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Redox-changes associated with the glutathione-dependent ability of the Cu(II)–GSSG complex to generate superoxide

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ABSTRACT

The intracellularly-occurring Cu(I)–glutathione complex (Cu(I)–$[GSH]_2$) has the ability to reduce molecular oxygen into superoxide. Removal of such radicals leads to the irreversible conversion of Cu(I)–$[GSH]_2$ into the redox-inactive Cu(II)–GSSG complex. The present study addressed the potential of reduced glutathione, ascorbate and superoxide to reductively regenerate Cu(I)–$[GSH]_2$ from Cu(II)–GSSG, and investigated the redox changes involved in such process. Results show that: (i) among the three tested reductants, only GSH is able to reduce the Cu(II) bound to GSSG; (ii) during the reduction of Cu(II)–GSSG, a Cu(I)–GSSG intermediate would be formed (supported here by Cu(I) and GSSG recovery data and by NMR studies); (iii) when GSH is present in a molar excess equal or greater than 1:3, the reduction of Cu(II)–GSSG into Cu(I)–$[GSH]_2$ is quantitative and complete. Under such conditions, the Cu(II)–GSSG complex acquires a superoxide-generating capacity which is identical to that seen with the Cu(I)–$[GSH]_2$ complex. Within cells, the concentrations of GSH are at least 2- to 3-fold order of magnitude higher than those expected for the Cu(II)–GSSG complex. Thus, we postulate that the interaction between GSH and Cu(II)–GSSG could be seen as a potential mechanism to regenerate continuously the Cu(I)–$[GSH]_2$ complex and thereby affect the ability of the latter to generate superoxide.

1. Introduction

The interaction between Cu^{2+} ions and reduced glutathione (GSH), leads to the swift reduction of the metal. In the presence of additional GSH molecules, the Cu^{+} ions formed bind GSH to form a Cu(I)–glutathione complex.[1–3] Such complex is believed to occur within cells exposed to an excess of copper, mostly under the form of Cu(I)–$[GSH]_2$.[4,5] Studies aimed to define the biological significance of the latter suggest that, within cells, the Cu(I)–glutathione complex would play an essential role as carrier of Cu(I) ions into the Cu^{2+}/Zn^{2+} superoxide dismutase,[1,6] metallothionein[7–9] and ceruloplasmin[10] molecules. In the latter case, the Cu(I)–glutathione complex has been postulated to be able to reverse the deleterious effects of some aceruloplasminemia mutations.[11]

Recently, our laboratory[12] demonstrated that the Cu(I)–$[GSH]_2$ complex reacts with molecular oxygen, in a continuous and totally reversible manner, reducing it into superoxide (Rx. 1). In such reaction, the reduction of oxygen should involve the obliged one-electron oxidation of some component of the Cu(I)–$[GSH]_2$ complex, the metal or the thiol, giving place to the formation of an 'intermediate oxidized form' of the complex (IOC).[13]

$$\mathrm{Cu(I)\text{—}[GSH]_2 + O_2 \rightleftarrows IOC + O_2^-} \qquad \text{(Rx.1)}$$

Considering the reversible character of Rx. 1, it could be assumed that the regeneration of Cu(I)–$[GSH]_2$ from IOC involves the necessary use of superoxide as the reductant species of the latter intermediate.[13] In fact, removal of superoxide from Rx. 1, by the single addition of an excess of SOD, leads to the rapid formation and accumulation of hydrogen peroxide molecules and a complex containing Cu(II) and oxidized glutathione (GSSG), which by means of NMR was earlier identified by us[14] as Cu(II)–GSSG (Rx. 2).

$$\mathrm{2IOC + 2O_2^- + 2H^+ \xrightarrow{SOD} 2Cu(II)\text{—}GSSG + H_2O_2 + O_2} \qquad \text{(Rx.2)}$$

The latter complex, whose occurrence has been reported in human red blood cells,[15] is unable to transfer copper to the copper-free enzymes.[1] Although Cu(II)–GSSG is not expected to be redox/active towards molecular oxygen, we earlier proposed[14] that in the presence of an excess of GSH, the Cu(II)–GSSG complex might acquire the ability to generate superoxide. The latter

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contention would be of biological interest since the concentration of GSH[16] largely exceeds the concentration of copper[17] and that of GSSG[16] expected to occur within cells. A similar scenario applies to ascorbate,[18] and possibly to superoxide, which are also recognized to function as reductants within cells.[19] In view of the potential de novo pro-oxidant implications associated with the redox consequences arising from the interaction between either GSH, ascorbate or superoxide, and the Cu(II)–GSSG complex, in the present study we investigated the feasibility of such interactions and characterize them in terms of the redox and chemical changes that affect both, the reductant and the reducible species within the Cu(II)–GSSG complex. Based on the results obtained in the present study, we postulate the possible mechanism(s) underlying the reductant-dependent capacity of the Cu(II)–GSSG complex to generate superoxide.

2. Results and discussion

Ability of biologically relevant reducing molecules to interact with the Cu(II)–GSSG complex. Early studies by Postal et al.[20] showed that the Cu(II)–GSSG complex exhibits a typical absorption peak at 625 nm. Taking advantage of such spectroscopic property we evaluated the ability of biologically relevant reductants ascorbate, reduced glutathione and superoxide, to interact with the Cu(II)–GSSG complex. Figure 1 compares the effects of increasing concentrations (0.25–2.0 mM) of either GSH or ascorbate on the concentration of a 2.0 mM Cu(II)–GSSG solution (assessed through its $OD_{625\ nm}$). As shown, the addition of GSH to such solution led to a concentration-dependent decrease in Cu(II)–GSSG levels. When added at 0.25 mM, GSH induced a 20% drop in Cu(II)–GSSG concentration; for an equimolar concentration, the addition of GSH led to a total drop of the concentration of this complex. Comparatively, ascorbate was largely unable to induce such an effect. Theoretically, the drop in Cu(II)–GSSG induced by GSH could involve either the reduction of Cu^{2+} into Cu^{+} ions, that of GSSG into GSH, or both. We found that, unlike Cu(II)–GSSG, the Cu(I)–$[GSH]_2$ complex exhibits no absorption at the 625 nm wavelength (not shown). Thus, if the disappearance of Cu(II)–GSSG was to involve its conversion into Cu(I)–$[GSH]_2$, it would produce no confounding effect in interpreting the above shown GSH-induced drop in $OD_{625\ nm}$.

To assess the feasibility of ascorbate and GSH to reduce the Cu(II)–GSSG complex, a comparative analysis, based on the standard reduction potentials (E°) of each of the potentially reacting couples was done. The latter allows predicting which couple would be thermodynamically capable of reducing another. According to data available in the literature, the E° values for the couples Cu^{2+}/Cu^{+}, GSSG/GSH and dehydroascorbate/ascorbate are: 0.17,[21] −0.26,[22] and 0.40 V,[23] respectively. Thus, the inability of ascorbate to reduce Cu(II)–GSSG could be explained, at least theoretically, by the fact that its E° is more positive than that of the Cu^{2+}/Cu^{+} and GSSG/GSH couples. The inability of ascorbate to reduce GSSG in the Cu(II)–GSSG complex is in line with the extremely low rate constant ($3.52 \times 10^{-5}\ M^{-1}\ s^{-1}$) at which ascorbate reduces GSSG.[24] In turn, the much negative E° value of the GSSG/GSH couple makes GSH thermodynamically capable of reducing Cu^{2+}. Results from Figure 1 suggest that GSH is indeed able to reduce Cu^{2+} ions and that its Cu(II)–reducing ability is not limited by the fact that such metal is bound to GSSG.

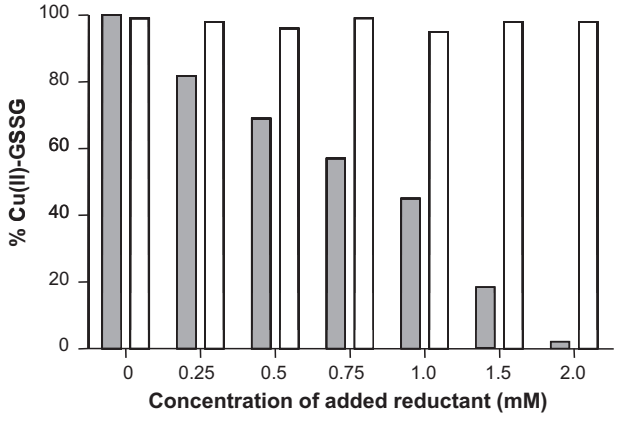

Figure 1. Potential reductive effect of GSH and ascorbate on the Cu(II)–GSSG complex. Increasing concentrations of GSH or ascorbic acid (0.25–2.0 mM) were added to solutions (prepared in phosphate buffer, pH 7.4) containing a fixed concentration of Cu(II)–GSSG (2 mM). The change in OD at 625 nm was registered 1 min after the addition of either GSH or ascorbic acid to Cu(II)–GSSG and the results are expressed as percentage of Cu(II)–GSSG complex detected (100% corresponds to the $OD_{625\ nm}$ of a 2 mM Cu(II)–GSSG solution). The bars in grey and white represent the results arising from the use of GSH and ascorbate, respectively.

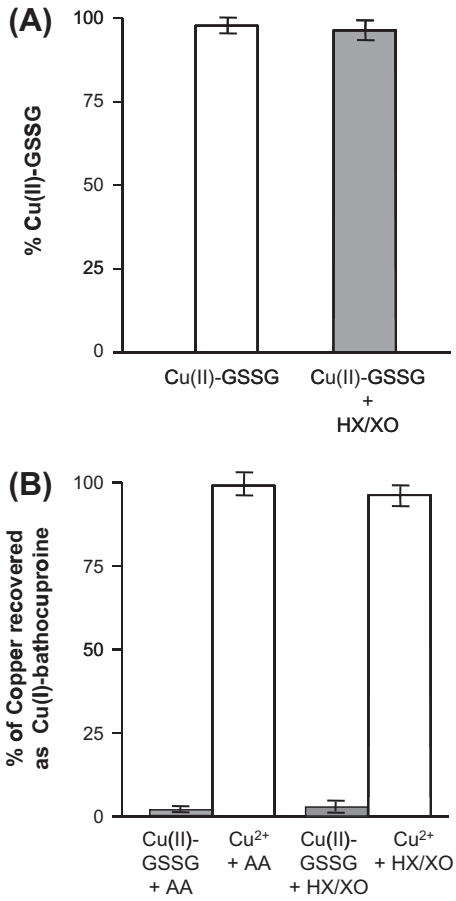

Figure 2. (A) Potential reductive effect of superoxide on the Cu(II)–GSSG complex. Hypoxanthine (2 mM) plus Xanthine oxidase (1.1 U/mL) HX/XO was added to a solution (prepared in phosphate buffer, pH 7.4) containing a fixed concentration of Cu(II)–GSSG (0.5 mM). The change in OD at 625 nm was registered and the results are expressed as percentage of Cu(II)–GSSG complex detected (100% corresponds to the $OD_{625\ nm}$ of a 2 mM Cu(II)–GSSG solution). (B) Reduction of free and GSSG-bound cupric by ascorbate and superoxide. A solution prepared (in phosphate buffer, pH 7.4) by mixing bathocuproine (2 mM) and ascorbic acid (2 mM), or the former plus hypoxanthine (2 mM) and xanthine oxidase (1.1 U/mL) HX/XO, was added to solutions containing either free or GSSG-bound Cu^{2+} ions (0.5 mM). The increase in OD at 480 nm was registered and the results, which were estimated using a molar absorption coefficient of $12.25\ mM^{-1}\ cm^{-1}$, are expressed as % of copper recovered as Cu(I)-bathocuproine.

Figure 2 depicts the result of exposing the Cu(II)–GSSG complex (at 0.5 mM concentration) to a mixture of hypoxanthine plus xanthine oxidase (HX/XO) as a source of superoxide. As shown in part A of such figure, after 60 min of incubation, the HX/XO mixture was totally unable to decrease the concentration of the complex (assessed through its absorption at 625 nm). To ascertain that the inability of superoxide to reduce Cu(II)–GSSG was not due to a lack or a limited Cu(II)–reducing power, the ability of this enzyme-dependent superoxide-generating system to reduce free Cu^{2+} ions was assessed using the Cu(I)-bathocuproine assay[25] (Fig. 2B). The assay was applied to a concentration of Cu^{2+} (0.5 mM), namely, identical to that of Cu(II)–GSSG employed in part A of Figure 2. As shown in part B of such figure, the above-used HX/XO mixture was able to reduce 100% of Cu^{2+} into Cu^{+} ions. A similar Cu^{2+}-reducing effect was also attained when instead of HX/XO, a 2 mM concentration of ascorbic acid was used. The lack of ability of superoxide to reduce the Cu(II)–GSSG complex is in apparent disagreement with the prediction made on the basis of comparing the E° values for the Cu^{2+}/Cu^{+} and O_2/O_2^- couples (0.17 vs −0.33 V,[26]). It should be noted, however, that such prediction is based on the direct use of E° values reported for these couples in their free and ionic form. Thus, the possibility exists that binding of GSSG to Cu(II) may, somehow, reduce the standard reduction potential of the metal couple making its reduction by superoxide thermodynamically unfavorable.

Considering that amongst the three tested reductants, only GSH effectively decreased the concentration of Cu(II)–GSSG, we addressed the redox changes that affect the latter complex during its interaction with GSH, in terms of both, a possible reduction of Cu(II) into Cu(I) and/or an oxidation of the GSH molecules. To assess the former changes, the reduction of Cu(II) induced by the addition of increasing concentrations of GSH was assessed (using the bathocuproine assay). Figure 3 depicts the results from incubating during 1 or 25 min, an 8 μM concentration of Cu(II)–GSSG with an equimolar, a threefold and a ninefold concentration of GSH. As soon as 1 min after its addition, the three concentrations of GSH were equally effective in inducing a total reduction of the Cu(II) bound to GSSG. However, when studied 25 min after an equimolar addition of GSH, the concentration of Cu(I) recovered at such time was only around one third of that present at min one. Thus, the interaction between equimolar concentrations of GSH and the Cu(II)–GSSG complex seems to have swiftly led to the formation of a Cu(I)-containing species that underwent a relatively fast re-oxidation along time. In the case of a 3- and a 9-fold GSH excess addition, the levels of Cu(I)-bathocuproine obtained initially remained largely unaltered 25 min after (Fig. 3). Thus, the in-excess added reduced glutathione would help to preserve the Cu(I)-containing species that formed immediately after the addition of GSH.

Earlier studies by other investigators[1,2] revealed that the Cu(I)–$[GSH]_2$ complex can be equally formed upon addition of GSH to Cu^{2+} ions in a 3:1 molar ratio or in a 2:1 ratio if Cu^{+} is used. Considering the ability of equimolar concentrations of GSH to swiftly reduce the Cu(II) bound to GSSG (shown in Fig. 3), it would seem reasonable to speculate that upon addition of a threefold molar excess of GSH to the Cu(II)–GSSG complex a Cu(I)–$[GSH]_2$ complex is also formed. Since the Cu(I)–$[GSH]_2$ complex is able to continually convert molecular oxygen into superoxide,[12] the ability of GSH molecules to reduce the Cu(II)–GSSG complex suggests that in the presence of an equimolar or a threefold excess of free GSH, both complexes, (Cu(I)–$[GSH]_2$ and Cu(II)–GSSG), exhibit a potential to generate superoxide.

Within cells, however, the above-referred complexes are likely to occur not only with a large molar excess (mM concentrations) of GSH but also in the presence of diverse superoxide-removing molecules, among which SOD is the most important.[27] Recent work conducted by us[13] indicates that in the presence of Tempol, a SOD-mimetic molecule,[28] the Cu(I)–$[GSH]_2$ complex undergoes a time-dependent irreversible oxidation into Cu(II)–GSSG (Rx. 2). In view of the latter, we investigated here the effect of adding Tempol to a mixture of GSH plus Cu(II)–GSSG on the concentration of Cu(I) susceptible to be recovered (after 25 min of incubation). As shown in Figure 3, relative to Tempol-free mixtures, the concentration of Cu(I)-bathocuproine recovered after such time was reduced in all

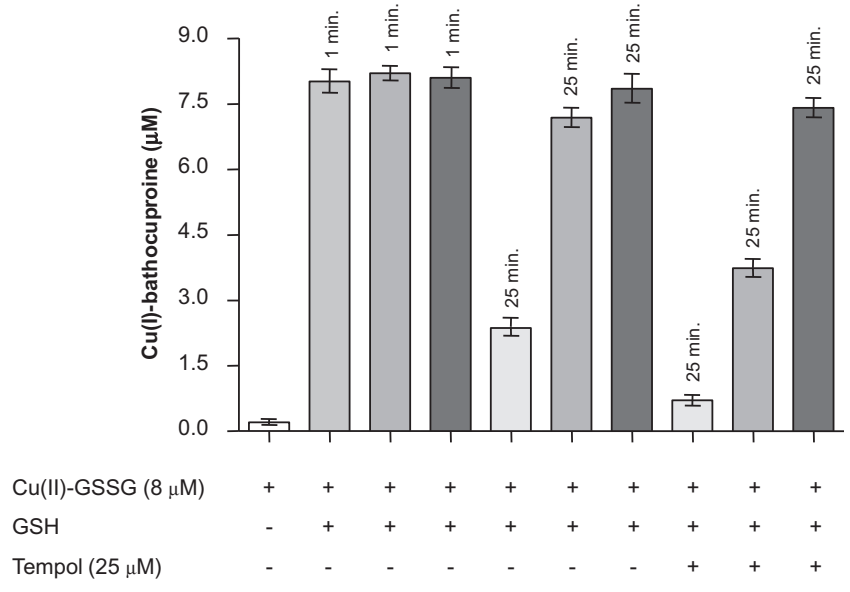

Figure 3. Effect of Tempol on the recovery of Cu(I) generated during the interaction between Cu(II)–GSSG and GSH. To evaluate the effect of Tempol on the recovery of Cu(I) that results from the addition of GSH to Cu(II)–GSSG, solutions (prepared in phosphate buffer, pH 7.4) containing Cu(II)–GSSG (8 μM) and increasing concentrations of GSH (8, 24 and 72 μM) were incubated in the absence or presence of Tempol (25 μM). Recovery of Cu(I) was evaluated using bathocuproine (500 μM). The increase in OD at 480 nm of incubation of the above-referred mixture was registered after 1 and 25 min and the results were estimated using a molar absorption coefficient of 12.25 mM^{-1} cm^{-1}. Results are expressed as Cu(I)-bathocuproine concentration (μM). The bars light grey, medium grey and dark grey represent the results arising from the use of GSH at 8, 24 and 72 μM, respectively.

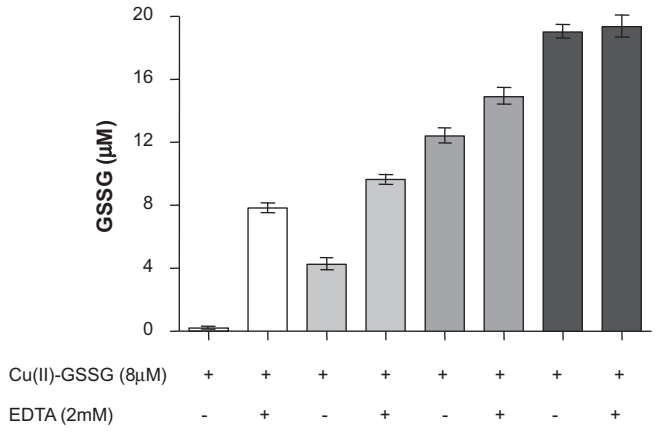

Figure 4. Recovery of free and Cu(II)–bound GSSG during the interaction between Cu(II)–GSSG and GSH. Mixtures of solutions (prepared in phosphate buffer, pH 7.4) containing increasing concentrations of GSH (8, 24 and 72 μM) and a fixed concentration of Cu(II)–GSSG (8 μM) were incubated for 25 min. Subsequently, the decrease in OD at 340 nm that resulted from adding to such mixtures, NADPH plus glutathione reductase both, in absence and presence of EDTA (2 mM), was registered. Results are expressed as GSSG concentration (μM). The bars light grey, medium grey and dark grey represent the results arising from the use of GSH at 8, 24 and 72 μM, respectively.

mixtures. The relative magnitude of the Cu(I)-lowering effect of Tempol was, however, maximal for the 1:3 and minimal for the 1:9 mixtures. The latter results are in line with the above interpretation that addition of an excess of GSH to the Cu(II)–GSSG complex leads to the formation of Cu(I)–$[GSH]_2$ and suggest that upon conditions leading to its oxidation (Tempol-mediated), the larger GSH excess (1:9) would secure a continuous re-generation of Cu(I)–$[GSH]_2$ from Cu(II)–GSSG.

To gain further understanding on the redox changes that follow the interaction between the added GSH and the Cu(II)–GSSG complex, we investigated whether such interaction is associated with a de novo formation of GSSG. As shown in Figure 4, no GSSG levels were detected when an 8 μM Cu(II)–GSSG solution was directly assayed through the NADPH/glutathione reductase assay. However, when assayed in the presence of 2 mM EDTA, a chelating agent which releases the GSSG bound to Cu(II),[13] a total of 8 μM GSSG was quantitated. The latter value corresponds exactly to the concentration of GSSG that formed part of the Cu(II)–GSSG complex. When GSH was equimolarly added to the 8 μM Cu(II)–GSSG solution, after 25 min of incubation, the concentration of GSSG recovered in the absence of EDTA was near 4 μM. Such value reflects, most likely, the concentration of GSH molecules (8 μM) that were consumed during the reduction of the Cu(II) that was bound to GSSG (as suggested in Rx. 3). These newly formed GSSG molecules would be in their free state since they were readily quantified in absence of EDTA.

$$8Cu(II)\text{—}GSSG + 8GSH \rightarrow 8Cu(I)\text{—}GSSG + 4GSSG + 8H^{+} \qquad (Rx.3)$$

However, the addition of an equimolar concentration of GSH to the Cu(II)–GSSG complex raised the concentration of GSSG (recoverable in the presence of EDTA) from 4 to over 8 μM. Thus, it can be assumed that, at least an additional 4 μM GSSG concentration was generated from an EDTA-releasable form of copper. We propose that the latter compound corresponds to Cu(II)–GSSG, since EDTA is not expected to react with Cu(I) and the GSSG contained in the

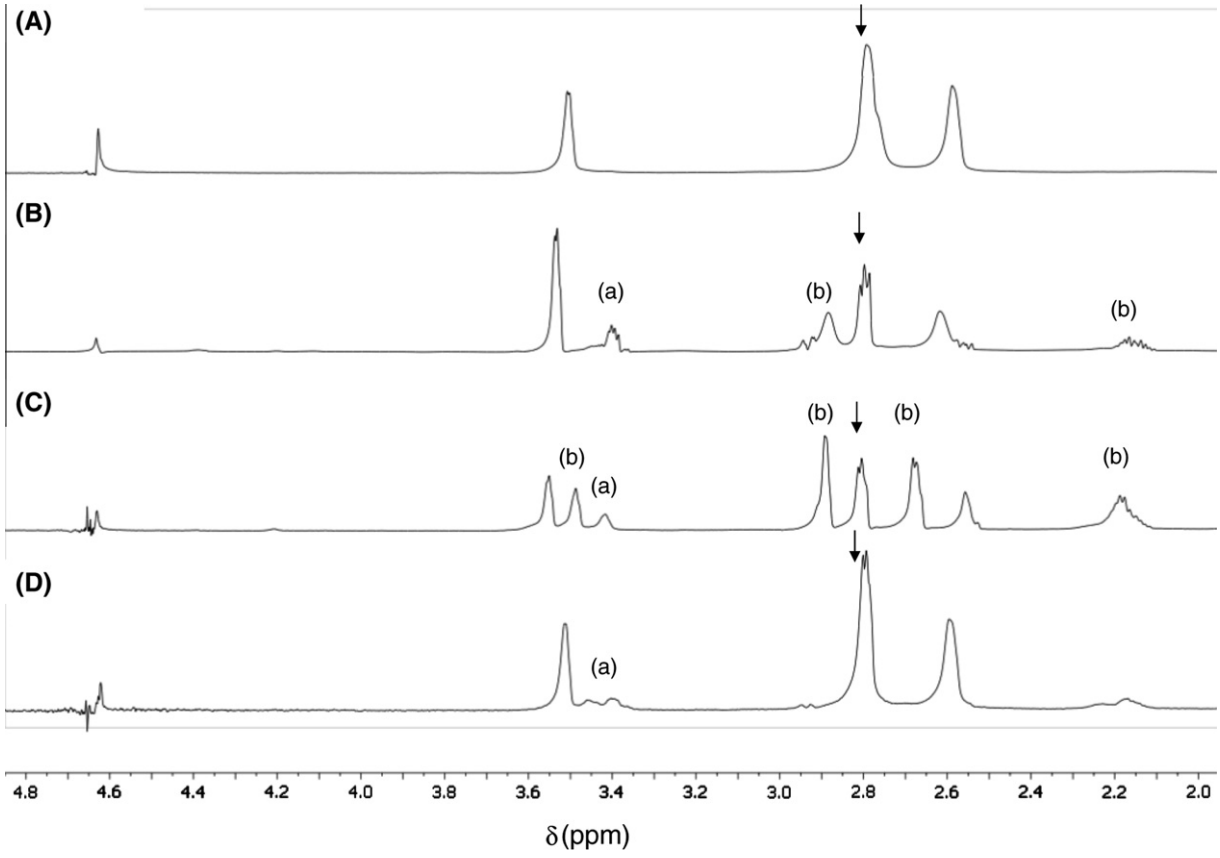

Figure 5. Changes in the Cu(II)–GSSG ^{1}H NMR spectra induced by the addition of GSH. Spectra of the Cu(II)–GSSG complex (1 mM) alone (A) or, plus GSH added at 3 mM (B) or 9 mM (C). Spectrum of the Cu(I)–$[GSH]_2$ complex (1 mM, (D)). All solutions were prepared in phosphate buffer, pH 7.4. Assignments: (a) represents traces of GSSG and (b) excess of GSH. Arrows represent β-CH_2 of the cysteine containing in GSH.

former complex is totally assayable by the EDTA-coupled NADPH/glutathione reductase assay.[13] Accordingly, the Cu(I)–GSSG complex postulated to be formed in Rx. 3 would undergo—along the 25 min of incubation—an oxidative conversion into Cu(II)–GSSG; this contention is partially supported by the data presented in Figure 3 on Cu(I)-bathocuproine levels for the equimolar mixture.

Regarding the experiments in which GSH was added to the Cu(II)–GSSG complex at a threefold excess, Fig. 4 shows that the concentration of GSSG assayed in absence of EDTA was close to 12 μM. As explained before, a total of 4 μM GSSG would be accounted for by the GSH molecules that are expected to be consumed during the reduction of the Cu(II) bound to GSSG, as in Rx. 3; assuming that in Rx. 3 a total of 8 μM Cu(I)–GSSG were indeed formed, the other 8 μM GSSG would be accounted for by GSSG molecules that were displaced from the Cu(I)–GSSG complex by the 16 μM in-excess added GSH molecules, as in Rx. 4. Relative to GSSG, GSH molecules would have a greater affinity for Cu(I).[29]

$$8\mathrm{Cu(I)}{-}\mathrm{GSSG} + 16\mathrm{GSH} \rightarrow 8\mathrm{Cu(I)}{-}[\mathrm{GSH}]_2 + 8\mathrm{GSSG} \qquad (\mathrm{Rx.4})$$

When GSSG was assayed in the presence of EDTA, the interaction between Cu(II)–GSSG and GSH, the latter added at a threefold excess, led to the generation of an amount of GSSG which is only slightly higher than that obtained in the absence of the chelating agent (Fig. 4). This result suggests that under the latter experimental conditions, the Cu(I)–$[GSH]_2$ formed in Rx. 4 would indeed undergo some degree of oxidation.[13,14] On the other hand, in the presence of a ninefold GSH excess, the concentration of GSSG assayed in the absence or presence of EDTA was almost identical, near 20 μM. Relative to the threefold GSH excess condition, the higher recovery of GSSG obtained with the ninefold GSH excess may be due to the here-postulated ability of the in-excess GSH molecules to reduce Cu(II)–GSSG, regenerating (at their oxidative expense) the Cu(I)–$[GSH]_2$ complex.

According to the results on Cu(I)-bathocuproine and GSSG levels (Figs. 3 and 4) obtained with the equimolar, 3- and 9-fold GSH excess, the interaction between GSH and Cu(II)–GSSG would always lead to the initial formation of a Cu(I)-containing complex (as proposed in Rx. 3). To elucidate, in each case, the possible chemical nature of the Cu(I)-containing complex formed during the interaction between GSH and the Cu(II)–GSSG complex, we conducted ^{1}H NMR experiments. Figure 5 depicts the spectrum of the Cu(II)–GSSG complex (1 mM in part A) and that resulting from mixing such complex with a three or a ninefold GSH excess (parts B and C, respectively). The peaks in the Cu(II)–GSSG spectrum show a considerably line broadening and featureless peaks with completely unresolved J-couplings which is a symptom of protons with very short transversal relaxation time that are in the vicinity of paramagnetic species[30] and it fully corresponds to that previously reported for the Cu(II)–GSSG complex by us.[14] However, when GSH was added at a 3- or 9-fold excess, more species are formed but the signals become considerably narrower and some J-couplings are much better resolved, these characteristics strongly suggests a chemical reaction that promotes the loss of paramagnetic Cu(II). The spectra obtained for the latter two mixtures (parts B and C) has a key peak at ~2.8 ppm (indicated by arrows in Fig. 5) that shows similar characteristics with that in

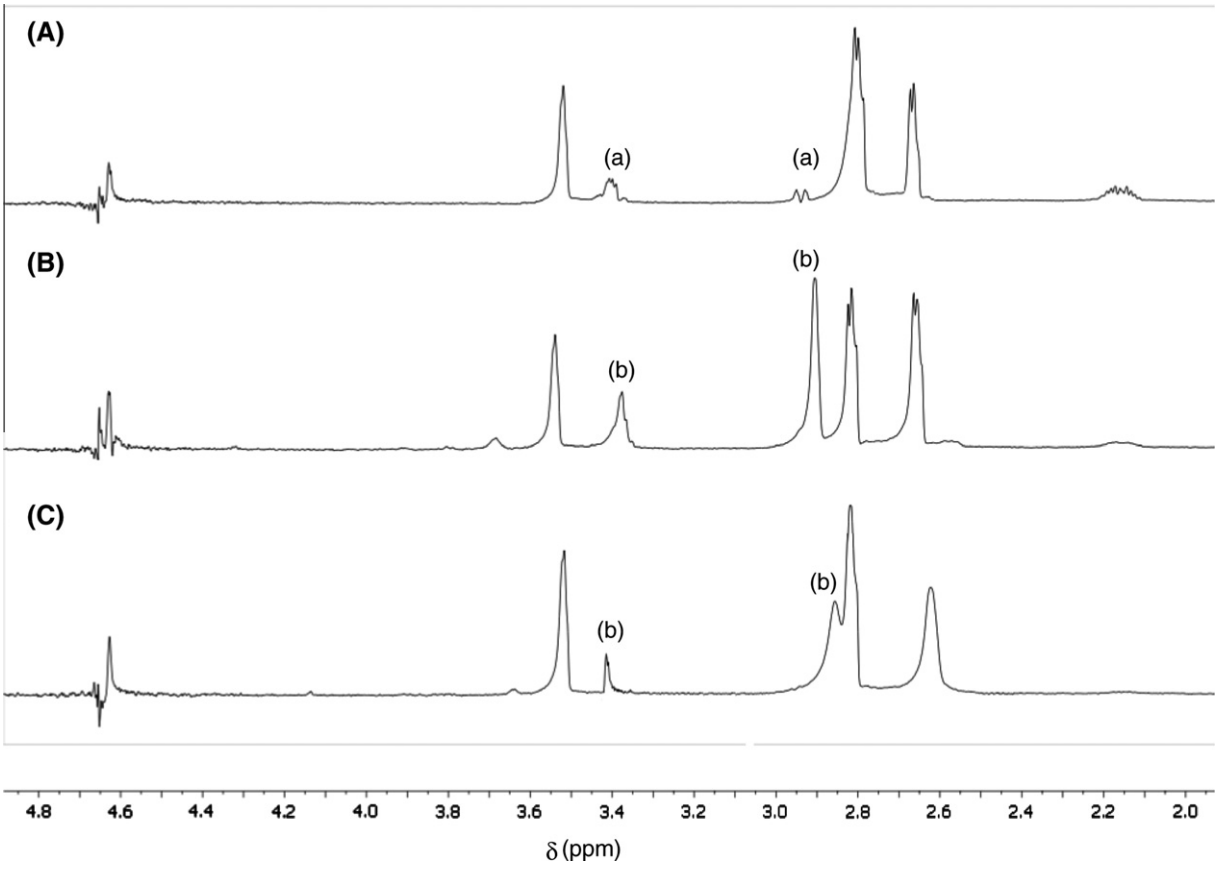

Figure 6. Changes in the Cu(II)–GSSG ^{1}H NMR spectra induced by the addition of ascorbate and GSH. Spectrum (A) corresponds to a mixture of Cu(II)–GSSG complex (1 mM) plus GSH (1 mM). Spectrum (B) corresponds to a solution prepared by mixing: Cu(II) (1 mM) plus ascorbic acid (3 mM) and GSSG (1 mM). Spectrum (C) corresponds to a mixture of Cu(II)–GSSG complex (1 mM) plus ascorbic acid (3 mM). All solutions were prepared in phosphate buffer, pH 7.4. Assignments: (a) represents traces of GSSG and (b) excess of ascorbic acid.

the spectrum of Cu(I)–$[GSH]_2$ (part D), very different from the featureless and broad peak seen at that chemical shift in the spectrum of Cu(II)–GSSG complex (part A). On the other hand, the spectrum resulting from mixing equimolar concentrations of GSH plus Cu(II)–GSSG (shown in part A of Fig. 6) depicts relatively good resolved peaks (namely, non paramagnetic copper) while it also exhibits chemical shifts that are typically associated with the presence of GSSG.[20] This spectrum is closely comparable to that shown in part B (Fig. 6), which was obtained after adding GSSG (1 mM) to a mixture of Cu^{2+} (1 mM) plus ascorbic acid (3 mM). In the latter mixture, we ascertained that just prior to the addition of GSSG, ascorbate had fully reduced Cu^{2+} into Cu^{1+} ions (data not shown). In turn, when the same experiment was conducted by adding ascorbic acid to a preformed mixture of Cu^{2+} plus GSSG (1 mM each), the characteristics of the spectrum obtained (part C in Fig. 6) was coincidental with that of the Cu(II)–GSSG complex (shown in Fig. 5, part A), confirming the initially shown lack of ability of ascorbate to reduce Cu(II)–GSSG (Fig. 1). In contrast to ascorbate, GSH is capable of reducing the Cu(II) in such complex leading, in all studied cases, to the formation of a Cu(I)-containing complex; based on the present NMR data, we propose that, in the case of the equimolar addition of GSH to Cu(II)–GSSG, the complex formed is Cu(I)–GSSG, as suggested in Rx. 3, and that in the case of adding a 3- and a 9-fold GSH excess, the Cu(I)–$[GSH]_2$ complex is formed predominantly, as suggested in Rx. 4.

Finally, based on the previously established ability of the Cu(I)–$[GSH]_2$ complex to catalyze the reduction of molecular oxygen,[12] we decided to investigate whether the Cu(I)–GSSG complex, presumably formed by the direct interaction between equimolar concentrations of GSH and Cu(II)–GSSG, is also capable of generating superoxide. The latter, was addressed using DHE as an oxidizable superoxide-sensitive probe.[31,32] As shown in Figure 7, the addition of an equimolar concentration or a threefold GSH excess to the Cu(II)–GSSG complex were found to be equally effective in inducing the DHE oxidation. These effects, that were evident and maximal already after 1 min of adding GSH, are in line with the total and also earlier (after one min) reduction of Cu(II) into Cu(I) seen before upon the addition of both types of mixtures to the Cu(II)–GSSG complex (Fig. 3). Interestingly, the addition of SOD to the equimolar mixture of GSH plus Cu(II)–GSSG (Fig. 7), or an threefold molar excess of GSH (not shown) totally prevented DHE oxidation, suggesting that the DHE-oxidizing ability of the putatively formed Cu(I)–GSSG complex is totally attributable to superoxide. Compared to what was seen one min after GSH addition, the ability of both Cu(I)-containing complexes (i.e., Cu(I)–GSSG generated as in Rx. 3 and Cu(I)–$[GSH]_2$ as in Rx. 4) to induce DHE oxidation was lower 25 min after. A greater decrease in the DHE-oxidizing capacity was seen for the equimolar mixture. Such result is not only consistent with the also comparatively lower recovery of Cu(I) observed at such time for the same mixture in Figure 3, but furthers the interpretation that the GSH-dependent ability of the Cu(II)–GSSG complex to function as a potential source of superoxide is extended by the presence of a threefold GSH excess. In fact, such ability of the Cu(II)–GSSG complex was almost totally conserved 25 min after the addition of a ninefold GSH excess (not shown).

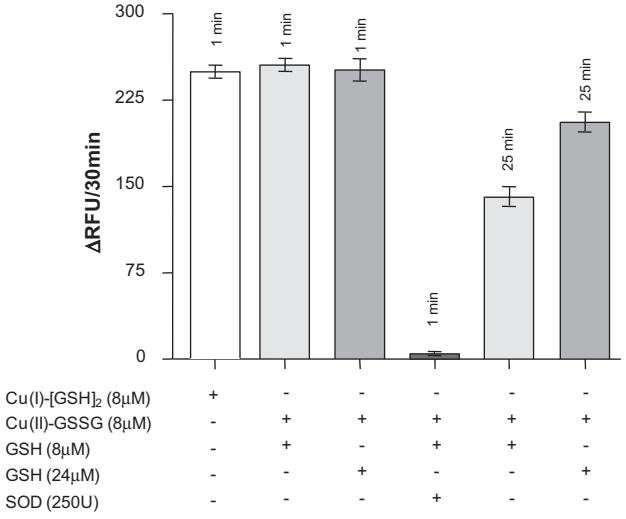

Figure 7. GSH-dependent generation of superoxide by the Cu(II)–GSSG complex. The generation of superoxide was assessed using DHE (50 μM) as probe. DHE oxidation was measured in solutions (prepared in phosphate buffer, pH 7.4) containing increasing concentrations of GSH (8 and 24 μM) and a fixed concentration of Cu(II)–GSSG (8 μM). Thirty minutes after mixing GSH and Cu(II)–GSSG, the increase in fluorescence resulting from DHE oxidation was registered. The results are expressed as the difference in relative fluorescence units (ΔRFU/30 min) that results from subtracting the fluorescence of DHE alone from that of mixtures of DHE plus the GSH/Cu(II)–GSSG.

3. Conclusions

Based on the use of various biologically-relevant reducing molecules, we concluded that GSH is the only reducing molecule able to reduce the Cu(II)–GSSG complex; data provided supports the interpretation that during such reduction, a Cu(I)–GSSG intermediate would be formed. When GSH is present in a molar excess equal or greater than 1:3, the reduction of Cu(II)–GSSG into Cu(I)–$[GSH]_2$ is quantitative and complete, and the former complex acquires a superoxide-generating capacity which is identical to that seen with the Cu(I)–$[GSH]_2$ complex. Based on the here-presented evidence, we propose that under biological conditions, where micromolar concentrations of the Cu(II)–GSSG complex are likely to face millimolar concentrations of GSH, the former molecule is likely to occur mainly under the form of Cu(I)–$[GSH]_2$, constituting thereby a continuous source of superoxide.

4. Experimental

4.1. Chemicals and reagents

Cupric chloride ($CuCl_2{\cdot}2H_2O$), reduced glutathione (GSH), oxidized glutathione (GSSG), L-ascorbic acid (AA), glutathione reductase (GR; EC 1.6.4.2. from baker's yeast), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), superoxide dismutase (SOD; EC 1.15.1.1. from bovine erythrocytes), hypoxanthine (HX), xanthine oxidase (XO; E.C. 1.17.3.2. from bovine milk), bathocuproine disulfonic acid (BCS), deuterium oxide (HOD) and DMSO were all purchased from Sigma–Aldrich. Dihydroethidium (DHE) and 4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl (Tempol) were purchased from Calbiochem. EDTA was purchased from Bio-Rad Laboratories. All aqueous solutions were prepared in Chelex-100-treated sodium phosphate buffer (20 mM; pH 7.4).

4.2. Preparation of copper–glutathione complexes

The Cu(I)–$[GSH]_2$ complex was prepared as previously described[12] 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. The Cu(II)–GSSG complex was prepared by direct mixing of $CuCl_2$ and GSSG in a 1:1 molar ratio as previously described.[20,33] Unless indicated otherwise, both complexes were prepared and used always freshly.

4.3. Determination of the Cu(II)–GSSG complex

Cu(II)–GSSG was assessed as described by Postal et al.[20] taking advantage of its spectroscopic properties. The increase in $OD_{625\ nm}$ associated with the formation of Cu(II)–GSSG was monitored at 625 nm using an Unicam Heλios α spectrophotometer. The assay was initiated after the addition of samples containing one of the following reducing agents: AA (0.25–2.0 mM), GSH (0.25–2.0 mM) or HX (2 mM)/XO (1.1 U/mL), to a cuvette containing Cu(II)–GSSG complex (2 mM). Results were estimated using a molar absorption coefficient of 60 M^{-1} cm^{-1} and expressed as percentage of the Cu(II)–GSSG complex detected (100% corresponds to the $OD_{625\ nm}$ of a 2 mM Cu(II)–GSSG solution).

4.4. Determination of cuprous ions

Cu(I) was assessed as described by Moffett et al.[25] employing the Cu(I)-bathocuproine assay. The increase in $OD_{480\ nm}$ associated with the formation of the Cu(I)–$[BCS]_2$ complex was monitored at 30° C, using an Unicam Heλios α spectrophotometer. The assay was initiated after the addition of either free Cu^{2+} ions ($CuCl_2 \times 2H_2O$; 0.5 mM) or GSSG-bound Cu^{2+} ions (Cu(II)–GSSG; 0.5 mM) to a solution prepared by mixing 2 mM bathocuproine plus either AA (2 mM) or HX (2 mM)/XO (1.1 U/mL) as reducing agents. Results were estimated using a molar absorption coefficient of 12.25 mM^{-1} cm^{-1} and expressed as % of copper recovered as Cu(I)-bathocuproine (Fig. 2B).

Figure 3, the assay was initiated after the addition of bathocuproine (500 μM) to a cuvette containing increasing concentrations of GSH (8, 24 and 72 μM) plus a fixed concentration of Cu(II)–GSSG (8 μM), incubated during 1 or 25 min, in absence or in presence of Tempol (25 μM). Results were expressed as micromolar concentration of Cu(I)-bathocuproine.

4.5. Determination of free- and bound- to Cu(II) oxidized glutathione

GSSG was assessed as described by Tietze,[34] employing the NADPH/glutathione reductase assay. The decay in $OD_{340\ nm}$ associated with the formation of nicotinamide adenine dinucleotide phosphate, oxidized form ($NADP^+$) was monitored at 30° C using an Unicam Heλios α spectrophotometer. The assay was initiated after the addition of samples freshly prepared containing NADPH (0.2 mM) and glutathione reductase (2 U/mL) in presence or absence of EDTA (2 mM) to a cuvette containing increasing concentrations of GSH (8, 24 and 72 μM) plus a fixed concentration of Cu(II)–GSSG (8 μM), incubated during 25 min. The results from applying these assays are expressed as micromolar concentration of GSSG and represent free GSSG (in absence of EDTA) or the sum of, free GSSG plus GSSG released by EDTA from the Cu(II)–GSSG complex (in presence of EDTA).

4.6. Nuclear magnetic resonance (NMR) studies

^1H NMR spectra of solutions prepared by mixing the Cu(II)–GSSG complex (1 mM) plus either GSH (1; 3 or 9 mM) or AA (3 mM) (all in 20% deuterated, sodium phosphate buffer, 20 mM, pH 7.4) were acquired on a Varian INOVA 750-MHz spectrometer. Controls were carried out using: a pre-formed Cu(II)–GSSG complex (1 mM), the Cu(I)–$[GSH]_2$ complex (1 mM), or a solution prepared by mixing Cu(II) (1 mM) plus AA (3 mM) and GSSG (1 mM).

All the proton spectra were acquired under the same conditions at 278 K. The spectra were acquired with the soft-watergate pulse sequence[35] that provided a satisfactory suppression of the strong water proton resonance at ~4.7 ppm. Each spectrum was acquired with 128 scans, with the carrier position placed at the frequency of the water signal to suppress and a spectral width of ~16 ppm. The time domain was 16 k data points. The spectra were referenced automatically in the spectrometer respect to the deuterium lock signal of D_2O. The NMR spectra were processed and analyzed with Mestre-C software (Mestrelab inc.).

4.7. Dihydroethidium oxidation assay

Superoxide was assessed as described by Zhao et al.,[32] employing the dihydroethidium (DHE) oxidation assay. The oxidation of DHE was monitored fluorimetrically in a 96-well plate using a Multi-Mode Microplate Reader (Synergy™ HT). Excitation and emission wavelengths were 470 and 590 nm, respectively. Freshly prepared DHE, dissolved in DMSO, was added (50 μM) to wells containing increasing concentrations of GSH (8 and 24 μM) and a fixed concentration of Cu(II)–GSSG (8 μM). Incubations were carried out at 30 °C and readings of fluorescence were obtained after 30 min. When employed (as referred in the text), SOD was added to the wells at 250 U/well. Results were expressed as ΔRFU/30 min (delta relative fluorescence units) and represent the difference in RFU which results from subtracting the fluorescence of DHE alone from that of mixtures of DHE plus the GSH/Cu(II)–GSSG.

4.8. Data expression and analysis

Data points represent the means of at least three 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 Fig. 1. In the case of Figs. 2, 3, 4 and 7 (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.

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