating system, xanthine and xanthine oxidase, and to a lesser extent with glucose and glucose oxidase, a system producing superoxide, in addition to H<sub>2</sub>O<sub>2</sub>, with a rate dependent on the pH and O<sub>2</sub> concentration [24]. Lesser efficiency of glucose/glucose oxidase system is possibly because of a lower rate of superoxide production compared to xanthin/xanthine oxidase system. Presence of bovine erythrocyte SOD (300 U/ml) in the assay mixture abolished this activation whereas catalase was ineffective (Fig. 1B). Examination of kinetics of the enzyme activation revealed that steady state levels of superoxide (0.7-0.8

nmol/ml/min) generated by xanthine/xanthine oxidase activate the reduced IMS Cu,Zn-SOD at a rate of 66 units/mg enzyme/min (Fig. 1C). The enzyme specific activity was ultimately reached to a plateau of 3200 units/mg enzyme at 45 min. These findings suggest that both H<sub>2</sub>O<sub>2</sub> and superoxide may act as activators of IMS Cu,Zn-SOD. While superoxide can directly act upon the enzyme, H<sub>2</sub>O<sub>2</sub> requires additional mitochondrial component(s) to trigger activation of Cu,Zn SOD.

### **Superoxide generated by mitochondrial respiration activates Cu,Zn-SOD in intact mitochondria**

In order to verify whether superoxide generated during mitochondrial respiration can activate Cu,Zn-SOD in the IMS, superoxide production was promoted by antimycin in mitochondria respiring on state 4 (Fig. 2A). Mitochondrial superoxide production was dose-dependently increased with antimycin concentrations between 0.01 to 0.1 µg/mg mitochondrial protein, generating hydrogen peroxide ranging from  $0.30 \pm 0.03$  to  $0.65 \pm 0.04$  nmoles/min/mg (equivalent to superoxide ranging from  $0.60 \pm 0.06$  to  $1.35 \pm 0.08$  nmoles/min/mg). An inverse relationship was, however, found between the levels of mitochondrial superoxide production and the extent and duration of Cu,Zn-SOD activation in the IMS. Cu,Zn-SOD was more potently activated for a significantly longer duration when mitochondria generated lower levels of superoxide  $0.60 \pm 0.06$  nmoles/mg mitochondrial protein/min compared to a lesser and shorter duration of the enzyme activity when mitochondria generated high levels of superoxide  $1.35 \pm 0.08$  nmoles/mg mitochondrial protein/min (Fig. 2B). These findings suggest that mitochondrial respiration regulates Cu,Zn-SOD activity in the IMS.

### **TxrR inactivates IMS Cu,Zn-SOD.**

Presence of TxrR-1 in the IMS of rat liver mitochondria was detected by western blotting (Fig. 3). In order to determine potential biochemical pathway(s) implicated in the inactivation of IMS Cu,Zn-SOD, the enzyme was purified from mitochondria in an active, oxidized state by rupturing the outer membrane with digitonin alone. Incubation with TxrR-1 from rat liver significantly decreased activity of the oxidized Cu,Zn-SOD (Fig. 4A). The initial rate of inactivation of the oxidized enzyme was estimated 60 units/mg enzyme/min (Fig. 4B). Presence of Txr in the assay mixture did not potentiate inactivation of the oxidized enzyme by TxrR-1 (Fig. 4A). Furthermore, neither Txr nor GSH alone affected superoxide scavenging activity of the oxidized form of Cu,Zn-SOD. These findings suggest that mitochondrial TxrR-1 is implicated in the inactivation of IMS Cu,Zn-SOD.

CDNB is an alkylating agent that selectively inhibits TxrR-1 at low concentrations (250  $\mu$ M). Pre-incubation of purified IMS Cu,Zn-SOD, which was reduced with DTT and subjected to dialysis to remove excess of DTT, with CDNB at 37°C for 10 min did not affect activation of the reduced enzyme by xanthine/xanthine oxidase system (data not shown). This finding suggests that CDNB, at concentration used here, is unable to alkylate and inhibit IMS Cu,Zn-SOD. Addition of CDNB to intact mitochondria at 4<sup>th</sup> min of H<sub>2</sub>O<sub>2</sub> treatment substantially prolonged duration of H<sub>2</sub>O<sub>2</sub>-induced IMS Cu,Zn-SOD activity (Fig 4C). In a similar set of experiments, auranofin, a potent inhibitor of selenocystein-containing enzymes [25], comparably prolonged the enzyme activity induced by H<sub>2</sub>O<sub>2</sub> (data not shown). These findings suggest that TxrR-1 play a role in inactivation of Cu,Zn-SOD in intact mitochondria. However, pre-incubation of intact

mitochondria with CDNB completely abolished activation of IMS Cu,Zn-SOD by H<sub>2</sub>O<sub>2</sub> (Fig. 4C). It is likely that mitochondrial factor(s) involved in H<sub>2</sub>O<sub>2</sub>-induced activation of IMS Cu,Zn-SOD are sensitive to alkylation by CDNB under this experimental condition.

**IMS Cu,Zn-SOD protects against oxidative loss of aconitase activity in matrix.**

Rat liver mitochondria are known to produce superoxide when respiring with complex II-linked substrates on state 3 [26, 27]. Direct aerobic inactivation of matrix aconitase was also recently reported in yeasts lacking Cu,Zn-SOD. Incubation of rat liver mitochondria respiring on state 3 at 37 °C resulted in loss of aconitase activity by 33 ± 5% and 42 ± 4% after 5 and 15 min, respectively, (Fig. 5). However, activation of IMS Cu,Zn-SOD following incubation of intact mitochondria with H<sub>2</sub>O<sub>2</sub> (5 µM) significantly prevented loss of aconitase activity in mitochondrial matrix (Fig. 5).

**IMS Cu,Zn-SOD diminishes apoptotic capacity of mitochondria.**

CaCl<sub>2</sub> progressively promoted dissipation of Ψ<sub>m</sub> in mitochondria respiring at state 4 (Fig. 6A). Loss of Ψ<sub>m</sub> is known to precede the release of mitochondrial apoptotic factors, such as cytochrome c. Analysis of the mitochondria incubated with CaCl<sub>2</sub> for 30 min demonstrated significant loss of IMS cytochrome c accompanying with its release into extramitochondrial space (Fig. 6B, upper panel). Prior activation of IMS Cu,Zn-SOD by exposing mitochondria to H<sub>2</sub>O<sub>2</sub> (5 µM) significantly delayed loss of Ψ<sub>m</sub> and prevented cytochrome c release induced by CaCl<sub>2</sub> (Fig. 6). These findings suggest that active state of IMS Cu,Zn-SOD may regulate events leading to the loss of mitochondrial integrity and cell death.

## DISCUSSION

The findings of this study suggest that IMS Cu,Zn-SOD activity is regulated by mitochondrial respiratory chain and TxrR-1. Figure 7 illustrates a network of biochemical pathways, which may regulate or be affected by IMS Cu,Zn-SOD activity. It appears that IMS Cu,Zn-SOD is implicated in protecting intermediate metabolism against oxidative damage and regulating apoptosis.

### **Regulation of Cu,Zn-SOD activity in the IMS.**

In order to elucidate the biochemical pathways involved in reversible activation of IMS Cu,Zn-SOD, a series of experiments were conducted with intact mitochondria and the purified Cu,Zn-SOD reduced by DTT to an inactive state. While H<sub>2</sub>O<sub>2</sub> transiently activated Cu,Zn-SOD in intact mitochondria, it did not affect the activity of the reduced enzyme once it was purified from the IMS, suggesting that additional mitochondrial component(s) are necessary to catalyze the intra-subunit disulfide formation. In spite of localization of peroxiredoxin III in mitochondrial matrix and in the IMS, our findings suggest that peroxiredoxin III is not implicated in the activation of Cu,Zn-SOD. However, given that H<sub>2</sub>O<sub>2</sub> is readily diffusible across membranes, these findings suggest that IMS Cu,Zn-SOD is responsive to fluctuations of physiologically relevant concentrations of H<sub>2</sub>O<sub>2</sub> in a cell.

Mitochondria are the major source of superoxide. The mitochondrial inner membrane vectorially releases superoxide into the IMS [28] upon autoxidation of the ubisemiquinone at the outer coenzyme Q pool. Direct activation of IMS Cu,Zn-SOD by superoxide constitute a typical example of enzyme regulation in response to changes in the level of its substrate. The rate of Cu,Zn-SOD activation was, however, different

following exposure to H<sub>2</sub>O<sub>2</sub> and superoxide. Superoxide generated by X/XO or mitochondrial respiration activated Cu,Zn-SOD at a lower rate (0.6 μM/min) than H<sub>2</sub>O<sub>2</sub> (5 μM/min). Higher rate of enzyme activation by H<sub>2</sub>O<sub>2</sub> may reflect enzyme-catalysed nature of this reaction. However, temporal kinetics of the enzyme activation by H<sub>2</sub>O<sub>2</sub> and superoxide were similar in the intact mitochondria. Both H<sub>2</sub>O<sub>2</sub> and superoxide elicited a transient activation of Cu,Zn-SOD, although the extent and duration of enzyme activation was longer with superoxide than with H<sub>2</sub>O<sub>2</sub>. It is noteworthy that the activity of Cu,Zn-SOD was inversely correlated with the level of mitochondrial superoxide production, a similar phenomenon observed following exposure of intact mitochondria to exogenous H<sub>2</sub>O<sub>2</sub>. These findings suggest that IMS Cu,Zn-SOD is active in a narrow window of physiologically relevant concentrations of superoxide or H<sub>2</sub>O<sub>2</sub>. The underlying reason for the lower level of the enzyme activity in the presence of higher concentrations of superoxide or H<sub>2</sub>O<sub>2</sub> remains to be elucidated.

The transient nature of the enzyme activation could be also attributed to the biochemical pathways inactivating IMS Cu,Zn-SOD. One such enzyme detected in the IMS was TxrR-1. The rate of Cu,Zn-SOD inactivation (0.1 μM/min) was, however, slower than its activation with either H<sub>2</sub>O<sub>2</sub> or superoxide. Whether additional enzymatic pathways are implicated in inactivation of IMS Cu,Zn-SOD remained to be determined. Alternatively, faster inactivation of Cu,Zn-SOD in intact mitochondria could be potentially due to the substantially higher IMS TxrR-1 concentration compared to that under the assay conditions presented in Fig. 4 (20 U/ml). Moreover, CDNB and auranofin, selective TxrR-1 inhibitors, prolonged duration of H<sub>2</sub>O<sub>2</sub>-induced IMS Cu,Zn-SOD activation. These findings demonstrate that TxrR-1 is implicated in the inactivation

of IMS Cu,Zn-SOD. In agreement with these findings, eukaryotic Cu,Zn-SOD expressed in prokaryotic organisms was found active only when the cytosolic expression of TxrR-1 was prevented [29].

### **IMS Cu,Zn-SOD protects vital mitochondrial functions.**

Yeast mutant lacking or deficient in Cu,Zn-SOD presents direct aerobic inactivation of enzymes, such as aconitase, homoaconitase and succinate dehydrogenase, containing superoxide sensitive matricial 4Fe-4S cluster. These findings suggest that superoxide released into the IMS may pass mitochondrial inner membrane and damage matricial 4Fe-4S cluster containing enzymes even in the presence of Mn-SOD [30]. Superoxide (pK 4.8) is likely to be protonated at the external surface of inner membrane due to the protons extrusion, and diffuse into the matrix [31], or it may react with mitochondrial nitric oxide to produce peroxynitrite (pK 6.8), which can be then diffuse into the matrix [32]. Our findings indicate that active IMS Cu,Zn-SOD protects the matricial aconitase activity against superoxide-mediated inactivation.

It has long been known that Ca<sup>2+</sup> overload leads to the state of mitochondrial membrane permeability transition [15, 16] and the opening of a mitochondrial membrane pore (PTP) following increased mitochondrial production of reactive oxygen species [33]. The opening of PTP leads to cytochrome c release into cytosol, which then triggers apoptotic process [34]. Transient activation of IMS Cu,Zn-SOD significantly decelerated dissipation of mitochondrial  $\Delta\psi$  and decreased release of cytochrome c, suggesting that IMS Cu,Zn-SOD may function to prevent apoptosis during episodes of increased mitochondrial superoxide levels.



Taken altogether, these findings suggest that IMS Cu,Zn-SOD activity is reversibly regulated by H<sub>2</sub>O<sub>2</sub>, superoxide, and TxrR-1 and that the enzyme is implicated in protecting vital mitochondrial functions.

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## FIGURE LEGENDS

**Fig. 1- H<sub>2</sub>O<sub>2</sub> and superoxide promote activation of IMS Cu,Zn-SOD.** (A) Temporal kinetics of Cu,Zn-SOD activation by exogenous H<sub>2</sub>O<sub>2</sub> in intact mitochondria. Mitochondria were incubated with H<sub>2</sub>O<sub>2</sub> (5 μM) for indicated times at 37°C, washed with sucrose-Tris buffer, and incubated with digitonin in the presence of iodoacetamide for 1 hour at room temperature. \*Significantly different from the activity at 0 min, *P* < 0.05. (B) Superoxide, but not H<sub>2</sub>O<sub>2</sub>, directly activates purified IMS Cu,Zn-SOD. Cu,Zn-SOD was purified from mitochondria and subsequently reduced by DTT to inactive state. DTT was then removed by dialysis. The reduced enzyme (3 μM) was incubated with xanthine (50 μM) and xanthine oxidase (5 mU/ml), glucose (2mM) and glucose oxidase (2 mU/ml), peroxiredoxin III (20 μM) and H<sub>2</sub>O<sub>2</sub> (5 μM) , or H<sub>2</sub>O<sub>2</sub> alone in the presence or absence of catalase (1000 U/ml) or bovine erythrocyte SOD (300 U/ml) for 1 h at 37°C. \*Significantly different from the activity after incubation with XO, *P* < 0.05. (C) Temporal kinetics of the reduced Cu,Zn-SOD activation by superoxide. The reduced IMS Cu,Zn-SOD (3 μM) was incubated with xanthine (50 μM) and xanthine oxidase (5 mU/ml) for indicated times at 37°C. After incubations described in (A)-(C), Cu,Zn-SOD activity was measured by following reduction of XTT at 470 nm.

**Fig. 2- Mitochondrial respiration regulates Cu,Zn-SOD activity in the IMS.** (A) Kinetics of superoxide production by antimycin-treated mitochondria. Mitochondria (2 mg/ml) respiring at state 4 were incubated with indicated concentrations of antimycin in the presence of 1 mM *p*-hydroxyphenylacetate. Superoxide formation was indirectly measured by monitoring horseradish peroxidase-catalysed (5 U/ml) H<sub>2</sub>O<sub>2</sub>-dependent oxidation of *p*-hydroxyphenylacetate. \*Significantly different from H<sub>2</sub>O<sub>2</sub> production in

the absence of antimycin ( $0 \mu\text{g}/\text{mg}$ ),  $P < 0.05$ . (B) Temporal kinetics of Cu,Zn-SOD activation by superoxide generated during mitochondrial respiration. Mitochondria ( $2 \text{ mg}/\text{ml}$ ) respiring at state 4 were incubated with indicated concentrations of antimycin for the specified times at  $37^\circ\text{C}$ . Mitochondria were subsequently washed with sucrose-Tris buffer and incubated with digitonin in the presence of iodoacetamide for 1 hour at room temperature. Cu,Zn-SOD activity was measured by following reduction of XTT at  $470 \text{ nm}$ .

**Fig. 3. TxrR-1 is localized in the IMS of rat liver mitochondria.** TxrR-1 was detected by western blotting in both IMS and cytosolic fractions. Cytosolic contamination of IMS fractions was assessed by detection of  $\beta$ -actin.

**Fig. 4- TxrR-1 inactivates the oxidized IMS Cu,Zn-SOD.** Cu,Zn-SOD was purified from mitochondria in an active, oxidized state by rupturing the outer membrane with digitonin alone. (A) Inactivation of IMS Cu,Zn-SOD by TxrR-1. The oxidized enzyme ( $3 \mu\text{M}$ ) was incubated with TxrR-1 ( $20 \text{ U}/\text{ml}$ ), Txr ( $15 \text{ U}/\text{ml}$ ), their combination, or glutathion ( $5 \text{ mM}$ ) for  $60 \text{ min}$  at  $37^\circ\text{C}$ . NADPH ( $0.25 \text{ mM}$ ) was added to the reaction mixture when TxrR-1 was present. Cu,Zn-SOD activity was assayed by following reduction of XTT at  $470 \text{ nm}$ . \*Significantly different from the activity in the presence of TxrR-1 and Txr,  $P < 0.05$ . (B) Temporal kinetics of Cu,Zn-SOD inactivation by TxrR-1. The oxidized enzyme ( $3 \mu\text{M}$ ) was incubated with TxrR-1 ( $20 \text{ U}/\text{ml}$ ) and NADPH ( $0.25 \text{ mM}$ ) for the indicated times at  $37^\circ\text{C}$ . \*Significantly different from the activity at  $0 \text{ min}$ ,  $P < 0.05$ . (C) CDNB prolongs duration of IMS Cu,Zn-SOD activity in intact mitochondria. Mitochondria were incubated with  $\text{H}_2\text{O}_2$  ( $5 \mu\text{M}$ ) for indicated times at  $37^\circ\text{C}$ , washed with sucrose-Tris buffer, and incubated with digitonin in the presence of

iodoacetamide for 1 hour at room temperature. CDNB (250  $\mu$ M) was pre-incubated with mitochondria for 10 min (●) or it was added at 4th min of H<sub>2</sub>O<sub>2</sub> incubation (○). After incubations described in (A)-(C), Cu,Zn-SOD activity was measured by following reduction of XTT at 470 nm..

**Fig. 5. IMS Cu,Zn-SOD prevents oxidative loss of aconitase activity in matrix.**

Mitochondria (2 mg/ml) were incubated in the presence or absence of H<sub>2</sub>O<sub>2</sub> (5  $\mu$ M) at 37°C for 5 min. State 3 respiration was then initiated following addition of ADP (2 mM), MgCl<sub>2</sub> (4 mM), KH<sub>2</sub>PO<sub>4</sub> (5 mM) and succinate (10 mM) for the indicated times. Aconitase activity was subsequently measured as described in the Material & Methods. \*Significantly different from the activity at 0 min,  $P < 0.05$ .

**Fig. 6. IMS Cu,Zn-SOD decreases apoptotic capacity of mitochondria.** (A) Delay in the loss of  $\Psi_m$  induced by CaCl<sub>2</sub>. Mitochondria (1 mg/ml) were incubated at 37°C for 3 min in the presence (●) or absence (○, ▲, and □) of H<sub>2</sub>O<sub>2</sub> (5  $\mu$ M) followed by addition of vehicle (▲) or 12  $\mu$ M (○) or 25  $\mu$ M (● and □) CaCl<sub>2</sub> to dissipate  $\Psi_m$ . Rhodamine 123 uptake was monitored to measure  $\Psi_m$ . (B) Prevention of mitochondrial cytochrome c release induced by CaCl<sub>2</sub>. Mitochondria (1 mg/ml) were incubated at 37°C for 3 min in the presence or absence of H<sub>2</sub>O<sub>2</sub> (5  $\mu$ M) followed by addition of 25  $\mu$ M CaCl<sub>2</sub> for 30 min. The supernatants were then concentrated by 10-fold and cytochrome c was detected by western blotting in both the mitochondrial pellets (lower panel) and the supernatants (upper panel).

**Fig. 7. Putative biochemical pathways regulating IMS Cu,Zn-SOD activity and impact of its active state on mitochondrial functions.** Cu,Zn-SOD is inactive in the IMS of rat liver mitochondria. An increase in the tonus of superoxide and/or H<sub>2</sub>O<sub>2</sub> in



mitochondrial matrix and/or cytosol activates Cu,Zn-SOD in the IMS. Upon activation, IMS Cu,Zn-SOD prevents oxidative loss of matrix aconitase activity, delays dissipation of mitochondrial inner membrane potential, and prevents cytochrome c release into extramitochondrial space possibly by scavenging the excess superoxide, and hence decreasing intramitochondrial superoxide levels. TrxR-1 catalyzes reduction of the critical disulfide group and inactivate IMS Cu,Zn-SOD. Cu,Zn-SOD: Cu,Zn-superoxide dismutase; PP: permeability pore; TrxR-1: thioredoxin reductase-1.



Figure 2.

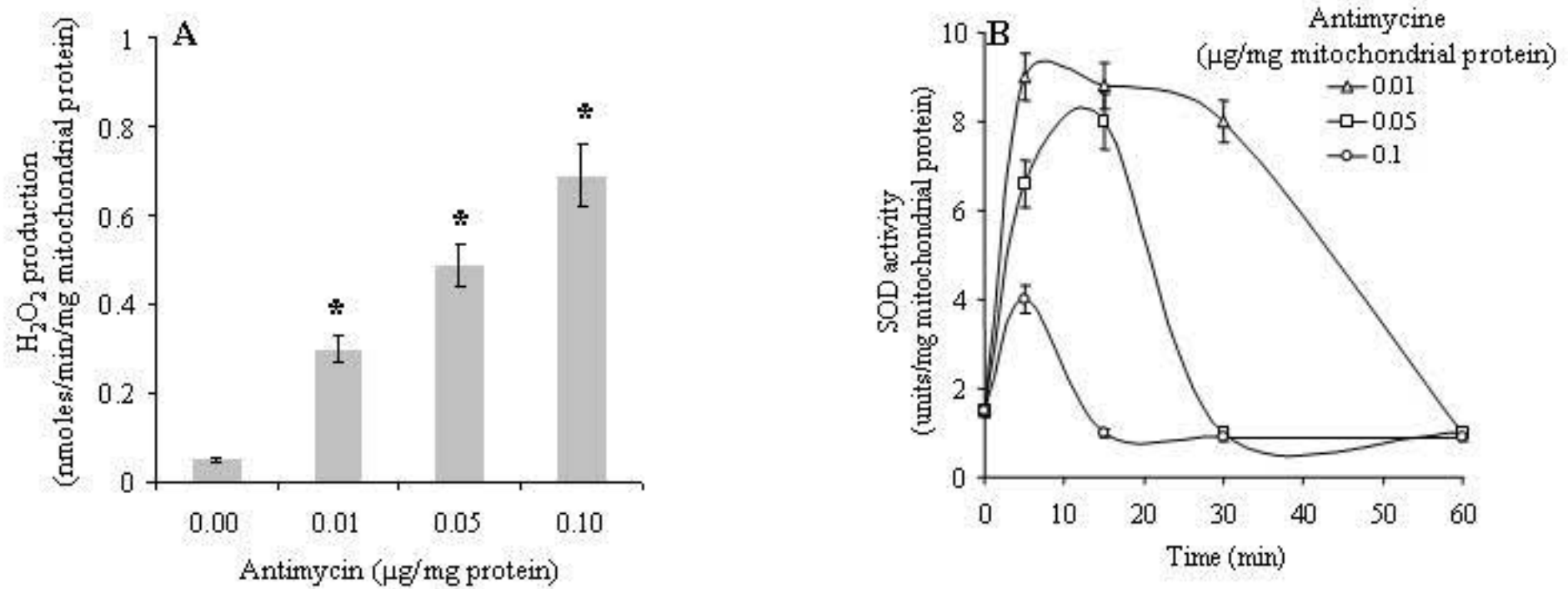


Figure 3.

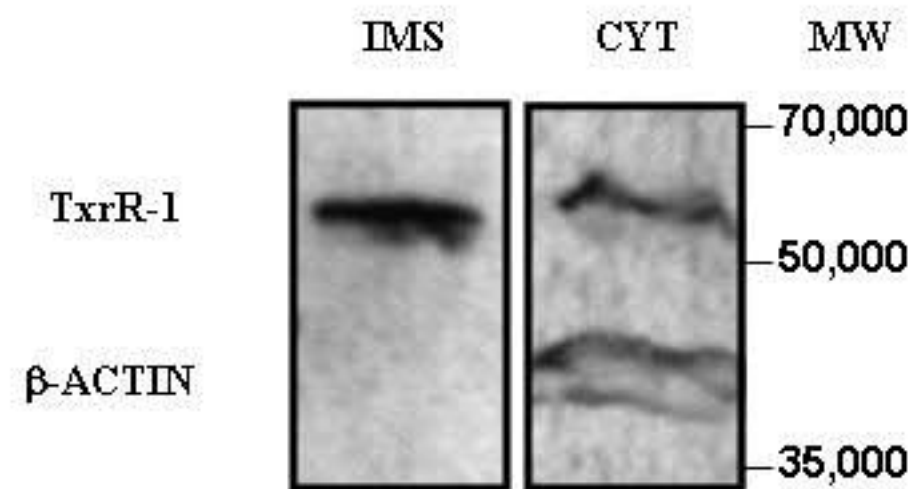


Figure 4.

