



## Cu(II)–disulfide complexes display simultaneous superoxide dismutase- and catalase-like activities



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### ABSTRACT

Superoxide is a potentially toxic by-product of cellular metabolism. We have addressed here the in vitro ability of complexes formed between copper(II) ions and various biologically-occurring disulfides (RSSR: oxidized glutathione, cystine, homocystine and  $\alpha$ -lipoic acid) to react with superoxide. The studied complexes were found to react with superoxide (generated by a xanthine/xanthine oxidase system) at rate constants ( $k_{\text{Cu(II)-RSSR}}$ ) close to  $10^6 \text{ M}^{-1} \text{ s}^{-1}$ , which are three orders of magnitude lower than that reported for superoxide dismutase (SOD) but comparable to that of several other copper-containing complexes reported as SOD mimetics. The interaction between the tested Cu(II)–RSSR and superoxide, led to the generation and recovery of concentrations of hydrogen peroxide and oxygen that were, respectively, below and above those theoretically-expected from a sole SOD mimetic action. Interestingly, oxygen was generated when the Cu(II)–RSSR complexes were directly incubated with hydrogen peroxide. Taken together, these results reveal that the Cu(II)–RSSR complexes not only have the capacity to dismutate superoxide but also to simultaneously act like catalase mimetic molecules. When added to superoxide-overproducing mitochondria (condition attained by its exposure to diclofenac), three of the tested complexes were able (2–4  $\mu\text{M}$ ), not only to totally restore, but also to lower below the basal level the mitochondrial production of superoxide. The present study is first in reporting on the potential of Cu(II)–disulfide complexes to act as SOD and catalase like molecules, suggesting a potential for these types of molecules to act as such under physiological and/or oxidative-stress conditions.

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### 1. Introduction

Copper is an essential micronutrient, it is found as cofactor of several enzymes and is involved in various redox-essential metabolic reactions [1,2]. When present under the form of free Cu(II) ions, the metal has the potential to interact with a number of cell-occurring thiols, amongst which reduced glutathione (GSH) [3–8], cysteine, gamma-glutamylcystine, cysteinyl-glycine, homocystine [6] and dihydrolipoic acid [9] are included. The interaction of Cu(II) ions with each of these thiols leads to the stoichiometric formation of Cu(I) ions. When occurring in a molar excess, some of these thiols are able to subsequently bind the cuprous ions formed, to generate the corresponding Cu(I)–thiol complexes. Formation of such complexes has been clearly shown for cysteine [10], homocystine [11,12], dihydrolipoic acid [9] and GSH [4]. Studies focusing on the interaction between Cu(I) and GSH have shown that binding of the metal leads to the formation of a Cu(I)–

[GSH]<sub>2</sub> complex [13–15]. Work conducted by Freedman et al. [4] early suggested that such complex can be formed within copper-exposed cells. Although the Cu(I)–[GSH]<sub>2</sub> complex was initially thought to be redox-inactive [16], work conducted by our laboratory has showed that upon interacting with molecular oxygen, the complex is able to actively generate superoxide [7,8], functioning thereby as a potentially relevant pro-oxidant towards biological targets [17]. Unlike the complex formed between Cu(I) and the GSH molecule, no superoxide-producing activity has been found for the complexes formed between Cu(I) ions and cysteine, homocystine, cysteinyl-glycine or gamma-glutamylcystine [6]. In the case of the complex formed with GSH, removal of the superoxide anions generated during its interaction with molecular oxygen has been shown to result on the oxidative conversion of Cu(I)–[GSH]<sub>2</sub> into a Cu(II)–GSSG [8]. Interestingly, in an early work on the redox properties of the latter complex, Jouini et al. [18] suggested that, within a pH range of 7–9, Cu(II)–GSSG would exhibit a superoxide-dismutase activity. Although, the interaction between thiols like homocystine, cysteine or dihydrolipoic acid with Cu(II) ions does not result in the formation of superoxide-generating complexes [6], the sole reduction of the metal by these thiols gives place to the formation of their corresponding disulfides (RSSR) [10,19,20]. Noteworthy, in the presence of Cu(II) ions,

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homocystine [21], cystine [22] and  $\alpha$ -lipoic acid [20] have all been reported to form Cu(II)–RSSR complexes with the metal.

In the present study we have investigated further the purported ability of Cu(II)–GSSG complex to dismutate superoxide anions [18], and evaluated whether such ability extends to complexes formed between Cu(II) ions and cystine (Cyss), homocystine (HCyss) and  $\alpha$ -lipoic acid (ALA) (Scheme 1). For each of the here-studied complexes, we determined kinetic rate constants for their reaction with superoxide and evaluated the formation of each of the products expected to occur as result of their postulated superoxide dismutase-like activity. Based on the results obtained along the study, we also describe and characterize here, for first time, a catalase-like activity that these Cu(II)–RSSR complexes were found to exhibit. Finally, using superoxide-overproducing mitochondria (condition attained by its exposure to diclofenac) as a model of oxidative stress, we provide evidence that, under a biologically-relevant condition, some of the studied complexes are potentially able to behave as potent antioxidants.

## 2. Experimental

### 2.1. Chemicals and reagents

Cupric chloride ( $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ), GSSG, HCyss, Cyss, ALA, superoxide dismutase (SOD; EC 1.15.1.1 from bovine erythrocytes), catalase (CAT; EC 1.11.1.6 from bovine liver), xanthine (X), xanthine oxidase (XO; E.C. 1.17.3.2 from bovine milk), cytochrome *c* (Cyt *c*; from bovine heart), bovine serum albumin (BSA), DMSO, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), xylenol orange and ammonium iron(II) sulfate were all purchased from Sigma-Aldrich. Dihydroethidium (DHE), hydrogen peroxide 30% (Perhydrol®) and Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) were purchased from Calbiochem. All aqueous solutions were prepared in Chelex-100-treated sodium phosphate buffer (20 mM; pH 7.4).

### 2.2. Preparation of copper–disulfide complexes

The Cu(II)–RSSR complexes were prepared, as previously described by Jouini et al. [18] for Cu(II)–GSSG, by mixing  $\text{CuCl}_2$  and RSSR in a 1:1 molar ratio, respectively. Whenever referring to a given concentration

of such complexes, it should be understood that it reflects the concentration of copper used in its preparation. All complexes were used within 2 h after being prepared.

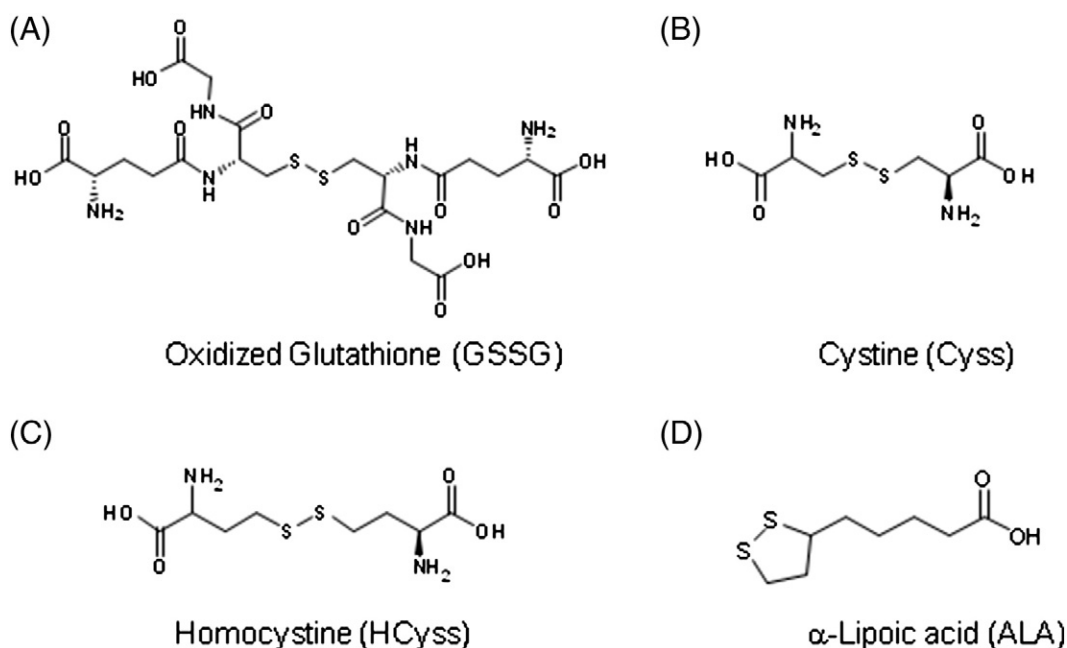
Preliminary identification of the complexes was obtained from IR and Raman spectra (see Supplementary data, Tables S1 and S2). The IR spectra were recorded using a Bruker Model Vector 22 spectrophotometer in the region  $450\text{--}3500\text{ cm}^{-1}$ . The Raman spectra were measured with a Renishaw micro-Raman system (RM1000) using as excitation the 632.8 nm laser line. This instrument was equipped with a Leica microscope, and an electrically cooled CCD camera. The signal was calibrated by using the  $520\text{ cm}^{-1}$  line of a Si wafer and the resolution was set to  $4\text{ cm}^{-1}$ . The solid samples were obtained with  $50\times$  objective, laser power of 2.0 mW, and 1 scan of 10 s. The liquid samples were obtained with  $20\times$  objective, laser power of 3.0 mW, and 1 scan of 10 s.

### 2.3. Generation and detection of superoxide

Superoxide was generated enzymatically through the xanthine (X) plus xanthine oxidase (XO) system and was assessed through the Cyt *c* reduction assay, as previously described by Van Gelder [23]. Cyt *c* reduction assayed monitoring the increase in absorbance at 550 nm, using an Agilent-8453 UV–vis spectrophotometer. In brief, Cyt *c* (100  $\mu\text{M}$ ) was added to a solution containing xanthine (500  $\mu\text{M}$ ) and xanthine oxidase (1.37 mU/mL) in phosphate buffer, 20 mM, pH 7.4, incubated at 25 °C. Such mixture (X/XO) gave place to a superoxide production rate of about  $0.4\text{ }\mu\text{M min}^{-1}$ .

The initial rates of Cyt *c* reduction, in the presence ( $V_c$ ) and in the absence ( $V_0$ ) of the Cu(II)–RSSR complexes, were obtained as the slope of plots of absorbance at 550 nm vs. time at the initial times (data obtained from monitoring the first 60 s of the reaction). The kinetic rate constants ( $k_{\text{Cu(II)–RSSR}}$ ) were determined by competition kinetics where cytochrome *c* was the target molecule according to the previously reported by Spasojevc et al. [24]. In addition, the concentration of the target molecule (Cyt *c*) was high enough to reach the zero order kinetics limits [25].

Previously, with the aim to discard the inactivation of the xanthine oxidase by the tested Cu(II)–RSSR complexes, the concentration of uric acid was determined by spectrophotometry ( $\lambda = 295\text{ nm}$ ,  $\epsilon_M =$



Scheme 1. Chemical structures of biologically relevant low molecular weight disulfides (RSSR).

$11.0 \text{ M}^{-1} \text{ cm}^{-1}$ ) [26] in the presence and absence of such complexes. A maximal inhibition of XO activity, of less than 10%, was observed when the complexes were added at a  $100 \mu\text{M}$  concentration (not shown).

#### 2.4. Detection of $\text{H}_2\text{O}_2$

Hydrogen peroxide was determined using the Fox's assay [27,28]. This method is based on the oxidation of ferrous to ferric ion which binds xylenol orange generating a chromophore complex with a visible absorption band at 560 nm. A Fox's working solution was prepared as following: 1 mL of a solution containing ammonium iron(II) sulfate ( $25 \text{ mM}$ ) in sulfuric acid  $0.25 \text{ M}$  was added to 100 mL of a water solution containing xylenol orange ( $125 \mu\text{M}$ ) and sorbitol ( $100 \text{ mM}$ ). An aliquot of the sample was added to the Fox's working solution (in a proportion of 1:10, respectively) and 20 min after, the optical density was recorded (at  $25 \text{ }^\circ\text{C}$ ) using a Multi-Mode Microplate Reader (Synergy™ HT).

#### 2.5. Oxygen consumption experiments

The percentage of oxygen in solution for mixtures containing X ( $500 \mu\text{M}$ ), XO ( $4.26 \text{ mU/mL}$ ) in the absence and in the presence of SOD ( $1000 \text{ U/mL}$ ) or Cu(II)-RSSR complexes was continually monitored (during 0–20 min at  $26 \text{ }^\circ\text{C}$ ) using a Clark-type oxygen electrode (Yellow Spring Instrument, model 5300A). In control experiments (run at  $26 \text{ }^\circ\text{C}$ ), no changes in the basal oxygen level were observed during 20 min when GSSG, Cyss, HCYss, ALA (at a  $20 \mu\text{M}$  concentration) or  $\text{CuCl}_2$  (at  $10 \mu\text{M}$ ) were added. The concentration of oxygen dissolved (S) in the mixtures, was determined by using the following Eq. (1):

$$S = \left( \frac{\alpha}{22.414} \right) \left( \frac{P-p}{p} \right) \left( \frac{r\%}{100} \right) S \quad (1)$$

Where  $\alpha$  is the absorption coefficient of  $\text{O}_2$  ( $0.02783$  at  $26 \text{ }^\circ\text{C}$ ),  $p$  is the vapor pressure of water ( $25.09 \text{ mm Hg}$  at  $26 \text{ }^\circ\text{C}$ ),  $P$  is the barometric pressure, and  $r\%$  is the percentage of oxygen in the air.

#### 2.6. Hydrogen peroxide consumption experiments

Catalase activity was assayed polarographically by monitoring the conversion of hydrogen peroxide into oxygen with a Clark type oxygen electrode (5300A). In a typical experiment, the  $\text{H}_2\text{O}_2$  solution was thermostated and continually stirred, and upon attaining a steady baseline, the Cu(II)-RSSR complexes were added. The oxygen concentrations along time were collected and the initial rates calculated by linear regression using data from the first 5 min of the reaction. The effects of the Cu(II)-RSSR complexes on the level of oxygen were tested in a range of concentrations (which depended on each complex), following their addition to a solution (conserved at  $26.0 \pm 0.1 \text{ }^\circ\text{C}$ ) containing hydrogen peroxide ( $2 \text{ mM}$ ) in phosphate buffer ( $20 \text{ mM}$ ) at pH 7.4.

#### 2.7. Isolation of mitochondria from rat duodenum epithelium

Mitochondria were isolated from rat duodenum epithelium by differential centrifugation as described previously [29]. Briefly, frozen samples of rat epithelium duodenum were sonicated for 30 min, and centrifuged ( $14,000 \text{ g}$  at  $4 \text{ }^\circ\text{C}$  for 10 min). The pellet was resuspended in a buffer consisting of  $250 \text{ mM}$  sucrose,  $1 \text{ mM}$  EDTA (ethylene diamine tetraacetic acid),  $10 \text{ mM}$  HEPES and  $1 \text{ mg/mL}$  BSA (fraction V), pH 7.4 and homogenized in a Teflon homogenizer. The homogenate was centrifuged ( $2000 \text{ g}$  at  $4 \text{ }^\circ\text{C}$  for 10 min), the supernatant kept aside and the pellet re-extracted as above. Finally, the two supernatants were combined and centrifuged ( $14,000 \text{ g}$  at  $4 \text{ }^\circ\text{C}$  for 10 min). The resulting pellet was resuspended in a buffer containing  $250 \text{ mM}$  sucrose,  $1 \text{ mM}$  EDTA and  $10 \text{ mM}$  HEPES and kept at  $4 \text{ }^\circ\text{C}$  for further experiments.

#### 2.8. Mitochondrial superoxide production assay

Mitochondrial superoxide production was monitored as the oxidation of the fluorescent probe dihydroethidium, as reported previously [30]. Briefly, rat duodenal epithelium-isolated mitochondria were incubated for 30 min with DHE ( $10 \mu\text{M}$ ). After incubation, mitochondria were centrifuged for 10 min ( $14,000 \text{ g}$ ,  $4 \text{ }^\circ\text{C}$ ) and resuspended in phosphate buffer ( $20 \text{ mM}$ , pH 7.4) containing NADH ( $65 \mu\text{M}$ ), coenzyme Q ( $32.5 \mu\text{M}$ ), diclofenac ( $500 \mu\text{M}$ ) and each of the corresponding Cu(II)-RSSR complexes. After 20 min, mitochondria were centrifuged again ( $14,000 \text{ g}$  at  $4 \text{ }^\circ\text{C}$  for 10 min) and resuspended in Triton X-100 ( $0.03\%$  in phosphate buffered saline) in order to lysate them. Ten minutes after the addition of Triton, the fluorescence at  $470\text{Ex}/590\text{Em}$  was quantified. Protein content was also measured according to the method of Bradford [31].

#### 2.9. Data expression and analysis

Data points represent the means of at least 3 independent experiments, each conducted in triplicate. For the sake of simplicity and since the standard deviation values represented less than 5% of the means, these were omitted from Figs. 1–4. In the case of Fig. 5 (plotted as bar graphs), however, since some of the means exhibited standard deviations greater than 5%, the latter were included. When evaluated, statistical significance of the difference between points was assessed using the Student's  $t$  test. Differences at  $p < 0.05$  were considered significant. GraphPad Prism 4 was used as statistical software.

### 3. Results and discussion

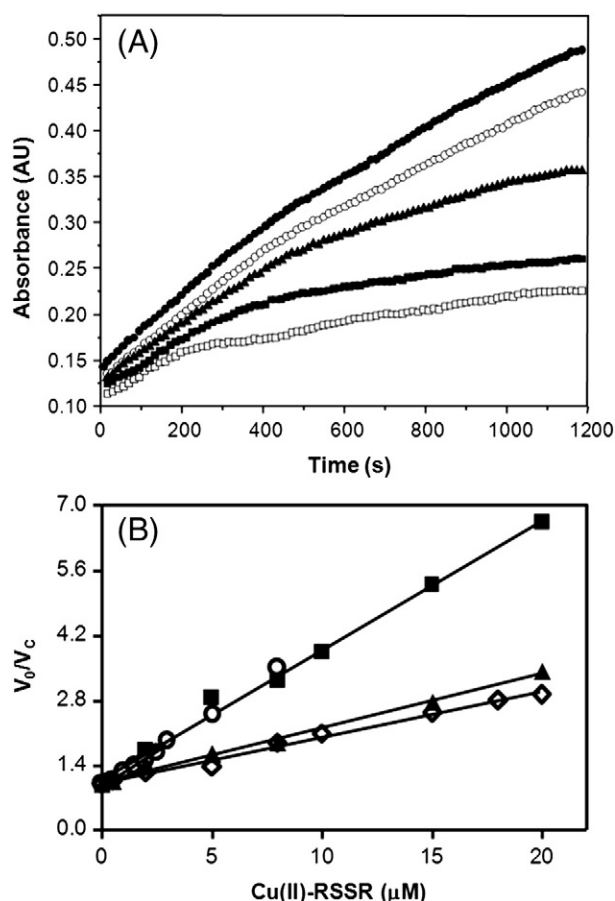
#### 3.1. Ability of the Cu(II)-RSSR complexes to remove superoxide

To evaluate the ability of the complexes formed between Cu(II) ions and the disulfides GSSG, Cyss, HCYss and ALA (Scheme 1) to interact with superoxide, we first determined the kinetic rate constant of their reaction. The kinetic rate constants ( $k_{\text{Cu(II)-RSSR}}$ ) were determined by competition kinetics using Cyt  $c$  as the target molecule and a mixture of xanthine plus xanthine oxidase (X/XO) as a standard system to generate superoxide [24].

Fig. 1(A) depicts a concentration-dependent ( $0.5$  to  $3.0 \mu\text{M}$ ) inhibitory effect of the Cu(II)-GSSG complex on the reduction of Cyt  $c$  induced by X/XO. A similar concentration-dependent behavior was seen when the three other Cu(II)-RSSR complexes were tested (data not shown). For the kinetics analysis, the inhibition of Cyt  $c$  ( $100 \mu\text{M}$ ) reduction was assessed as previously described by [32]. The initial rates were calculated in the presence ( $V_C$ ) and absence ( $V_0$ ) of the Cu(II)-RSSR complexes. As shown in Fig. 1(B), a plot of  $V_0/V_C$  versus the concentration of each complex yielded a straight line, with a slope  $k_{\text{Cu(II)-RSSR}}/k_{\text{Cyt}c}$  [Cyt  $c$ ], according to Eq. (2).

$$\frac{V_0}{V_C} = 1 + \frac{k_{\text{Cu(II)-RSSR}}[\text{Cu(II)-RSSR}]}{k_{\text{Cyt}c}[\text{Cyt}c]} \quad (2)$$

The terms  $k_{\text{Cyt}c}$  and  $k_{\text{Cu(II)-RSSR}}$  are the kinetic rate constants for the reaction between superoxide and Cyt  $c$  ( $2.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ) [33] and that between superoxide and Cu(II)-RSSR, respectively. The kinetic rate constant values  $k_{\text{Cu(II)-RSSR}}$  were calculated from data shown in Fig. 1(B) and are summarized in Table 1. Although the estimated rate constants values are three orders of magnitude lower than those obtained for the reaction between SOD and superoxide ( $\approx 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ) [34], they are comparable to those reported for well-characterized, biologically active (manganese-based) SOD mimetics like EUK-8 ( $k \sim 8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ) [35] and MnTMAP ( $k \sim 2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ) [36]. For comparison purposes we also determined the SOD-like activity of the known SOD mimetic MnTBAP [37]. As shown in Table 1 the kinetic rate constant for the reaction between



**Fig. 1.** (A). Effect of increasing concentrations of Cu(II)-GSSG on the reduction of cytochrome *c* (Cyt *c*) induced by a xanthine/xanthine oxidase system. Cyt *c* (100  $\mu\text{M}$ ) was incubated with xanthine (500  $\mu\text{M}$ ) and xanthine oxidase (1.37 mU/mL). The increase in absorbance at 550 nm, due to the reduction of Cyt *c*, was registered during 20 min. The symbols represent the results obtained in the absence ( $\bullet$ ), or presence ( $\circ$ ) 0.5  $\mu\text{M}$ , ( $\blacktriangle$ ) 1.5  $\mu\text{M}$ , ( $\blacksquare$ ) 2.0  $\mu\text{M}$  and ( $\square$ ) 3.0  $\mu\text{M}$  of the complex. (B). Plot of  $V_0/V_c$  vs. Cu(II)-RSSR concentration ( $\mu\text{M}$ ) for each of the tested complexes. The symbols represent the results of experiments conducted in the presence of the tested complexes: ( $\diamond$ ) Cu(II)-Cyss, ( $\blacktriangle$ ) Cu(II)-HCyss, ( $\circ$ ) Cu(II)-GSSG and ( $\blacksquare$ ) Cu(II)-ALA.

**Table 1**

Kinetic rate constants ( $k_{\text{Cu(II)-RSSR}}$ ) and  $I_{50}$  values for the reaction between superoxide and the Cu(II)-RSSR complexes, prepared at three different molar ratios. The rate constants were determined by competition kinetics using cytochrome *c* (Cyt *c*) as the target molecule and a mixture of xanthine plus xanthine oxidase, and estimated using Eq. (2). The  $I_{50}$  values correspond to the concentration of complex for which  $V_0 = 2V_c$ , as described in the text.

Cu(II)-RSSR complexes	$k_{\text{Cu(II)-RSSR}}$ ( $\text{M}^{-1} \text{s}^{-1}$ )	$I_{50}$ ( $\mu\text{M}$ )
<i>Metal to disulfide ratio of 1:1</i>		
Cu(II)-GSSG	$7.8 \times 10^6$	$3.6 (\pm 0.1)$
Cu(II)-Cyss	$2.6 \times 10^6$	$9.9 (\pm 0.2)$
Cu(II)-HCyss	$3.1 \times 10^6$	$9.7 (\pm 0.1)$
Cu(II)-ALA	$7.3 \times 10^6$	$3.3 (\pm 0.1)$
<i>Metal to disulfide ratio of 1:2</i>		
Cu(II)-GSSG	$6.2 \times 10^6$	$4.6 (\pm 0.2)$
Cu(II)-Cyss	$1.9 \times 10^6$	$10.3 (\pm 0.1)$
Cu(II)-HCyss	$1.4 \times 10^6$	$17.9 (\pm 0.2)$
Cu(II)-ALA	$6.1 \times 10^6$	$4.2 (\pm 0.1)$
<i>Metal to disulfide ratio of 1:3</i>		
Cu(II)-GSSG	$6.0 \times 10^6$	$5.1 (\pm 0.1)$
Cu(II)-Cyss	$1.8 \times 10^6$	$10.8 (\pm 0.2)$
Cu(II)-HCyss	$2.3 \times 10^6$	$10.3 (\pm 0.1)$
Cu(II):ALA	$7.3 \times 10^6$	$4.3 (\pm 0.2)$
<i>Others</i>		
MnTBAP	$9.4 \times 10^5$	$52.9 (\pm 0.2)$

superoxide and MnTBAP ( $9.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ) was found to be very similar to that reported in the literature ( $k \sim 1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ) [38]. Noteworthy, the kinetic rate constant value estimated in the present study for MnTBAP was always lower than the values estimated for each of the studied Cu(II)-RSSR complexes. In addition to the kinetic rate constants, Table 1 provides data on the ability of the Cu(II)-RSSR complexes to inhibit by 50% the rate of reduction of Cyt *c* ( $I_{50}$ ). The  $I_{50}$  value is the concentration of complex for which  $V_0 = 2V_c$ . Comparatively, the SOD-like activities (expressed in terms of  $I_{50}$ ) of these complexes were high and close to the  $I_{50}$  values reported for other analogous Cu(II)-containing complexes [39–41]. As shown in Table 1, the Cu(II)-GSSG and Cu(II)-ALA complexes were the most active in terms of their  $k_{\text{Cu(II)-RSSR}}$  and  $I_{50}$  values. Understanding the structure-related determinants on which reside the higher activity of these two Cu(II)-RSSR complex is beyond the scope of the present work. However, as suggested by Ramadan [41] for other Cu(II)-containing complexes, it is likely that for a given Cu(II)-RSSR complex, a greater activity could relate to a more favorable accessibility for the superoxide anions and to a higher flexibility of the ligands around copper(II) to allow a facile reduction to copper(I).

Under biologically-relevant conditions, the concentration of (free) Cu(II) ions is expected to be very low [42,43] and always below that of any of the here studied disulfides [44,45]. Thus, we extended the characterization of the interaction between superoxide and the Cu(II)-RSSR complexes to mixtures where the disulfides occur in a slight molar excess (2:1 and 3:1) see Supplementary data, Figs. S1–S2. According to the results on the inhibition of Cyt *c* reduction for the tested molar ratios no significant differences are observed in the  $k_{\text{Cu(II)-RSSR}}$  and  $I_{50}$  values (Table 1).

### 3.2. Ability of the Cu(II)-RSSR complexes to generate hydrogen peroxide

Compounds with authentic SOD-like activity not only should interact with superoxide but also catalyze the generation of  $\text{H}_2\text{O}_2$  and the regeneration of  $\text{O}_2$  during the dismutation reaction. Thus, we assessed the formation of the two latter species during the reaction of superoxide with each of the tested Cu(II)-RSSR complexes.

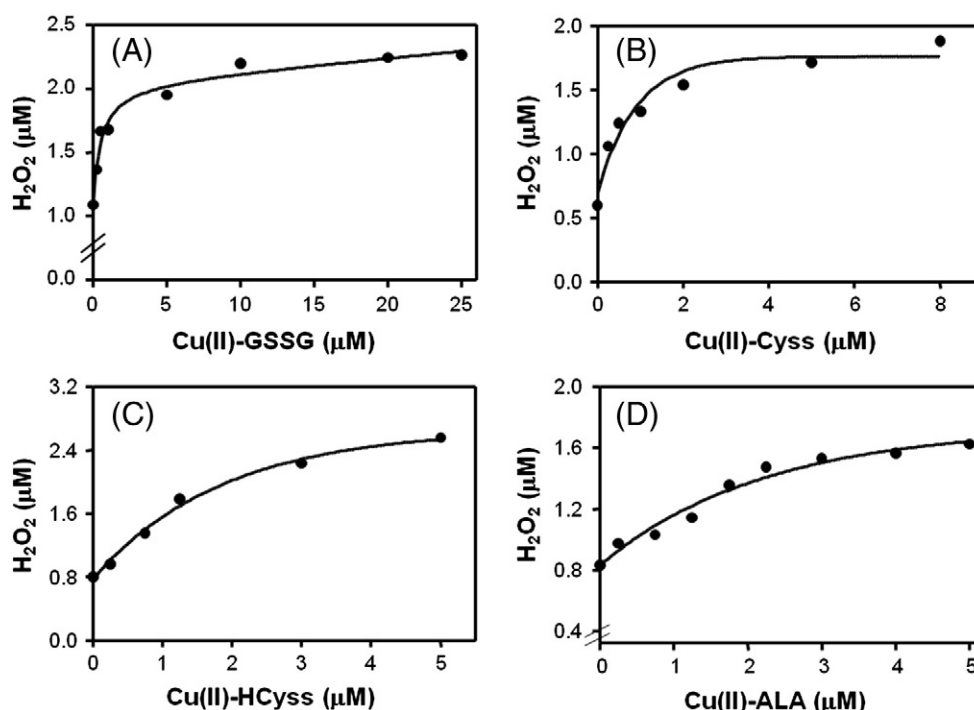
As shown in Fig. 2 (A–D), the addition of increasing concentrations of each of the complexes to a solution containing X/XO incremented, in all cases, concentration-dependently the basal levels of  $\text{H}_2\text{O}_2$ . In the case of the Cu(II)-GSSG (Fig. 2(A)), an apparently lower  $\text{H}_2\text{O}_2$ -generating efficiency was associated with concentrations of the complex greater than 5  $\mu\text{M}$ . No increment in the production of  $\text{H}_2\text{O}_2$  was evident for the 10–25  $\mu\text{M}$  concentration range. A comparable loss of  $\text{H}_2\text{O}_2$ -generating efficiency can also be observed for the three other complexes.  $\text{H}_2\text{O}_2$  production associated to the highest concentrations of the four studied complexes was substantially abolished (by over 80%) by catalase (not shown).

In line with the above-seen absence of differences in the capacity of the Cu(II)-containing complexes to inhibit the reduction of Cyt *c* when these were prepared using a slight molar excess of the disulfides (2:1 and 3:1), no differences were found when the same complexes (at a 1  $\mu\text{M}$  concentration) are compared, in terms of their capacity to generate  $\text{H}_2\text{O}_2$  (see Supplementary data, Table S3). It should be noted, however, that at such low micromolar concentration, the complexes are already able to generate a concentration of  $\text{H}_2\text{O}_2$  that is over half that induced by the addition of SOD (1000 U/well). While the latter comparison may simply reflect the higher kinetic rate constant of SOD towards superoxide, the comparatively lower concentration of  $\text{H}_2\text{O}_2$  associated with the complexes might also reflect a putative  $\text{H}_2\text{O}_2$ -degrading activity of the latter (this aspect is experimentally supported and discussed below).

### 3.3. Ability of the Cu(II)-RSSR complexes to generate oxygen

To further support the proposed capacity of the Cu(II)-RSSR complexes to act as SOD mimetic, we measured the levels of  $\text{O}_2$  generated

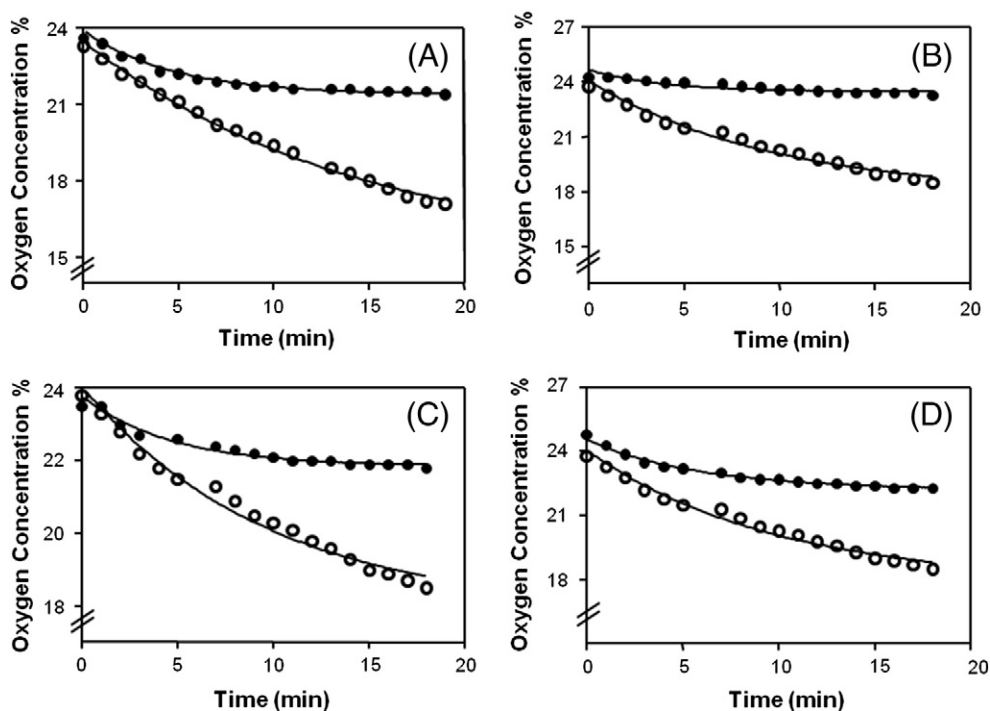




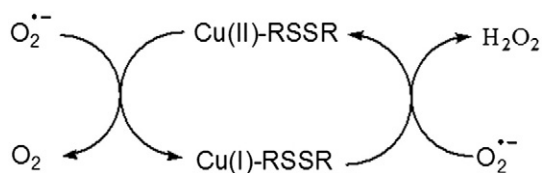
**Fig. 2.** Effect of the Cu(II)–RSSR complexes on the generation of hydrogen peroxide. Mixtures of solutions (prepared in phosphate buffer, pH 7.4) containing increasing concentrations of the Cu(II)–RSSR complexes and a mixture of X plus (XO) were incubated for 5 min (25 °C). Subsequently, the increase in OD at 560 nm was registered. Results are expressed as  $\text{H}_2\text{O}_2$  concentration ( $\mu\text{M}$ ). The graphs represent the results of each of the complexes, assessed at a different concentration range: (A) Cu(II)–GSSG (0.25–25  $\mu\text{M}$ ); (B) Cu(II)–ALA (0.25–8.0  $\mu\text{M}$ ); (C) Cu(II)–Cys (0.25–5.0  $\mu\text{M}$ ) and (D) Cu(II)–HCys (0.25–5.0  $\mu\text{M}$ ).

by these complexes during their reaction with superoxide. As shown in Fig. 3 (A–D), the sole addition of X/XO to a Clark-cuvette time-dependently lowered the percentage of oxygen concentration from an initial average value of 24% (288  $\mu\text{M}$ ) to approximately 17% (204  $\mu\text{M}$ )

after 20 min. As expected, addition of SOD to such system led to a slowing of the decay; the percentage of oxygen concentration reached after 20 min was near 20% (not shown). When the tested complexes were added (each at their most efficient superoxide-dismutating



**Fig. 3.** Changes in oxygen concentration induced by a solution containing X/XO and Cu(II)–RSSR complexes. Oxygen concentration was continuously monitored (0–20 min) in a phosphate buffer solution (pH 7.4) containing a mixture of 500  $\mu\text{M}$  of X plus 4.26 mU/mL of XO both, in the absence (open circles) and in the presence of Cu(II)–RSSR (close circles). Five minutes after the addition of X/XO the complexes were added (namely time zero). Results are expressed as oxygen concentration (%). The graphs represent the results of each of the complexes, assessed at different concentrations: (A) Cu(II)–GSSG (5  $\mu\text{M}$ ); (B) Cu(II)–ALA (2  $\mu\text{M}$ ); (C) Cu(II)–Cys (2  $\mu\text{M}$ ) and (D) Cu(II)–HCys (1.25  $\mu\text{M}$ ).



**Scheme 2.** Proposed mechanism for the dismutation of superoxide induced by the various Cu(II)-RSSR complexes.

**Table 2**

Recovery of oxygen after the interaction between the tested Cu(II)-RSSR complexes superoxide radicals generated by a X/XO system. The number of micromoles of molecular oxygen lost was estimated according to the Eq. (1) (see Experimental section) using data from Fig. 3. Percentages of oxygen recovery were estimated after multiplying by 100 the value that results from subtracting the ratio between the number of micromoles of oxygen lost in the presence and absence of each of the tested Cu(II)-RSSR complexes from 1.0.

Compounds tested	Loss of oxygen ( $\mu\text{mol}$ ) in the absence of Cu(II)-RSSR	Loss of oxygen ( $\mu\text{mol}$ ) in the presence of Cu(II)-RSSR	Recovery of oxygen (%)
Cu(II)-GSSG	0.20	0.08	60.0
Cu(II)-ALA	0.24	0.10	58.3
Cu(II)-HCyss	0.24	0.11	54.2
Cu(II)-Cyss	0.12	0.04	66.7
SOD	0.16	0.08	50.0

concentration; as from data in Fig. 2), a significant slowing of the rate of decay of oxygen concentration was also observed. As known, SOD converts 2 mol of superoxide into 1 mol of molecular oxygen (Rx. 1).



Thus, if the complexes are acting as SOD mimetic-like molecules, their maximal slowing effect on the decay of oxygen would not be expected to surpass a recovery of 50% of the X/XO induced loss oxygen

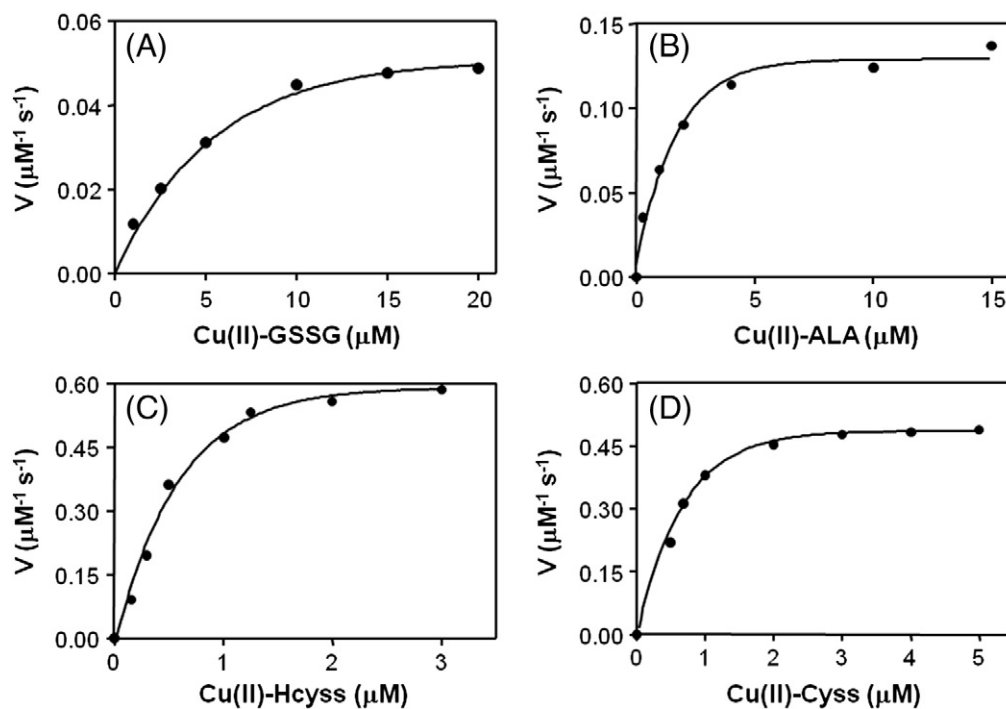
molecules. As a possible mechanism for the here-proposed superoxide dismutating action of the Cu(II)-RSSR complexes, we postulate a redox cycling reaction where, initially, the Cu(II) ion contained in each of the complexes would undergo a reduction by 1 mol of superoxide, to form 1 mol of molecular oxygen and 1 mol of a Cu(I)-RSSR complex intermediate (Scheme 2). Subsequently, the latter species would be readily oxidized by a second mole of superoxide, to regenerate the Cu(II)-RSSR complex and form 1 mol of hydrogen peroxide.

Based on data plotted in Fig. 3 (A–D), we estimated the percentages of recovery of molecular oxygen that followed the addition of each of the complexes to the X/XO containing system (Table 2). Interestingly, the recoveries of oxygen (i.e.; the slowing in the oxygen decay curves) were, in all cases, larger than 50%. While the maximal oxygen recovery effect (of near 67%) was seen for the Cu(II)-Cyss complex, and minimal recovery effect (of 54%) was seen for the Cu(II)-HCyss complex. Since the actual recovery of oxygen was estimated to surpass the 50% expected from Rx. 1, we decided to evaluate the possibility that these complexes may, in addition to their SOD-like action, display also a catalase-like activity.

#### 3.4. Ability of the Cu(II)-RSSR complexes to react with hydrogen peroxide

To distinguish between a putative  $\text{H}_2\text{O}_2$ -degrading activity of the Cu(II)-RSSR complexes, as suggested by data from Fig. 2, where a lower  $\text{H}_2\text{O}_2$ -generating efficiency was observed at the higher concentrations of the complexes, and a putative catalase-like activity of the same complexes, as suggested by the above-50% recoveries of oxygen from superoxide (Table 2), we directly assessed the ability of the complexes to convert hydrogen peroxide into molecular oxygen. Towards such end, the initial rates of oxygen production for the reaction between  $\text{H}_2\text{O}_2$  and Cu(II)-RSSR (disproportion reactions) were measured under pseudo first-order conditions, in excess of  $\text{H}_2\text{O}_2$  (2 mM).

As seen in Fig. 4 (A–D), the initial rates of oxygen production ( $v$ ) show a lineal behavior for the lower range of concentrations of each of the complexes [Cu(II)-RSSR], suggesting a first-order dependence for

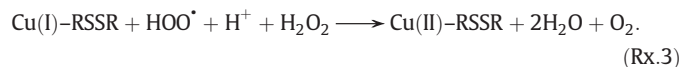


**Fig. 4.** Effect of Cu(II)-RSSR complexes on the initial rate of conversion of  $\text{H}_2\text{O}_2$  into oxygen. Cu(II)-RSSR complexes were added to solutions containing a fixed concentration of hydrogen peroxide (2 mM). The increase in the oxygen concentration resulting from the decomposition of  $\text{H}_2\text{O}_2$  was registered using a Clark electrode and the results are expressed as initial rates. Further details are described in the Experimental section. The graphs represent the results of each of the complexes: (A) Cu(II)-GSSG; (B) Cu(II)-ALA; (C) Cu(II)-HCyss and (D) Cu(II)-Cyss.

their reaction with  $\text{H}_2\text{O}_2$ . In turn, at the higher concentration range, the reaction rates became independent of the concentration of the complexes. When comparing each of the Cu(II)–RSSR complexes, at a concentration of  $1 \mu\text{M}$ , differences of over thirty-fold were estimated in the initial oxygen production rates. For instance, while for the Cu(II)–GSSG complex, the  $v$  value is  $0.012 \mu\text{M/s}$ , a  $\approx 0.4 \mu\text{M/s}$  value is estimated for the Cu(II)–Cyss complex. Interestingly, Cu(II)–Cyss was also found to be the most active in terms of its ability to promote the recovery of oxygen (67%, as shown in Table 2). However, since in the reaction of conversion of superoxide into oxygen, the Cu(II)–Cyss complex presented the lowest kinetic rate constant value ( $k$ ; Table 1), we suggest that the high recovery of oxygen (seen for this complex in Table 2) would, presumably reflect, a greater catalase-like compared to a SOD-like activity. In contrast, the complexes Cu(II)–GSSG and Cu(II)–ALA, which showed the highest rate constant values in their reaction with superoxide (Table 1), were found to exhibit the lowest initial rate values in their reaction with  $\text{H}_2\text{O}_2$  (Fig. 4). Such results suggest that a SOD-mimetic activity would predominate in the action of these two complexes.

Interestingly, we observed that when the ability of the complexes to convert hydrogen peroxide ( $2 \text{ mM}$ ) into oxygen is assessed at a  $10 \mu\text{M}$  concentration, the initial rate values obtained are closely comparable to those reported when other catalase mimetic complexes [46] were assessed using also  $10 \mu\text{M}$  concentration but a five-fold higher concentration of  $\text{H}_2\text{O}_2$ .

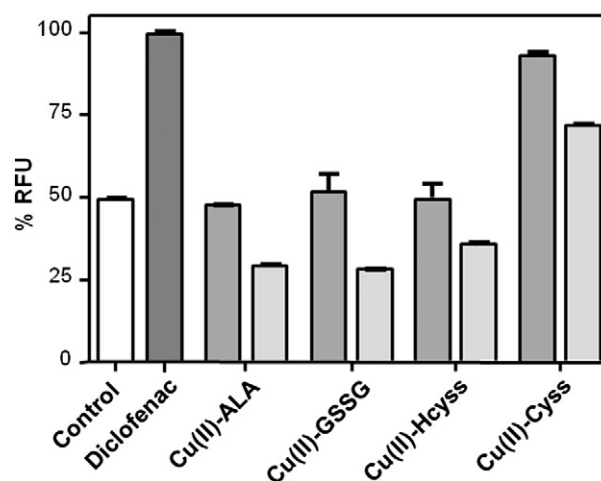
As initially proposed by Ramadan [41] for other metal-containing complexes, as a possible mechanism to explain the disproportionation of  $\text{H}_2\text{O}_2$  by the here-studied Cu(II)-containing complexes, we show below (Rx. 2 and Rx. 3) two redox cycling reactions involving the copper ion:



Establishing the validity of the above-proposed mechanism would be most interesting but is beyond the scope of the present work. Its assessment would demand spectroscopic studies on the transient formation of the Cu(I)–RSSR intermediate (Rx. 2), the conservation of the Cu(II)–RSSR complexes and quantitative recovery studies on the molar disappearance of  $\text{H}_2\text{O}_2$  and generation of molecular oxygen.

### 3.5. Efficacy of the Cu(II)–RSSR complexes on the protection of superoxide-generating mitochondria

To determine whether the tested Cu(II)–RSSR complexes are able to act as SOD mimetic in a biologically-relevant system, their ability to remove superoxide generated within superoxide-producing mitochondria was tested. Towards that end, rat duodenal epithelium isolated mitochondria, pre-loaded with the superoxide-sensitive fluorescent probe dihydroethidium, were incubated with  $500 \mu\text{M}$  diclofenac. As recently reported by us [29], the inhibition of mitochondrial complex I by non-steroidal anti-inflammatory drugs, including diclofenac, markedly elevates the mitochondrial generation of superoxide. Diclofenac-exposed mitochondria were incubated in the absence and presence of different Cu(II)–RSSR concentrations ( $2$  and  $4 \mu\text{M}$ ). As seen in Fig. 5, the basal production of superoxide, which was doubled by diclofenac, was totally restored by the addition of a  $2 \mu\text{M}$  concentration of the following complexes: Cu(II)–ALA, Cu(II)–GSSG and Cu(II)–HCySS. Interestingly, when tested at a  $4 \mu\text{M}$  concentration, these complexes lowered the production superoxide below the basal level. At such concentration, Cu(II)–Cyss was only marginally effective. Based on the above-described ability of the Cu(II)–RSSR complexes to inhibit the reduction of Cyt *c* (Fig. 1) and to convert superoxide into hydrogen peroxide (Fig. 2), we postulate that the effectiveness of these complexes to inhibit DHE oxidation



**Fig. 5.** Protection against DHE-oxidation by the addition of Cu(II)–RSSR to superoxide-generating mitochondria. DHE ( $100 \mu\text{M}$ ) was added to diclofenac ( $500 \mu\text{M}$ ) treated-mitochondria incubated in the absence or presence of the tested Cu(II)–RSSR complexes. The increase in fluorescence intensity at  $580 \text{ nm}$  was registered after  $15 \text{ min}$ . Results were expressed as percentage of Relative Fluorescence Units (RFU). For each Cu(II)–RSSR concentration added: dark gray bars and light gray bars represent solutions containing the complexes in a concentration of  $2 \mu\text{M}$  or  $4 \mu\text{M}$ , respectively.

within mitochondria would be primarily attributable to a SOD mimetic action. Cu(II)–Cyss and Cu(II)–HCySS do not differ in terms of their  $k_{\text{Cu(II)-RSSR}}$  and  $I_{50}$  values (Table 1). Thus, one might speculate that the relatively lower ability of former complex to inhibit DHE oxidation would reflect, rather than differences in their intrinsic SOD mimetic potential, differences in their intra-mitochondrial availability to interact with superoxide. Noteworthy, the effectiveness of Cu(II)–ALA, Cu(II)–GSSG and Cu(II)–HCySS to lower the production of superoxide below the basal level (when tested at a  $4 \mu\text{M}$  concentration) suggests that the SOD mimetic potential of these complexes would comprise the possibility to act also reducing the production of this ROS under physiological conditions. Future work aimed at understanding the precise molecular mechanisms by which Cu(II)–RSSR complexes are able to protect mitochondria is required, and might potentially unravel novel strategies to produce new therapeutic agents that mimic their “mitoprotective” mode of action.

## 4. Conclusions

The complexes formed between copper(II) ions and several biologically-occurring disulfides (RSSR: oxidized glutathione, cystine, homocystine and  $\alpha$ -lipoic acid) were found to simultaneously display superoxide dismutase- and catalase-like activities. The antioxidant properties of these Cu(II)–RSSR complexes were seen at very low micromolar concentrations. Since current evidence indicates that copper(II) ions are unlikely to occur as such within cells, the actual possibility of having the above-referred Cu(II)–RSSR complexes formed intracellularly remains, at this point, as an hypothetical one. However, beyond their debatable intracellular formation, the also here-shown ability of some of these complexes to act as effective antioxidants when added directly onto intact superoxide-generating mitochondria, warrants studying further their potential to act as SOD- and catalase-like molecules under other oxidative-stress conditions.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jinorgbio.2013.09.006>.

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