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# The Cu(I)–glutathione complex: factors affecting its formation and capacity to generate reactive oxygen species

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**Abstract** $Cu^{2+}$ ions and reduced glutathione (GSH) swiftly interact to form the physiologically occurring Cu(I)–$[GSH]_2$ complex. Prompted by the recently reported ability of this complex to generate superoxide radicals from molecular oxygen, the present study addressed how the concentration of $Cu^{2+}$ and GSH, the pH, and the temperature affect the formation of the Cu(I)–$[GSH]_2$ complex and its capacity to generate superoxide radicals and hydrogen peroxide. Increasing concentrations of $Cu^{2+}$ and GSH, added at a fixed molar ratio of 1:3, led to a proportionally greater production of superoxide anions, hydrogen peroxide, and oxidized glutathione (GSSG). GSSG formation was found to closely reflect the formation of Cu(I)–$[GSH]_2$. Biologically relevant changes in pH (e.g., from 6.8 to 7.7) and temperature (from 22 to 37 °C) did not affect the formation of the Cu(I)–$[GSH]_2$, as assessed by GSSG production. However, production of superoxide radicals increased as the pH values were incremented. An opposite effect was observed regarding hydrogen peroxide production. The ability of a freshly prepared Cu(I)–$[GSH]_2$ complex (assayed within a minute from its formation) to generate superoxide radicals was incremented by as the temperature was increased. Such ability, however, correlated inversely with the temperature when, before assaying for superoxide, the earlier referred preparation was incubated during 30 min in the presence of oxygen. Under the latter condition, hydrogen peroxide linearly accumulated in time, suggesting that an increased autodismutation underlies the apparent time-dependent "aging" of the capacity of the complex to generate superoxide.

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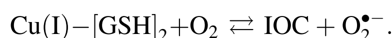
## Introduction

Cu(I)-glutathione is a biologically occurring complex that forms from the interaction between $Cu^{2+}$ ions entering the cell and cytosolic molecules of reduced glutathione (GSH) [1–3]. In fact, following the exposure of either hepatoma cells (HAC) [1, 2] or intestinal epithelial cells (Caco-2) [3] to high concentrations of copper, most of the metal is recovered bound to GSH molecules, probably forming a Cu(I)–$[GSH]_2$ complex. Although the biological role of this complex has not been fully established, it appears to function as a Cu(I)-carrier to cupro-enzymes such as Cu, Zn-superoxide dismutase, and ceruloplasmin [4, 5], and also to the copper-storing protein, metallothionein [1–3, 6].

In non-cellular systems, the Cu(I)–$[GSH]_2$ complex swiftly forms when $Cu^{2+}$ ions and GSH are mixed in a molar ratio equal to or greater than 1:3 [5, 7–11]. Its formation has been characterized by $^1$H-NMR (nuclear magnetic resonance) and by EPR (electron paramagnetic resonance) techniques [5, 8].

The Cu(I)–glutathione complex was for long thought to be redox-inactive toward molecular oxygen [5, 12–14]. Recent studies [15, 16], however, have revealed that, rather than being redox-inactive, the Cu(I)–$[GSH]_2$ complex does react with oxygen, behaving as a continuous source of

superoxide radicals $\left(O_2^{\bullet-}\right)$. Its interaction with oxygen appears to imply a reversible reaction, in which superoxide is formed from oxygen at the expense of oxidizing the Cu(I)–$[GSH]_2$ complex, and that the latter form of the complex (an intermediate oxidized form; IOC) would be able to react back rapidly with $O_2^{\bullet-}$ to regenerate molecular oxygen and the reduced Cu(I)–$[GSH]_2$ complex. This concept is represented by the following reaction



$$Cu(I)-[GSH]_2+O_2 \rightleftarrows IOC + O_2^{\bullet-}.$$

However, as can be anticipated from the above mentioned reaction, the regeneration of Cu(I)–$[GSH]_2$ from its putative IOC (reversibility of the reaction) will take place only if the experimental conditions do not favor the autodismutation of the $O_2^{\bullet-}$ formed in the reaction. Autodismutation, which disturbs the original equilibrium and displaces the afore mentioned reaction toward the right, would lead to the formation of hydrogen peroxide ($H_2O_2$). As such, the Cu(I)-containing Cu(I)–$[GSH]_2$ complex is unable to catalyze the reduction of the $H_2O_2$ molecules, generated during the autodismutation of superoxide, into hydroxyl radicals [15]. Its ability to generate superoxide, and under some conditions hydrogen peroxide, is, however, of potential toxicological interest since the latter species are capable of interacting with a large number of biologically relevant target molecules [17–20].

In view of the potential biological and toxicological importance of the ability of the Cu(I)–$[GSH]_2$ complex to generate superoxide radicals and hydrogen peroxide, in the present study, we address some of the physicochemical factors that can affect both the formation of such complex and the generation of the two oxidizing species referred earlier, superoxide and hydrogen peroxide. Specifically, we investigate the influence that factors such as the initial concentration of the complex, time elapsed after its preparation, and pH and temperature of the media have on the generation of such oxidizing species.

## Experimental

### Chemical reagents

Acetaminophen, cupric chloride ($CuCl_2 \times 2H_2O$), cytochrome *c* (Cyt *c*; bovine heart), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR; EC 1.6.4.2 from baker's yeast), bis-*N*-methylacridinium nitrate (lucigenin), $\beta$-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), catalase (CAT; EC 1.11.1.6 from bovine liver), superoxide dismutase (SOD; EC 1.15.1.1 from bovine erythrocytes), and dimethylsulfoxide (DMSO) were all purchased from Sigma–Aldrich. Hydrogen peroxide (30% w/w) was purchased from Merck. Unless indicated otherwise, all solutions employed in this study were prepared in Chelex-100-treated sodium phosphate buffer (20 mM).

### Preparation of the Cu(I)–$[GSH]_2$ complex

The Cu(I)–$[GSH]_2$ complex was prepared as previously described [15, 16] by mixing $CuCl_2$ and GSH in a 1:3 molar ratio, respectively. Whenever referring to a given concentration of such complex, it should be understood that it reflects the concentration of copper used in its preparation.

### Determination of oxidized glutathione

GSSG was quantified as described by Tietze [21], employing the NADPH/glutathione reductase assay. The decay in $OD_{340nm}$ associated with the formation of $\beta$-Nicotinamide adenine dinucleotide 2′-phosphate, oxidized form ($NADP^+$), was monitored at 30 °C using an Unicam He$\lambda$ios $\alpha$ spectrophotometer.

The assay was initiated after the addition of samples containing freshly prepared NADPH (0.2 mM) and glutathione reductase (GR, 2 U/mL) to a set of cuvettes containing increasing concentrations of a fixed molar ratio 1:3 of $[Cu^{2+}]$/[GSH], from 4/12 to 50/150 μM. Results were estimated using a molar absorption coefficient of 6.22 $mM^{-1}$ $cm^{-1}$ [22] and expressed as micromolar concentration of GSSG. Control was carried out using GSSG.

### Assays for superoxide radical detection

#### *Cytochrome c reduction assay*

Superoxide radical was detected as described by Van Gelder et al. [23], employing Cytochrome *c* (Cyt *c*) as a sensitive and specific probe for this free radical. The increase in $OD_{550nm}$ associated with the reduction of Cyt *c* was monitored at 30 °C in a 96-well plate using a Multi-Mode Microplate Reader (Synergy$^{TM}$ HT), as carried out previously by Carrasco-Pozo et al. [24]. The assay was initiated after the addition of samples containing increasing concentrations of the Cu(I)–$[GSH]_2$ complex (4–50 μM) to a solution containing Cyt *c* (50 μM). Results were estimated using a molar absorption coefficient of 21.1 $mM^{-1}$ $cm^{-1}$ [23] and expressed as micromolar concentration of superoxide produced. The exceptions are for Fig. 2 and Table 1, where the results are expressed as initial rate of Cyt *c* reduction (μM/s and μM/min, respectively). Control experiments were carried out using GSH instead of the complex.

*Lucigenin chemiluminescence assay*

Superoxide radical was also detected as described by McCord and Fridovich [25], employing Lucigenin (Luc), as chemiluminescent probe. The luminescence obtained associated with the reduction of Luc, was registered at 22 s intervals, for a counting period of 112 s and was monitored at 30 °C in a 96-well plate using a Multi-Mode Microplate Reader (Synergy™ HT), as carried out previously by Carrasco-Pozo et al. [24]. The assay was initiated after the addition of Luc (15 µM) to a set of solutions containing increasing concentrations of the Cu(I)–$[GSH]_2$ complex (4–50 µM). It is important to note that a low concentration of Luc was used, to avoid its redox cycling, as reported previously by Skatchkov et al. [26].

Results were expressed as delta area under the curve ($\Delta AUC = AUC_{complex} - AUC_{basal}$). Control experiments were carried out using GSH instead of the complex.

Assays for determination of hydrogen peroxide

*Reflectoquant assay*

Hydrogen peroxide concentration was determined by Reflectoquant Peroxide test strips (Merck, Germany). The assay was initiated after the immersion of the reaction zone of the analytical test strip in the solution containing the Cu(I)–$[GSH]_2$ complex (from 4 to 50 µM) for 2 s, at 25 °C. The formation of $H_2O_2$ was assessed from 5 to 150 min after the preparation of the complex. The results were obtained in mg/L and expressed as micromolar concentration. Stock solutions of $H_2O_2$ were used to calibrate the Reflectoquant assay.

*Fluorimetric assay*

Hydrogen peroxide ($H_2O_2$) was also quantified as described by Jie et al. [27], employing acetaminophen as hydrogen peroxide reacting agent. The fluorescence obtained (excitation and emission wavelengths were 298 and 333 nm, respectively), associated with the oxidation of acetaminophen in acidic medium, was monitored at 30 °C in a 96-well plate using a Multi-Mode Microplate Reader (Synergy™ HT). The assay was initiated after the addition to a 10-mL volumetric flask of 1 mL of acetaminophen (0.01 M), 0.6 mL of sulfuric acid (2 M), and 5 mL of a solution containing either the Cu(I)–$[GSH]_2$ complex (4–40 µM) or $H_2O_2$ (2–50 µM). The flask was placed in a boiling-water bath for 40 min. After cooling, the final volume of 10 mL was attained with water and the fluorescence was measured. Results were obtained as delta relative fluorescence units ($\Delta RFU = RFU_{complex} - RFU_{basal}$) and expressed as micromolar concentration, using $H_2O_2$ as standard. The only exception is that of Table 1, which the results are expressed as initial rate of acetaminophen oxidation (µM/min).

**Table 1** The relationship between increasing concentrations of the Cu(I)–$[GSH]_2$ complex (4–50 µm) and the initial rate of Cyt *c* reduction and acetaminophen oxidation

| Concentration of Cu(I)–$[GSH]_2$ (µM) | Initial rate of reduction of Cyt *c* (µM/min) | Initial rate of oxidation acetaminophen (µM/min) |
|---|---|---|
| 4 | 1.9 | 0.043 |
| 6 | 7.8 | 0.049 |
| 8 | 12.5 | 0.056 |
| 10 | 18.7 | 0.069 |
| 20 | 21.3 | 0.092 |
| 30 | 23.5 | 0.156 |
| 40 | 25.4 | 0.188 |
| 50 | 26.1 | – |

Data expression and analysis

Data points in the figures and values in the tables represent the mean of at least three independent experiments, each conducted in quadruplicate. The SD of such data is not included as these generally represented less than 10% of the mean. When appropriate, data were processed by an analysis of variance (ANOVA), and statistical significance was evaluated using the Student's *t*-test. Differences at $p < 0.05$ were considered to be significant. GraphPad Prism 4 was used as statistical software.

## Results and discussion

Effect of increasing concentrations of a fixed molar ratio of $Cu^{2+}$ ions and GSH on the formation of the Cu(I)–glutathione complex

To further characterize the reaction between copper ions ($Cu^{2+}$) and reduced glutathione (GSH) in a fixed molar ratio 1:3, respectively, we addressed the relationship between the concentration of these species and the generation of oxidized glutathione (GSSG), as a form to evaluate the formation of the Cu(I)–$[GSH]_2$ complex [15, 16].

As shown in Fig. 1, increasing concentrations of a fixed molar ratio 1:3 $[Cu^{2+}]$/[GSH] (from 4/12 to 50/150 µM) resulted in linearly proportional increments in the concentration of GSSG, measured through the oxidation of NADPH in the presence of GR. Specifically, data from Fig. 1 indicate that 1 mol of the mixture $Cu^{2+}$ plus GSH

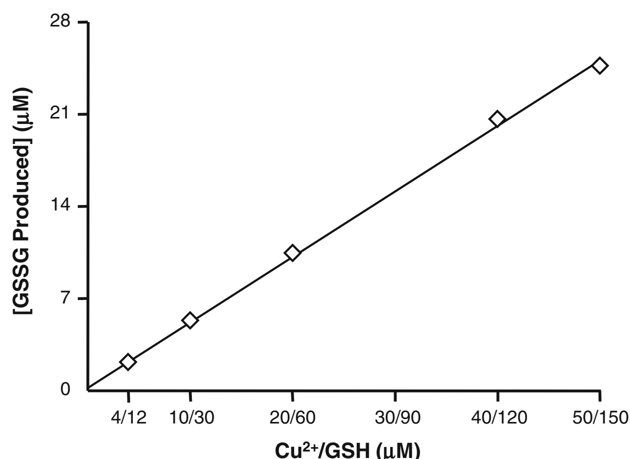



**Fig. 1** Oxidized glutathione formation induced by increasing concentrations of $Cu^{2+}$/GSH. The effect of increasing concentrations of a fixed molar ratio $Cu^{2+}$ and GSH, 1:3, respectively (4/12–50/150 µM), on the levels of production of oxidized glutathione (GSSG) was evaluated using a NADPH/GR system. Results are expressed as micromolar concentration of GSSG

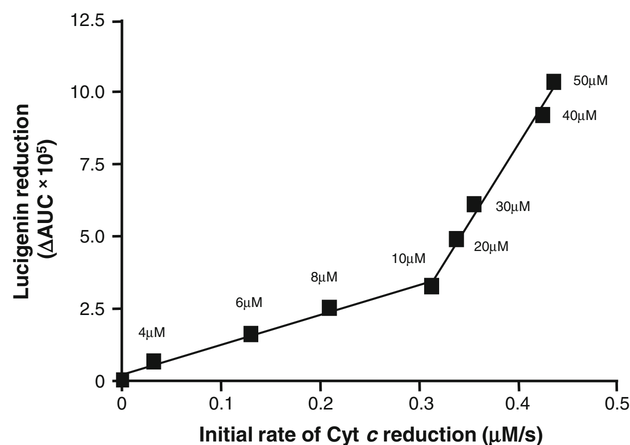



**Fig. 2** Correlation between the initial rates of Cyt *c* reduction and lucigenin-dependent chemiluminescence to determine production of superoxide radicals by increasing concentrations of the Cu(I)–$[GSH]_2$ complex. Cyt *c* (50 µM) or lucigenin (15 µM) were added to solutions containing increasing concentrations of the Cu(I)–$[GSH]_2$ complex (4–50 µM). Results represent the initial rate of Cyt *c* reduction, expressed as (µM/s), and chemiluminescence levels for lucigenin reduction, expressed as the difference in area under the *curve* (ΔAUC), as explained in Experimental section

produces 0.5 mol of GSSG, at 25 °C in a phosphate buffer (pH 7.4).

Such production of GSSG is consistent with the fact that, in order to form 1 mol of the Cu(I)–$[GSH]_2$ complex, 1 mol of $Cu^{2+}$ plus 3 mol of GSH is needed, and as a result of their mixture, 1 mol of GSH would be immediately consumed during the reduction of the added $Cu^{2+}$ [15, 16]. The latter process would result in the formation of 0.5 mol of GSSG.

## Effect of increasing concentrations of the Cu(I)–glutathione complex on its capacity to generate reactive species

Mindful of the recently reported capacity of the Cu(I)–$[GSH]_2$ complex to generate superoxide radicals $\left(O_2^{\bullet -}\right)$ and hydrogen peroxide ($H_2O_2$) [15, 16], we studied the effect of increasing concentrations of the complex on the rate of formation of the reactive species referred to earlier.

The dependence of the formation of $O_2^{\bullet -}$ as a function of the concentration of the Cu(I)–$[GSH]_2$ complex was evaluated using the well-known reductive reaction (SOD inhibited) of $O_2^{\bullet -}$ with both cytochrome *c* (Cyt *c*) [28] and lucigenin (Luc) [29]. Figure 2 shows that the Cu(I)–$[GSH]_2$ complex promotes the reduction of both probes in a concentration-dependent manner. These reductions were totally inhibited by SOD (250 U/mL; not shown). However, in Fig. 2, two phases were clearly observed. The first phase between 4 and 10 µM of the Cu(I)–$[GSH]_2$ complex, and the second one between 10 and 50 µM. Both phases showed good correlations between both methodologies with correlation coefficients of 0.997 and 0.994, for Cyt *c* and Luc assays, respectively (not shown). In spite of the good correlation found, different slopes were estimated for each phase. Interestingly, we found that the slope of the first phase (4 to 10 µM) was almost 20% of the slope of the second phase (10 to 50 µM). As can be seen in Fig. 2 and Table 1, in the lower concentrations of Cu(I)–$[GSH]_2$ complex-range, when the concentration of such complex was increased 2.5-fold (from 4 to 10 µM), the rate of Cyt *c* reduction increased 9.75 times. However, in the second phase, when the concentration of the complex varied in the same ratio (2.5), from 20 to 50 µM, the rate of Cyt *c* reduction increased only 1.22 times. These results would imply that at high Cu(I)–$[GSH]_2$ complex concentrations (10–50 µM), Cyt *c* would be assessed a lower fraction of the $O_2^{\bullet -}$ generated by the complex than that at low Cu(I)–$[GSH]_2$ complex concentrations (4–10 µM).

Taking into account that the autodismutation of superoxide radicals depends on the square of its concentration ($V_{Autodism;}$ Eq. 1; [30]), it would be interesting to speculate that at high Cu(I)–$[GSH]_2$ complex concentrations, the autodismutation process of $O_2^{\bullet -}$ would be more favorable than the reaction between $O_2^{\bullet -}$ and Cyt *c* ($V_{Cyt\ c\text{–}Red;}$ Eq. 2; [31]).

$$V_{Autodismut.} = k_1\left[O_2^{\bullet -}\right]\left[O_2^{\bullet -}\right];\quad k_1 = 2.4\times10^5\ M^{-1}s^{-1} \quad (1)$$

$$V_{Cyt\,c\text{–}Red.} = k_2\left[O_2^{\bullet -}\right][Cyt\,c];\quad k_2 = 2.6\times10^5\ M^{-1}s^{-1} \quad (2)$$

However, this effect would be minimized when the production of $O_2^{\bullet -}$ by the complex was evaluated employing Luc as probe. This is due to the fact that the

rate constant for the reaction of $O_2^{\bullet-}$ with Luc is at least three orders of magnitude ($V_{\text{Luc-Red;}}$ Eq. 3; [32]) larger than that reported for the interaction between $O_2^{\bullet-}$ and Cyt *c*.

$$V_{\text{Luc-Red.}} = k_3\left[O_2^{\bullet-}\right][\text{Luc}]; \quad k_3 = 1.0 \times 10^8\ \text{M}^{-1}\text{s}^{-1} \qquad (3)$$

Thus, the higher slope obtained at higher complex concentrations (Fig. 2) could be explained, at least theoretically, by the large value of rate constant reported for the reaction between Luc and $O_2^{\bullet-}$ in comparison with the autodismutation reaction.

The interpretation that an increment in the production of superoxide by the complex results in an increase in the rate of autodismutation was confirmed when studying the generation of hydrogen peroxide. The latter was assessed from the interaction between increasing concentrations of the Cu(I)–$[GSH]_2$ complex and molecular oxygen in a Cyt *c*- or Luc-free medium by both methodologies: KIT Reflectoquant and the acetaminophen oxidation.

Figure 3a, b show that, employing both assays (KIT Reflectoquant and acetaminophen oxidation, respectively), increasing concentrations of the Cu(I)–$[GSH]_2$ complex were associated with the generation of correspondingly increasing concentrations of $H_2O_2$. Interestingly, the concentration of $H_2O_2$ estimated by the KIT Reflectoquant method (Fig. 3a) is very similar to those estimated from the acetaminophen oxidation assay (Fig. 3b). Furthermore, the estimation of $H_2O_2$ by both methodologies was totally inhibited by CAT (40 U/mL). On the other hand, through the acetaminophen oxidation assay it was possible to follow the formation of $H_2O_2$ as a function of the time elapsed after preparation of the Cu(I)–$[GSH]_2$ complex (in the presence of oxygen), namely "preincubation time" (Fig. 3c). As shown in Fig. 3c, the dependence of $H_2O_2$ was not linear with the preincubation time of the complex, evidencing a noticeable downward curvature. If 150 min of incubation is considered as the time of the maximum $H_2O_2$ produced, it seems that 0.5 mol of $H_2O_2$ is formed for each mole of the Cu(I)–$[GSH]_2$ complex (Fig. 3c).

Table 1 shows the initial rate of reduction of the $O_2^{\bullet-}$-dependent Cyt *c* and the initial rate of oxidation of the $H_2O_2$-dependent acetaminophen, as a function of the Cu(I)–$[GSH]_2$ complex concentration. As seen in Fig. 2 and Table 1, employing the Cyt *c* reduction assay, two well-defined phases are observed depending on the concentration of the complex. While when the oxidation of acetaminophen is used as a $H_2O_2$-dependent method, the initial rate of acetaminophen oxidation increases in a proportional way with complex concentration (Table 1). It is important to point out that the oxidation of acetaminophen, induced by increasing concentration of the complex, was evaluated in a Cyt *c*-free medium. Thus, we postulate that under this condition, the autodismutation reaction would be favoured at higher complex concentrations, while at lower concentrations, superoxide reacts preferently with Cyt *c*. Nevertheless, for $H_2O_2$ quantification, a superoxide-interceptor free system was used and all the superoxide generated participates in $H_2O_2$ production.

## Effect of pH on the formation of the Cu(I)–glutathione complex and its capacity to generate reactive species

The effect of the pH on the formation of the Cu(I)–$[GSH]_2$ complex was studied through the generation of GSSG, in a Cyt *c*- and Luc-free system.

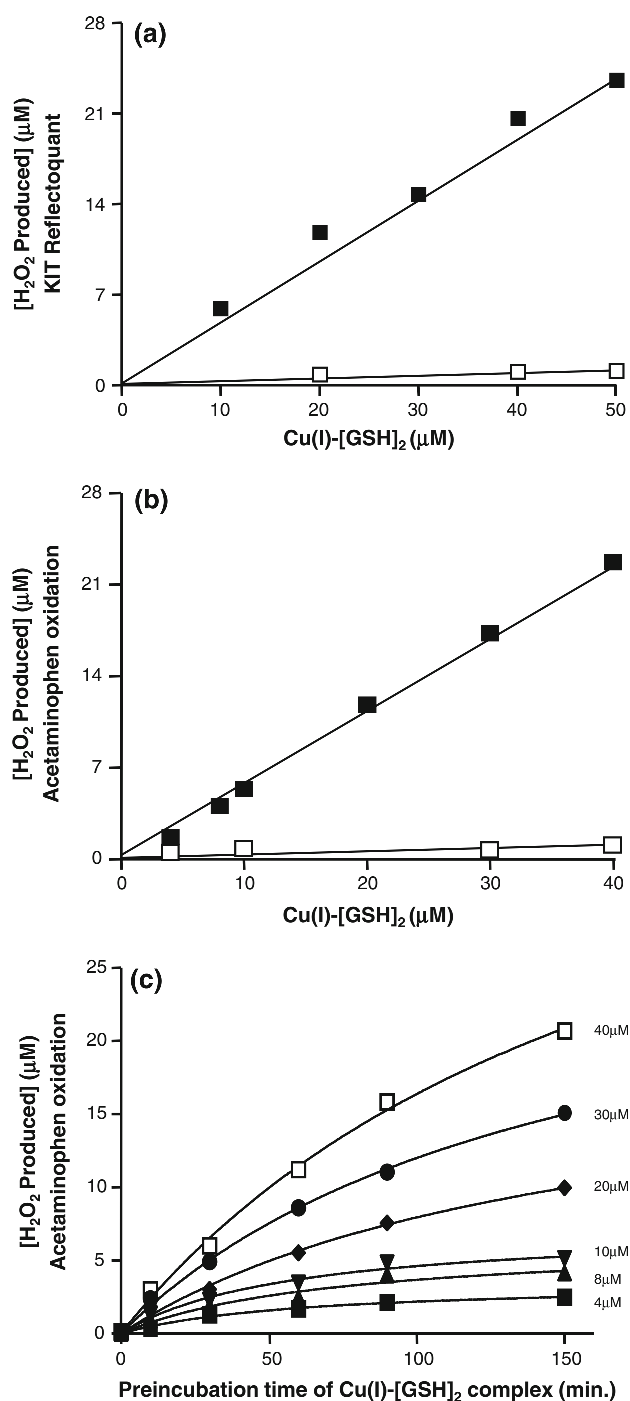
Copper ions ($Cu^{2+}$; 10 μM) and reduced glutathione (GSH; 30 μM) were mixed, at 25 °C and at different pH values (between 6.8 and 7.7). Under these experimental conditions, nearly 5 μM of GSSG was produced (Fig. 4a). Thus, the formation of GSSG remained unchanged despite the pH variation. The latter suggests that the pH did not affect the interaction between $Cu^{2+}$ and GSH in terms of its reduction and subsequent reaction to form the Cu(I)–$[GSH]_2$ complex.

On the other hand, the pH effect was also evaluated on the capacity of the complex (10 μM) to produce reactive oxygen species like superoxide radicals and hydrogen peroxide. The latter was evaluated through Cyt *c*- and Luc-reduction, and by acetaminophen oxidation, respectively.

In contrast to the results obtained for the generation of GSSG, the formation of $O_2^{\bullet-}$, assessed through Cyt *c* assay, was dependent on the pH of the preincubation medium (Fig. 4b).

The percentages expressed in Fig. 4b were estimated considering ideally an irreversible reaction for superoxide radicals generation by the Cu(I)–$[GSH]_2$ complex, meaning that each mole of complex produces 1 mol of superoxide radicals; therefore, 10 μM of the complex generates at most 10 μM of superoxide radicals, corresponding to a 100% of superoxide generation capacity of the complex. Thus, Fig. 4b shows that at a pH 7.4, the highest production of superoxide was detected (46%), and at a pH 6.8, the lowest production was observed (31%). Nevertheless, increasing pH from 7.4 to 7.7 did not increase the production of superoxide radicals; on the contrary, lower superoxide production was detected (39%). The results found in the pH range from 6.8 to 7.4 are consistent with those previously reported by other authors [33, 34] where using allopurinol and xanthine oxidase [34], as source of superoxide radicals, the amount of $O_2^{\bullet-}$ detected through Cyt *c* assay increased with pH.

Presumably, the lower reduction of the $O_2^{\bullet-}$-dependent Cyt *c* observed at pH 7.7 in our system (Fig. 4b) could be attributed to the dependence of Cyt *c* conformations on the pH of the media. Two conformations have been described according to two $pK_a$ values of Cyt *c* ($pK_{a1} = 7.4$ and $pK_{a2} = 9.1$). These conformations could mediate the




◀ **Fig. 3** **a** Hydrogen peroxide formation induced by increasing concentrations of the Cu(I)–$[GSH]_2$ complex. The relationship between increasing concentrations of the Cu(I)–$[GSH]_2$ complex (4–50 μM; preincubated for 150 min) and the formation of hydrogen peroxide in the media was evaluated using KIT Reflectoquant. Results are expressed as micromolar concentration of $H_2O_2$. The symbols represent: (*filled box*) Cu(I)–$[GSH]_2$ in the absence and (*square box*) Cu(I)–$[GSH]_2$ in the presence of CAT (40 U/mL). **b** Hydrogen peroxide formation induced by increasing concentrations of the Cu(I)–$[GSH]_2$ complex. The relationship between increasing concentrations of the Cu(I)–$[GSH]_2$ complex (4–40 μM, preincubated 150 min) and the formation of hydrogen peroxide in the media was evaluated using the oxidation of acetaminophen. Results are expressed as micromolar concentration of $H_2O_2$. The symbols represent: (*filled square*) Cu(I)–$[GSH]_2$ in the absence and (*square box*) Cu(I)–$[GSH]_2$ in the presence of CAT (40 U/mL). **c** Hydrogen peroxide formation induced by increasing concentrations of the Cu(I)–$[GSH]_2$ complex. Preincubation time dependence. The relationship between preincubation time of the Cu(I)–$[GSH]_2$ complex (5–150 min) and the formation of hydrogen peroxide produced by several concentrations of the Cu(I)–$[GSH]_2$ complex (4–40 μM) was evaluated using the oxidation of acetaminophen. Results are expressed as micromolar concentration of $H_2O_2$

reactivity of Cyt *c* toward superoxide radicals. Therefore, probably the lower superoxide radical detected at pH 7.7 using Cyt *c* as probe could be explained assuming that the conformation of Cyt *c* become less reactive toward superoxide radicals generated by the Cu(I)–$[GSH]_2$ complex [35, 36].

With the purpose to establish if the low superoxide radical detected at pH 7.7 is a consequence of Cyt *c* assay (conformation of Cyt *c* pH-dependent) or the low ability of the Cu(I)–$[GSH]_2$ complex to generate superoxide radicals, the reduction of the superoxide-dependent lucigenin, induced by the complex, was also studied. Figure 4c shows that higher pH values led to a higher Luc reduction, indicating either a higher capacity of the Cu(I)–$[GSH]_2$ complex to generate superoxide radicals or a higher stability of these radicals, in aqueous solution, at alkaline medium (p$K_a$ of $HO_2^{\bullet} = 4.8$) [37].

These results (Fig. 4c) are in accordance with the reduction of Cyt *c* mediated by superoxide radicals in the pH range between pH 6.8 and 7.4 (Fig. 4b). The latter confirms that the decrease in the superoxide detection observed at pH 7.7, under our experimental conditions, could be due to an artifactual underestimation related to the conformation of pH-dependent Cyt *c*.

The effect of pH on the hydrogen peroxide production induced by the complex, assessed through the oxidation of acetaminophen, is presented in Fig. 4d. Our results show that when pH increases, a decrease in the hydrogen peroxide production was observed. The amount of $H_2O_2$ generated at pH 6.8 is 2.4 times greater than at pH 7.7. These results are in accordance with the results depicted in Fig. 4c. Thus, the detection of superoxide radicals through the Luc assay increases as the values of pH increases; nevertheless, an inverse effect was observed when the formation of hydrogen peroxide was evaluated. The latter suggests that at more acidic pH, the autodismutation reaction was favored. This is in line with Marklund's work [38], where it is reported that the rate of the reaction of spontaneous autodismutation of superoxide radicals decreases 10-fold when pH increases by 1 unit. Interestingly, the maximum production of $H_2O_2$ by the Cu(I)–$[GSH]_2$ complex (10 μM), corresponding to 5 μM, at pH

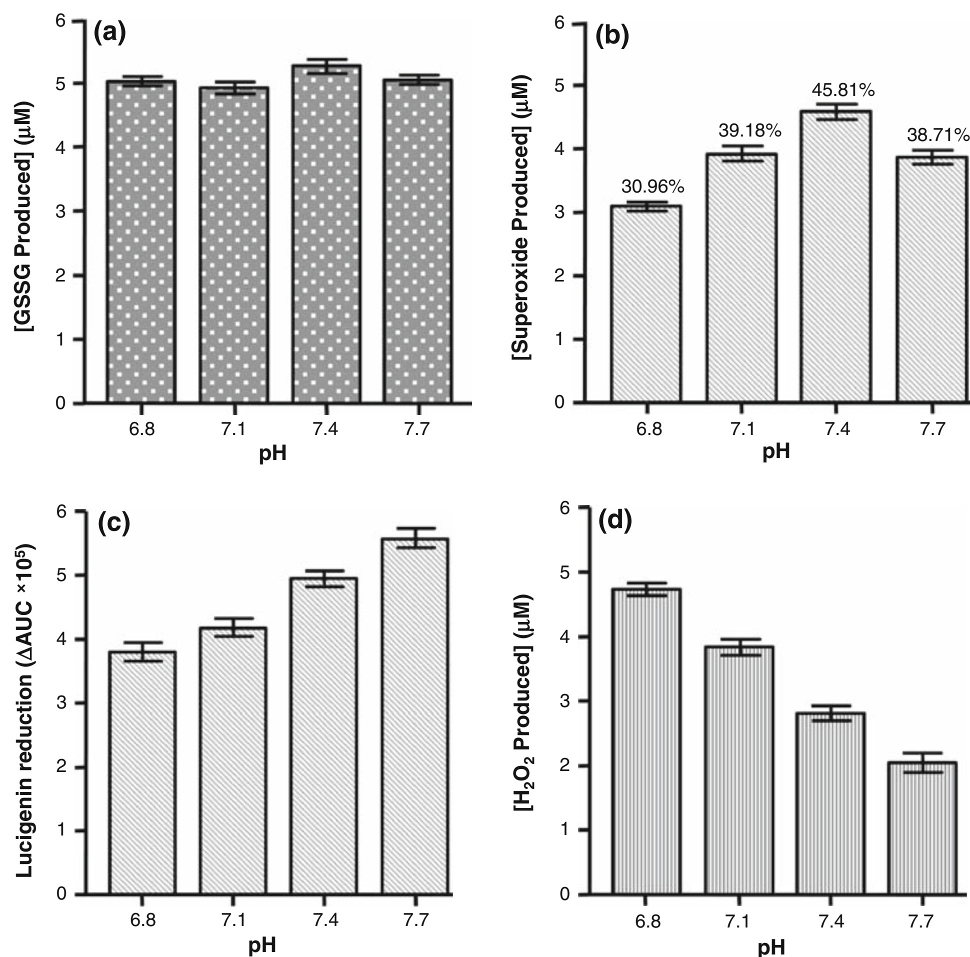



**Fig. 4** **a** Effect of pH on production of oxidized glutathione due to the formation of the Cu(I)–$[GSH]_2$ complex. The relationship between increasing pH values (6.8–7.7) and the production of oxidized glutathione (GSSG) due to the formation of a Cu(I)–$[GSH]_2$ complex (10 μM) was evaluated using a NADPH/GR system. Results are expressed as micromolar concentration of GSSG. **b** Effect of pH on production of superoxide radicals by the Cu(I)–$[GSH]_2$ complex. The relationship between increasing pH values (6.8–7.7) and the production of superoxide radicals by the Cu(I)–$[GSH]_2$ complex (10 μM) was evaluated through Cyt *c* reduction. The increase in optical density at 550 nm due to the reduction of Cyt *c* was registered 120 s after. Results are expressed as production of superoxide (μM). **c** Effect of pH on production of superoxide radicals by the Cu(I)–$[GSH]_2$ complex. The relationship between increasing pH values (6.8–7.7) and the production of superoxide radicals by the Cu(I)–$[GSH]_2$ complex (10 μM) was evaluated through lucigenin reduction. The resulting chemiluminescence was monitored during 112 s. Results represent the difference in the area under the curve (ΔAUC) described by the chemiluminescence levels. **d** Effect of pH on production of hydrogen peroxide by the Cu(I)–$[GSH]_2$ complex. The relationship between increasing pH values (6.8–7.7) and the production of hydrogen peroxide by the Cu(I)–$[GSH]_2$ complex (10 μM) was evaluated through acetaminophen oxidation. Results are expressed as micromolar concentration of hydrogen peroxide

6.8 was reached near 15 min of preincubation (Fig. 4d), while at pH 7.4, the maximum production of $H_2O_2$ was reached after 150 min of preincubation of the Cu(I)–$[GSH]_2$ complex (Fig. 3c).

## Effect of temperature on the formation of the Cu(I)–glutathione complex and its capacity to generate reactive species

Considering the recognized effect of the temperature on the production of reactive oxygen species [34, 39, 40], the influence of this parameter (from 22 to 37 °C) was investigated on the formation of the Cu(I)–$[GSH]_2$ complex and its capacity to produce $O_2^{\bullet -}$ and $H_2O_2$.

It is important to note that the formation of the complex was not affected by increase in temperature, under our experimental conditions. The latter was evaluated through an experiment conducted to determine the amount of GSSG, as reflection of complex formation. Results (not shown) indicate that the formation of GSSG did not vary as a function of the temperature. Probably, the latter may be due to the fast formation of the Cu(I)–$[GSH]_2$ complex (200 ms) [41], which does not distinguish a temperature effect under the experimental conditions used.

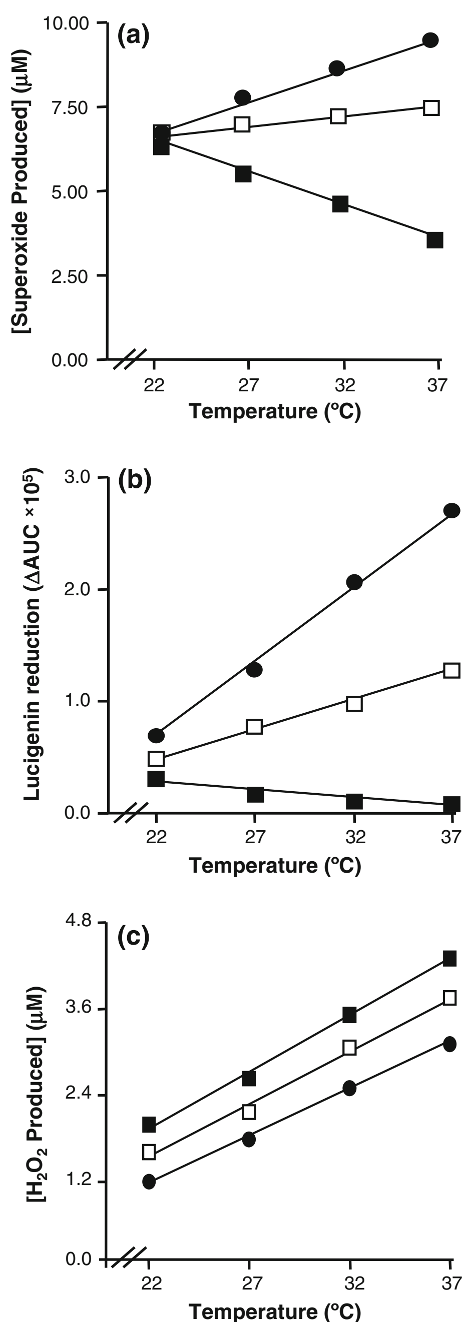



**Fig. 5** Effect of temperature on the formation of superoxide radicals and hydrogen peroxide by the Cu(I)–$[GSH]_2$ complex. Figures 5 show the effect of temperature (22–37 °C) on: **a** the formation of superoxide radicals (evaluated by Cyt *c* reduction); **b** the formation of superoxide radicals (evaluated by Luc reduction); and **c** the formation of hydrogen peroxide (evaluated by acetaminophen oxidation), by a fixed concentration of the Cu(I)–$[GSH]_2$ complex (10 µM). The symbols (*filled circle*), (*square box*), and (*filled square*) represent 1, 15, and 30 min of preincubation of the complex, respectively

The production of $O_2^{\bullet-}$ was assessed through the reduction of Cyt *c* and Luc, $O_2^{\bullet-}$-dependent, and the production of $H_2O_2$ was measured through the oxidation of acetaminophen, $H_2O_2$-dependent (Fig. 5a–c, respectively). Reduced glutathione and copper ions were preincubated at 1, 15, or 30 min, pH 7.4, and at different temperatures (between 22 and 37 °C). All assays such as reduction of Cyt *c* and Luc as well as oxidation of acetaminophen were conducted at 30 °C.

Figure 5a shows that the superoxide production, assessed through the Cyt *c* reduction, induced by the Cu(I)–$[GSH]_2$ complex varies as a function of the temperature. When the Cu(I)–$[GSH]_2$ complex was preincubated for 1 min, an increase of superoxide production was observed when temperature was increased. At preincubation time of 15 min, a slight increase in such production, from 22 to 37 °C, was observed. However, for a Cu(I)–$[GSH]_2$ complex preincubated for 30 min, the increment in temperature led to a drastic decrease in the capacity of this complex to generate superoxide radicals, assessed by the reduction of Cyt *c*. A similar tendency was also observed when the production of $O_2^{\bullet-}$ by the Cu(I)–$[GSH]_2$ complex was assessed through Luc reduction (Fig. 5b).

Thus, rise in temperature, at early preincubation times, led to a higher production of $O_2^{\bullet-}$ by the complex, observed through both assays (Cyt *c* and Luc). However, the opposite effect was observed at 30 min of preincubation. Probably, the latter suggest that at 30 min of preincubation, the spontaneous autodismutation reaction of superoxide radicals, and therefore the accumulation of $H_2O_2$, seems to be favored.

In fact in Fig. 5c at 30 min of preincubation, the formation of $H_2O_2$ evaluated through the oxidation of acetaminophen increases as temperature increases and is higher than those observed at 1 or 15 min of preincubation time of the Cu(I)–$[GSH]_2$ complex.

Interestingly, the accelerated formation and accumulation of $H_2O_2$, from interaction between Cu(I)–$[GSH]_2$ complex and molecular oxygen at high temperature, would be in line with previous work [16], which shows that the oxidation of the Cu(I)–$[GSH]_2$ complex is faster at 37 °C than at 25 °C, probably due to a condition that favors superoxide autodismutation.

## Conclusion

The present study demonstrates that the formation of the Cu(I)–$[GSH]_2$ complex led to a direct relationship with the amount of GSSG produced; however, such amount was not affected by changes in pH (6.8–7.7) and temperature (22–37 °C). Our studies also confirm the recently proposed capacity of the Cu(I)–$[GSH]_2$ complex, in aqueous media, to generate superoxide radicals and hydrogen peroxide [16]. It further demonstrates that such capacity is concentration-dependent and that, when a superoxide interceptor (such as Cyt *c* or Luc) is present, the superoxide radicals generated

by the complex react preferably with the interceptor (at complex concentrations lower than 10 μM). However, when the concentrations of the complex are larger than 10 μM, superoxide radicals react mainly between them to generate hydrogen peroxide. The only exception, in our study, is when Luc is used as superoxide interceptor.

In the absence of interceptors of superoxide radicals, a significant fraction of these radicals undergo autodismutation, leading to the accumulation of hydrogen peroxide in the media as a function of the concentration of the complex. In addition, the capacity of the complex to generate superoxide radicals increases with increasing pH values, but an inverse pH effect is observed in the production of hydrogen peroxide. Interestingly, our studies also reveal that at early preincubation times, the production of superoxide rises as temperature increases; however, when prolonging preincubation time to 30 min, less superoxide was detected due to a higher hydrogen peroxide production.

Thus, the earlier mentioned results should be considered to understand the possible role of the Cu(I)–$[GSH]_2$ complex on the generation of reactive species under physiological conditions.

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