Cu(I)–Glutathione complex: A potential source of superoxide radicals generation

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Cu^{2+} ions and GSH molecules interact to swiftly form the complex Cu(I)–glutathione. We investigated the potential capacity of such complex to reduce molecular oxygen. The addition of SOD to a solution containing Cu(I)–glutathione led to a sustained decline of the basal oxygen level. Such effect was partially reverted by the addition of catalase. The complex was able to induce the reduction of cytochrome *c* and the oxidation of dyhydroethidium into 2-hydroxyethidium. Both effects were totally blocked by SOD. The ability of the complex to generate superoxide radicals was confirmed by EPR spin-trapping. Cu(I)–glutathione induce no oxidation of fluorescein, a hydroxyl radical-sensitive probe. We conclude that in solutions containing the complex, oxygen is continually reduced into superoxide, and that—in absence of interceptors—the latter radicals are quantitatively re-oxidized into molecular oxygen. We suggest that by functioning as a continuous source of superoxide, the complex could potentially affect a broad range of susceptible biological targets.

1. Introduction

Reduced glutathione (GSH), γ-glutamyl-cysteinyl-glycine, is the single most abundant non-protein thiol-containing molecule within cells. While the intracellular concentration of GSH ranges from 2 to 8 mM, the tripeptide occurs extracellularly in concentrations ranging from 5 to 15 μM.[1–3] The occurrence of a thiol moiety in the GSH molecule endows it with the potential to act as a reductant by donating an electron to some endogenous acceptors, and to behave as a stabilizer of free radicals by donating a hydrogen atom to the latter species.[4,5] Although both modes of action could implicate an antioxidant effect, under conditions involving the co-occurrence of a transition metal, such as copper, the tripeptide might also promote a pro-oxidant effect through a metal-reducing action.[6–8] In the latter case, the reduction of Cu^{2+} ions by GSH R_x1 could give place to the formation of a redox-active species capable of catalyzing the subsequent reduction of molecular oxygen into superoxide anion R_x2, and that of hydrogen peroxide into hydroxyl radical[4,9] (R_x3; $k \approx 4.7 \times 10^3\ M^{-1}\ s^{-1}$).

$$2Cu^{2+} + 2GSH \longrightarrow 2Cu^{+} + GSSG \qquad (R_x1)$$

$$Cu^{+} + O_2 \rightleftharpoons Cu^{2+} + O_2{\cdot}^{-} \qquad (R_x2)$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + HO^{\cdot} + HO^{-} \qquad (R_x3)$$

Although the biological conditions which define the prevalence of an antioxidant versus a pro-oxidant action are not fully understood, there is evidence which implicates the interaction between GSH and Cu^{2+} ions in the promotion of both kinds of actions. For instance, GSH can enhance copper-dependent DNA cleavage in vitro,[10,11] probably as a result of redox-cycling of a stable copper–DNA complex.[12] In the case of LDL molecules, however, GSH strongly inhibits Cu^{2+}-dependent LDL oxidation.[13] In cells over-exposed to copper, the tripeptide has been found to largely ameliorate or even prevent the oxidative damage induced by the metal.[14–18] The protecting effects of glutathione against copper-induced cell damage are attributed not only to its antioxidant-related functions,[3,5] but also to the ability of the GSH molecule to interact directly with Cu^{2+} ions, sequestering the metal under a form which otherwise would indiscriminately bind to essential macromolecules.[19] In the presence of a GSH excess, the protection would involve a reduction of Cu^{2+} ions, which is followed by the formation of a Cu(I)–glutathione complex.[14–16] In non-cellular systems such complex is swiftly formed when GSH and Cu^{2+} ions are mixed in a molar ratio equal to or greater than 3:1.[6,20–23] Interestingly, Spear and Aust[24] observed that, depending on whether such ratio is lower or greater that 3:1, copper-dependent oxidative damage to DNA can be either exacerbated or totally prevented by GSH, respectively. The intracellular occurrence of the Cu(I)–glutathione complex has been reported in copper-exposed hepatoma cell lines.[14–16] Although the biological role of the complex has not been fully established, it is believed

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to play a role as copper-carrier to several copper-dependent enzymes[20,25] and to various copper-storing[14–16] and copper-transporting proteins.[25]

Interestingly, despite containing copper under the Cu(I) form, the copper–glutathione complex has been reported to be very stable in aqueous solutions even in the presence of oxygen.[20,21] According to the prevalent view, the cuprous ion in the complex is stabilized by the glutathione molecule in such a way that it prevents the metal from reacting with either oxygen[12] or hydrogen peroxide.[12,26] In the present study, we have undertaken experiments whose results contend with the view that the Cu(I)–glutathione complex is indeed redox-inactive towards oxygen. In fact, we provide evidence that, when present in aqueous solutions, the Cu(I)–glutathione complex continually reacts with molecular oxygen to generate superoxide anions, and that as a result of this, the complex can induce either the oxidation or the reduction of molecules which are redox-susceptible towards superoxide.

2. Results

The overall aim of the studies described below was to evaluate the hypothesis that the Cu(I)–glutathione complex, present in a pre-incubated (3:1) GSH plus Cu^{2+} mixture, reacts continually with molecular oxygen to generate superoxide anions.

2.1. Oxygen consumption experiments

According to above-stated hypothesis, the addition of a mixture containing the Cu(I)–glutathione complex to an aqueous solution containing a basal level of molecular oxygen should lead to a continuous decline in the concentration of oxygen dissolved in the solution. As shown in Figure 1, contrary to what was expected, the concentration of oxygen in the solution remained largely unaltered during, at least, the first 30 min after addition of the complex. Upon addition of SOD, however, the concentration of oxygen started to rapidly decline. This descent was steady, sustained and to some extent proportional to the amount of SOD (from 100–300 U/mL) added to the complex-containing solution. Since SOD

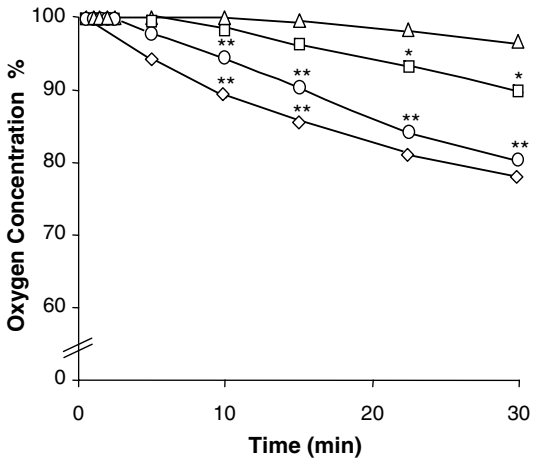

Figure 1. Changes in oxygen concentration in a solution containing the Cu(I)–glutathione complex. Oxygen concentration was continuously monitored (0–30 min) in a phosphate buffer solution (pH 7.4) containing a 15-min pre-incubated mixture of 900 μM GSH plus 300 μM Cu^{2+} both, in the absence (△) and in the presence of added SOD, (□)100 U/mL, (○) 200 U/mL or 300 U/mL (◇). SOD was added at the moment of assaying the concentration of oxygen in the mixtures. The symbol * represents the existence of a significant difference ($p < 0.05$) between the value obtained in the absence and that obtained in the presence of SOD. The symbol ** represents the existence of a significant difference ($p < 0.05$) between the signalled value and that obtained with the closest lower SOD concentration.

catalyzes the dismutation of two moles of superoxide into only one mol of oxygen and one mol of hydrogen peroxide R_x4, the decline in oxygen concentration seen after addition of SOD is consistent with the hypothesis that SOD-removable superoxide anions are being continually generated by the Cu(I)–glutathione containing solution.

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (R_x4)$$

2.2. EPR studies

Figure 2 A depicts a typical EPR spectrum of Cu^{2+} ions (5 mM). A G value of 2.051 was calculated. When Cu^{2+} ions were pre-incubated for 15 min in the presence of 15 mM GSH, no paramagnetic signal was detected (not shown). Likewise, no paramagnetic signal was seen when the time of pre-incubation of the GSH/Cu^{2+} mixture was prolonged from 15 to 300 min. To evaluate whether the GSH/Cu^{2+} mixture is capable of generating superoxide anions, the spin trap DMPO (100 mM) was added. Figure 2B, which depicts the DMPO-derived spectrum after having pre-incubated GSH (15 mM) plus Cu^{2+} (5 mM) for 15 min, reveals the presence of lines that are consistent with the trapping of $O_2^{\cdot-}$ radicals. The adduct formed between DMPO and $O_2^{\cdot-}$ is unstable and decomposes to produce, among several products, DMPO-OH, which is also generated by direct reaction with $HO^{\cdot}$.[27,28] The latter could account for the simultaneous occurrence of signals corresponding to both superoxide and hydroxyl radicals, as suggested by the EPR spectrum depicted in Figure 2B. The appearance of the latter signal was prevented when the GSH/Cu^{2+} mixture was incubated (15 min) in the presence of SOD (300 U/mL); SOD addition generated a spectrum similar to that obtained in absence of the complex (not shown).

Pursuant to demonstrating that the addition of SOD prevented the Cu(I)–glutathione complex from generating the previously mentioned DMPO adduct, we became interested in assessing whether, in the absence of DMPO, removal of superoxide radicals by SOD could result in the appearance of a paramagnetic properties of the complex. The spectrum in Figure 2C, obtained 45 min after incubating a mixture of GSH/Cu^{2+} (15 mM:5 mM) and SOD (200 U/mL), reveals the presence of a paramagnetic signal compatible with a Cu(II)-complex. This latter was found to be highly similar to the hyperfine spectrum obtained upon mixing Cu^{2+} ions plus GSSG (5 mM each), which results in the swift formation of the complex Cu(II)–GSSG (Fig. 2D).[29,30]

2.3. Dihydroethidium oxidation experiments

The postulated ability of the Cu(I)–glutathione complex to generate superoxide anions was also assessed by measuring the increase in fluorescence which arises from oxidizing dihydroethidium, a probe widely used to make evident the formation of such radicals.[31] As shown in Figure 3, the incubation of DHE (50 μM) with a 15 min pre-incubated GSH (75 μM) plus Cu^{2+} (25 μM) mixture, led to a time-dependent increase in fluorescence. Identical results were seen for Cu(I)–glutathione containing mixtures pre-incubated during either 15 or 90 min. The increase in fluorescence induced by the complex-containing mixture was virtually unaffected by the addition of catalase (100 U/mL) which removes the hydrogen peroxide resulting from the superoxide-dependent DHE oxidation. In turn, the addition of SOD (100 U/mL) to such mixture completely blocked such effect. No changes in fluorescence were detected when DHE was incubated with a mixture of Cu^{2+} (25 μM) plus GSSG (75 μM) (data not shown). Recent studies using HPLC have revealed that the oxidation of DHE induced by superoxide-generating systems (such as KO_2 or xanthine/xanthine

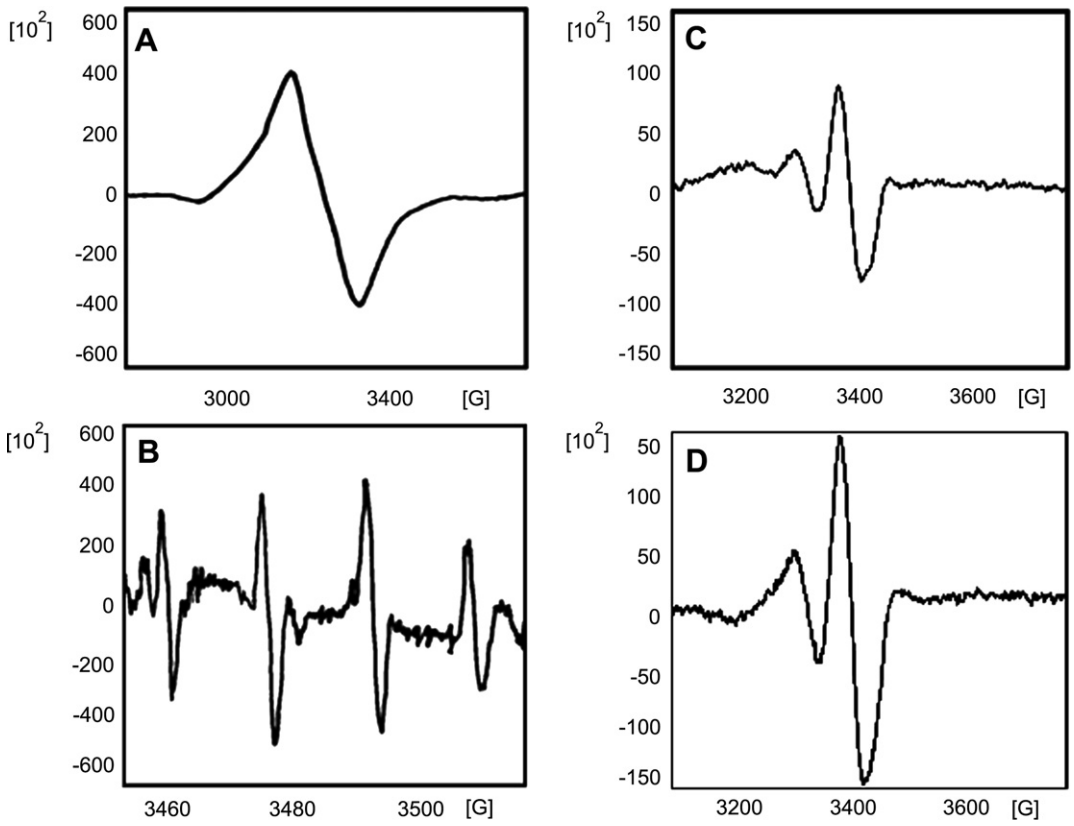

Figure 2. EPR spectra of Cu^{2+} ions and EPR spin-trapping of superoxide radicals. (A) Typical EPR spectrum of Cu^{2+} ions (5 mM); (B) spectrum resulting from adding DMPO (100 mM) to a sample containing the Cu(I)–glutathione complex (GSH 15 mM/Cu^{2+} 5 mM); (C) spectrum obtained 45 min after the addition of SOD (200 U/mL) to a GSH/Cu^{2+} mixture (15 mM:5 mM; pre-incubated during 15 min); (D) spectrum of a solution containing the Cu(II)–GSSG complex, prepared by mixing Cu^{2+} and GSSG, at 5 mM each. Experimental and instrumental EPR conditions were as described in Section 5.

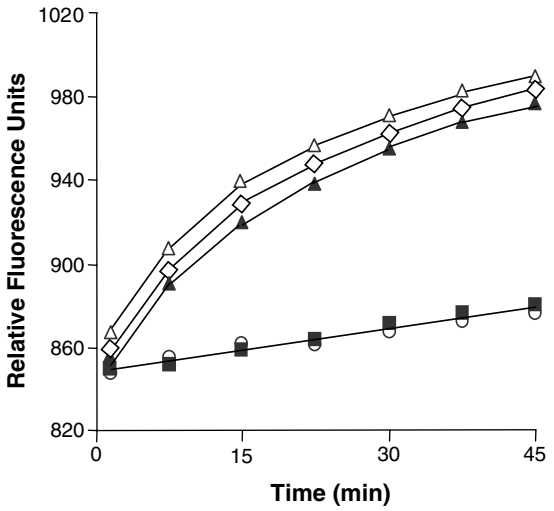

Figure 3. Dihydroethidium oxidation by the Cu(I)–glutathione complex. DHE oxidation products were monitored during 45 min and expressed as the increase in the relative fluorescence units (excitation 470 nm and emission 590 nm) that followed the addition of DHE (50 μM) to a solution containing the Cu(I)–glutathione complex (GSH 75 μM/Cu^{2+} 25 μM). Symbols represent the fluorescence of a solution containing DHE alone (○), or that resulting from adding DHE to a mixture of GSH plus Cu^{2+}, pre-incubated during either 15 min (△) or 90 min (◇). The symbols (■) and (▲) represent the addition of DHE to a mixture containing the Cu(I)–glutathione complex (pre-incubated during 15 min) in the presence of SOD or catalase (100 U/mL each), respectively.

oxidase), involves two different fluorescent products, 2-hydroxyethidium and ethidium.[32,33] While the generation of the former occurs specifically by interaction with superoxide, the latter is not linearly related to superoxide concentration. In fact, E^+ formation can be induced by other oxidants and/or secondarily in more complex pathways of DHE oxidation.[32,34] To obtain additional evidence on the postulated ability of the Cu(I)–glutathione complex to generate superoxide anions, the formation of E-OH during the incubation of DHE with a 3:1 GSH/Cu^{2+} mixture was assessed. After separation by HPLC, the fluorescence of DHE and its major reaction products (E-OH and E^+) is depicted in Figure 4A and B, respectively. The oxidation of DHE induced by the Cu(I)–glutathione complex led to a decrease in peak p1, corresponding to DHE, and to a concomitant increase in peaks p2 and p3. While p3 corresponded to ethidium (confirmed through the use of a standard), p2 was inferred to represent E-OH since only the increase in this peak was totally abolished by the addition of SOD to the incubation (not shown). The chromatogram in Figure 4B also shows a small peak p4, whose identity remains to be established. While its appearance in the chromatogram of the DHE plus GSH/Cu^{2+} mixtures was unaffected by the addition of SOD, its emergence could be induced by the sole addition of Cu^{2+} ions to DHE (data not shown).

The quantitative changes, expressed as fold-increments relative to the basal area (concentration) of E-OH and E^+, following the incubation of DHE with a GSH/Cu^{2+} mixture, are depicted in Figure 5. The complex-containing mixture induced a time-dependent increase in the concentration of both fluorescent products. However, in the case of E-OH, the increment was faster during the first 30 min of incubation, and after 90 min it had reached approximately 20-fold; in the case of E^+, the maximal increment was near 10-fold. While the addition of SOD to the incubation of DHE with the Cu(I)–glutathione complex virtually abolished the increment in E-OH concentration, the increment in E^+ was unaffected by SOD.

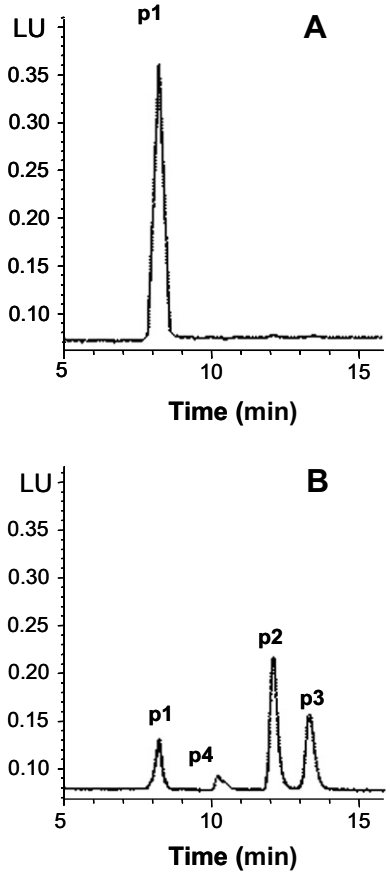

Figure 4. HPLC chromatogram of dihydroethidium and its major oxidation products. (A) peak corresponding to DHE (50 μM); (B) peaks resulting from incubating DHE for 90 min in the presence of the Cu(I)–glutathione complex (GSH 75 μM/Cu^{2+} 25 μM). Peaks p1, p2 and p3 correspond to DHE, 2-hydroxyethidium, and ethidium, respectively. Peak p4 was not identified. HPLC conditions were as described in Section 5.

2.4. Fluorescein oxidation experiments

To evaluate the possibility that the Cu(I)–glutathione complex could also generate hydroxyl radicals, the decrease in fluorescence resulting from the oxidation of fluorescein was measured. The addition of a 15 min pre-incubated (3:1) GSH/Cu^{2+} mixture to a system containing fluorescein did not affect the relative fluorescence values (Fig. 6). When hydrogen peroxide was added to the complex-containing mixture, a slight but non-significant decline ($p > 0.1$) in the fluorescence was observed. In contrast, a swift and substantial decrease in fluorescence was observed when hydrogen peroxide, Cu^{2+} and ascorbic acid (as Cu^{2+}-reducing agent) were added. Comparatively, in the absence of added peroxide, the copper–ascorbate mixture induced only a minor oxidation of fluorescein. The latter effect is likely to reflect the one-electron reduction of hydrogen peroxide molecules which are produced through the autodismutation of superoxide anions generated during the direct interaction between oxygen and ascorbate-reduced Cu^{+} ions.

2.5. Cytochrome *c*-reduction experiments

To further make evident the ability of the Cu(I)–glutathione complex to generate superoxide radicals, the capacity of the pre-incubated (30 μM:10 μM) GSH plus Cu^{2+} mixture to reduce cytochrome *c* was evaluated. As shown in Figure 7A, the addition of such mixture to a solution containing Cyt *c* led to a rapid and substantial reduction of the cytochrome (expressed as an increment in the OD_{550nm}). The initial rate of Cyt *c* reduction induced by the mixture was only slightly slower than that induced by the addition of a xanthine/xanthine-oxidase mixture, a well-recognized $O_2^{\cdot -}$-generator. The sole addition of GSH (30 μM) had, in turn, no Cyt *c*-reduction effect. An identical Cyt *c*-reducing capacity was observed when a GSH/Cu^{2+} (30 μM:10 μM) mixture pre-incubated during 60 min instead of 15 min was assessed (not shown). Figure 7B depicts the results from adding SOD to a complex-containing mixture

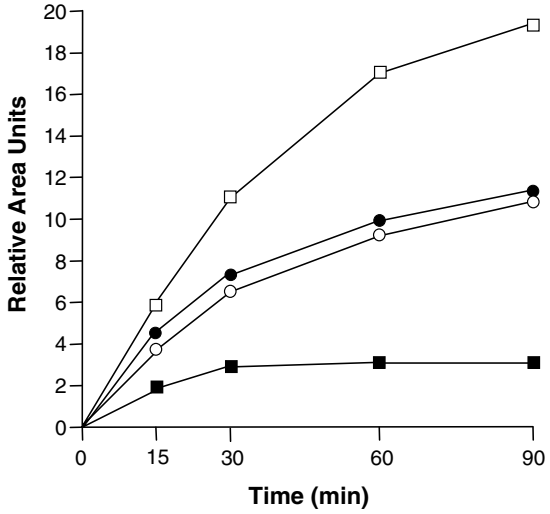

Figure 5. Oxidation-dependent changes in dihydroethidium fluorescence. DHE (50 μM) was added to a solution containing the Cu(I)–glutathione complex (GSH 75 μM/Cu^{2+} 25 μM) and the mixture was incubated during 0–90 min. Samples obtained along the incubation were assayed by HPLC for the two major DHE oxidation products: 2-hydroxyethidium and ethidium. Results are expressed as relative area units and represent the fold-number increase in the basal area (as defined by the area obtained after 1 min of incubation). The symbols correspond to the changes in 2-hydroxyethidium (squares) and ethidium (circles) which followed the incubation of DHE plus the complex both, in the absence (open symbols) or presence of 200 U/mL SOD (dark symbols).

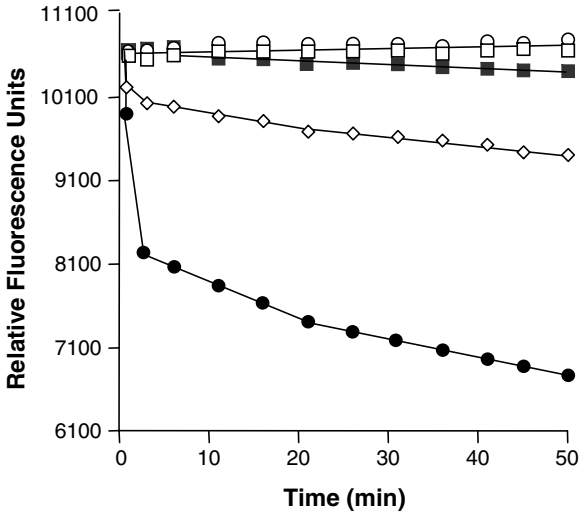

Figure 6. Oxidation-dependent changes in the fluorescence of fluorescein. Fluorescein (20 nM) was added to a solution containing the Cu(I)–glutathione complex (GSH 75 μM/Cu^{2+} 25 μM) and the mixture was incubated for 0–50 min. The decrease in fluorescence resulting from fluorescein oxidation was expressed as changes in the relative fluorescence units. The symbols correspond to a solution containing fluorescein alone (○), or to solutions containing fluorescein plus either Cu(I)–glutathione (□), Cu(I)–glutathione/H_2O_2 (■), ascorbic acid/Cu^{2+} (◇), or ascorbic acid/Cu^{2+}/H_2O_2 (●). Further details and the corresponding concentrations are described in Section 5.

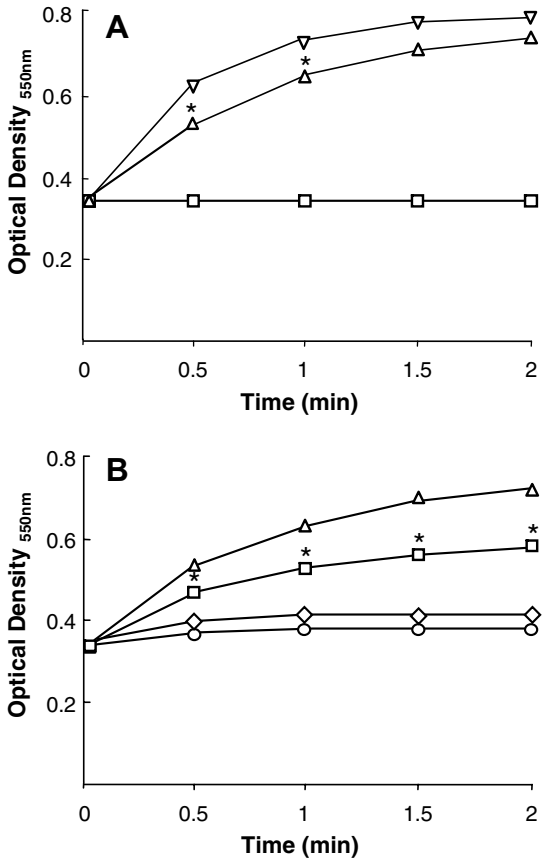

Figure 7. Superoxide-dependent reduction of cytochrome *c* by the Cu(I)–glutathione complex. Cytochrome *c* reduction was assessed by monitoring the increase in OD_{550nm} that followed the addition of Cyt *c* (50 μM) to a solution containing: (A) the Cu(I)–glutathione complex (△; GSH 30 μM/Cu^{2+} 10 μM), GSH alone (□; 30 μM), or a mixture of xanthine/xanthine oxidase (▽; 0.5 mM, 40 mU/mL); (B) the Cu(I)–glutathione complex alone (△; GSH 30 μM/Cu^{2+} 10 μM), or the same complex added SOD directly—namely, during the reduction assay—at 50 U/mL (□) or 150 U/mL (◇). The symbol (○) represents a system in which Cyt *c* was added to a Cu(I)–glutathione solution that—prior to the reduction assay—had been pre-incubated with SOD (50 U/mL) during 60 min. The symbol * represents the existence of a significant difference ($p < 0.05$) between the signalled value and all other experimental points.

on the ability of the latter to reduce Cyt *c*. The direct addition of 50 U/mL of SOD to the mixture led to a partial decrease in the extent of Cyt *c* reduction. Comparatively, the addition of 150 U/mL of SOD to such mixture totally prevented the ability of the latter to reduce Cyt *c*. However, when previous to the Cyt *c* assay, the complex-containing mixture was pre-incubated with 50 U/mL of SOD during 60 min, the resulting mixture failed to promote any reduction of Cyt *c*. Similarly, a total absence of Cyt *c*-reducing capacity was seen when the GSH plus Cu^{2+} mixture was incubated during 60 min in the presence of SOD plus catalase (50 U/mL each) (not shown).

3. Discussion

The formation and occurrence of the Cu(I)–glutathione complex has been well documented both, in non-cellular systems[6,20–23,35] as well as within cells exposed to copper excess.[14–16] From a redox point of view, the intracellular occurrence of Cu(I)–glutathione is of interest since this complex has been shown to be quite stable in aqueous solutions even in the presence of molecular oxygen.[20,21] In fact, early work by Ciriolo et al.[20] demonstrated that, when a solution containing the Cu(I)–glutathione complex was incubated at 37 °C under aerobic conditions, neither its ^{1}H NMR nor its EPR (Cu(II)-absent) spectra underwent changes, for at least 5 h after its preparation. To explain the stability of the Cu(I)–glutathione complex in oxygen-containing solutions, it has been proposed that upon chelating the metal, the glutathione molecule would stabilize the Cu(I) ion under a form that renders the complex redox-inactive towards oxygen.[12] Our study contends with this concept as it provides direct evidence for the ability of the complex to reduce oxygen. In fact, based on our results, we postulate that the Cu(I)–glutathione complex would continually react with molecular oxygen to generate superoxide anions R_x5.

$$\text{Cu(I)-complex} + O_2 \rightleftharpoons \text{Cu(II)-complex} + O_2^{\cdot -} \qquad (R_x5)$$

Initial support for the latter emerged from the demonstration that, in a solution containing the Cu(I)–glutathione complex, the basal level of oxygen started to decay only upon addition of SOD. Such a result can be construed as an indication that superoxide anions are being continually generated by the complex R_x5. Since SOD catalyzes the conversion of two moles of superoxide into one mole of oxygen and one of hydrogen peroxide (R_x4; $k \approx 2 \times 10^9\ M^{-1}\ s^{-1}$), the descent in oxygen concentration observed after SOD addition is likely to reflect only half of the actual extent of superoxide radicals formed. In accordance to R_x6 ($k \approx 2 \times 10^7\ M^{-1}\ s^{-1}$), the addition of catalase led to a partial reversal of the ongoing descent in oxygen concentration induced by the prior addition of SOD.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \qquad (R_x6)$$

To explain why in a solution containing the complex, the basal level of oxygen remains unaltered in the absence, but not in the presence of SOD, we propose that SOD-removable superoxide anions are permanently generated by the complex, and that in the absence of SOD, such radicals are continually and quantitatively re-oxidized into oxygen R_x5. It could be speculated that while the reduction of oxygen into superoxide anions involves the obliged oxidation of the complex, the re-generation of molecular oxygen would involve the use of superoxide as reductant of an 'oxidized form' of the complex. Consistent with the latter, in our EPR experiments we observed that the addition of SOD to a Cu(I)–glutathione containing solution results in a paramagnetic signal which reveals that a Cu(II)-containing complex has formed. The high similarity seen between the paramagnetic signal of the latter and that of a solution containing a preformed Cu(II)–GSSG complex,[29,30] strongly suggests that the structure of the 'oxidized form' of the complex postulated in this study corresponded to Cu(II)–GSSG.

Further evidence of the ability of the Cu(I)–glutathione complex to generate superoxide anions emerged from using DHE as a superoxide probe.[31] Noteworthy, the oxidation of this probe is not subjected to artefactual redox-cycling,[36] and it is unaffected by reductants such as glutathione[37] or oxidants such as hydrogen peroxide.[32] Coherent with the concept that superoxide radicals are generated by the Cu(I)–glutathione through the continuous reduction of molecular oxygen, we observed that this complex induced a marked increment in the fluorescence of DHE oxidation products. We confirmed that DHE oxidation was indeed caused by superoxide anions by showing a sustained increment in the formation of 2-hydroxyethidium, a metabolite whose formation is generated solely upon the interaction between DHE and superoxide.[32,33] The latter effect was totally abolished by the presence of SOD.

Additional support for the ability of the Cu(I)–glutathione complex to generate superoxide anions was obtained by the demonstration that the complex is also effective in inducing the reduction of cytochrome *c*. Likewise, as seen in the oxygen and DHE experiments, Cyt *c* reduction was also found to be both sustained in time and susceptible to be inhibited by SOD. Interestingly, we observed that SOD could induce a total inhibition of

Cyt *c* reduction, either when sufficient amounts of the enzyme were added together with the complex directly to the Cyt *c*-containing solution or when, amounts of SOD previously shown to be insufficient to induce total inhibition of Cyt *c* reduction, were pre-incubated with the complex and then added to the Cyt *c*-containing solution. In the latter case, a 60 min preincubation (instead of 3 min) was needed to attain total inhibition. Presumably, the need to prolong the preincubation appears to reflect that, at least when no superoxide interceptors such Cyt *c* are present, the complex generates (or releases) superoxide anions at a relatively slow rate. The preincubation of the complex with SOD during 60 min is likely to have provided enough time for the enzyme to dismutate the total amount of $O_2^{\cdot -}$ anions susceptible to be generated by the complex during such preincubation time period. According to our postulate, removal of $O_2^{\cdot -}$ anions by SOD would preclude the Cu(I)–glutathione complex from being regenerated from its 'oxidized form'. Since the effect of SOD was not altered by the co-addition of catalase, the loss of ability of the SOD-pre-incubated complex to reduce Cyt *c* is not attributable to hydrogen peroxide accumulation.

In addition to the use of molecules susceptible to undergo oxidation or reduction as probes for evidence of the formation of superoxide anions, the present study also supports the formation of such radicals by showing their direct spin-trapping with DMPO. Although the resulting EPR spectrum suggests that some hydroxyl radicals might also be formed (aN = 14,8 G), the latter would be rather attributable to the unstable character of DMPO-OOH adduct, known to easily undergo decomposition into the product generated by the reaction between the DMPO and HO· radicals.[27] In addition, the GSH molecules present in the 3:1 GSH/Cu^{2+} mixture, could themselves induce a two-electron reduction of DMPO-OOH into DMPO-OH.[28] Most importantly, however, we observed that the formation of the DMPO spectrum generated by the Cu(I)–glutathione could be totally prevented when SOD was added (not shown). On the other hand, results from experiments conducted with fluorescein, a highly sensitive probe for hydroxyl radicals[38] but insensitive to superoxide anions,[39] would rule out the possibility that, in addition to generating superoxide, the complex also generates hydroxyl radicals. The lack of ability of the Cu(I)–glutathione complex to reductively decompose hydrogen peroxide into HO· observed by us confirms results by others investigators.[12,24,26,40] However, the current study demonstrates that the purported redox stabilization of Cu(I) in the complex does not limit the ability of the metal to react with molecular oxygen. While we agree with the reputed stability of the complex in oxygenated aqueous solutions,[20,21] we believe that such stability does not involve a redox-inactivity of the complex towards oxygen.

4. Conclusions

Based on the use of SOD, various superoxide-susceptible probes, and an agent capable of spin-trapping superoxide radicals, we conclude that in solutions containing the Cu(I)–glutathione complex, oxygen is continually reduced into superoxide, and that—in the absence of superoxide interceptors—the latter radicals would be quantitatively re-oxidized into molecular oxygen. At this point, it would seem reasonable to assume that the formation of superoxide occurs in a reversible manner and with a very fast back reaction, much like recently proposed for mitochondrial formation of superoxide.[41] The latter contention provides a basis to explain the undisputable stability of the Cu(I)–glutathione complex under aerobic conditions.[20,21] More importantly, however, we consider that the redox-activity of the complex towards oxygen reported here could imply that, by functioning as a continuous source of superoxide radicals, the complex could participate or affect a broad range of susceptible target molecules. Future studies to further characterize the redox-activity of the Cu(I)–glutathione complex should contemplate conditions relevant to those occurring in biological environments.

5. Materials and methods

5.1. Chemicals and reagents

Cupric chloride ($CuCl_2{\cdot}2H_2O$), cytochrome *c* (Cyt *c*; bovine heart), reduced glutathione, oxidized glutathione (GSSG), superoxide dismutase (SOD; EC 1.15.1.1 from bovine erythrocytes), catalase (EC 1.11.1.6 from bovine liver), xanthine oxidase (EC 1.1.3.22 from buttermilk), ethidium bromide, fluorescein sodium salt, DMPO (5,5-dimethyl-1-pyrroline *N*-oxide), ascorbic acid, hydrogen peroxide, DMSO, acetonitrile and trifluoroacetic acid were all purchased from Sigma–Aldrich. Dihydroethidium (DHE) and xanthine were purchased from Calbiochem. All aqueous solutions were prepared in Chelex-100-treated sodium phosphate buffer (120 mM; pH 7.4).

5.2. Oxygen consumption experiments

The Cu(I)–glutathione complex was prepared as previously described,[20] by mixing in sodium phosphate buffer, $CuCl_2$ and GSH to a final concentration of 300 μM and 900 μM, respectively. The 3:1 molar GSH excess mixture was pre-incubated (during 15 min at 22 °C) in order to secure the formation of the Cu(I)–glutathione complex.[20] The concentration of oxygen in a Cu(I)–glutathione complex-containing solution was monitored (during 0–30 min at 22 °C), using a Clark-type oxygen electrode (Yellow Spring Instrument, model 5300). In some experiments, various amounts of SOD (100–300 U/mL) were added to the above solution. In control experiments (run at 22 °C), no changes in the basal oxygen level of a solution containing no-complex were observed during 30 min when either SOD (300 U/mL), catalase (100 U/mL), GSH (900 μM) or $CuCl_2$ (300 μM) were added.

5.3. Electron paramagnetic resonance (EPR) studies

The presence or absence of paramagnetic signals in a solution containing either $CuCl_2$ alone (5 mM; thereafter Cu^{2+}), or a mixture of Cu^{2+} (5 mM) plus GSH (15 mM) pre-incubated (at 22 °C) during either 15 or 300 min, was assessed using EPR. The mM concentrations of $CuCl_2$ and GSH used in this study provide well resolved and highly reproducible EPR spectra. In some experiments, SOD (200 U/mL) was added. For comparative purposes, the EPR spectrum of a preformed Cu(II)–GSSG complex,[29,30] which was prepared by previous mixing Cu^{2+} and GSSG (1:1), was assessed. To investigate the possible formation of reactive oxygen species, such as $O_2^{\cdot -}$ or HO·, by the latter mixture, the spin-trap DMPO (100 mM) was added when starting the EPR recordings. Spectra were recorded in a Bruker ECS 106 spectrometer, using an X band (9.85 GHz), a rectangular cavity and 50 kHz field modulation at 22 °C, under the following conditions: frequency, 9.79 GHz; centre field, 3180 gauss; amplitude modulation, 0.9 gauss; microwave power 25 mW; time constant 20 ms, time scan 40 s.

5.4. Dihydroethidium oxidation experiments

The oxidation of DHE was monitored fluorimetrically in a 96-well plate using a Synergy HT multilector. Excitation and emission wavelengths were 470 nm and 590 nm, respectively. Freshly prepared DHE, dissolved in DMSO, was added (50 μM) to wells containing mixtures of Cu^{2+} (25 μM) plus GSH (75 μM) pre-incubated

(22 °C) during either 15 min or 90 min. Readings were carried out at 30 °C during 0–45 min. When used, catalase or SOD were added alone to a final activity of 100 U/mL, and GSSG to a concentration of 75 μM. A control of DHE alone (50 μM) was included.

5.5. HPLC analysis of DHE and DHE-derived products

DHE, ethidium (E^+), and 2-hydroxyethidium (EOH) were separated as described by Fink et al.[37] using an HPLC system (HP Agilent 1100 Series) equipped with a C-18 reverse phase column (Agilent 4.6 × 150 mm) and a fluorescence detector. Fluorescence was monitored at 580 nm (emission) and 480 nm (excitation). The mobile phase was composed of a gradient containing 60% acetonitrile and 0.1% trifluoroacetic acid. DHE and its oxidation products were separated by a linear increase in acetonitrile concentration from 37% to 47% over 25 min at a flow rate of 0.2 mL/min. Mixtures containing the Cu(I)–glutathione complex (prepared by pre-incubating 15 min, 22 °C) and a mixture of GSH and Cu^{2+} (75 μM:25 μM) were added into DHE (50 μM) and incubated for various lengths of time (from 0 to 90 min). When used, SOD was added at 200 U/mL.

5.6. Fluorescein oxidation experiments

The oxidation of fluorescein was monitored fluorimetrically in a 96-well plate using a Synergy HT multilector. Excitation and emission wavelengths were 485 nm and 520 nm, respectively. Fluorescein, as a freshly prepared solution, was added (20 nM) to wells containing mixtures of GSH (75 μM) plus Cu^{2+} (25 μM) pre-incubated (22 °C) during 15-min. Readings were carried out at 30 °C during 0–50 min. Controls were carried out using fluorescein alone (20 nM) and a mixture of Cu^{2+}/ascorbic acid/hydrogen peroxide (25 μM/100 μM/250 μM).

5.7. Cytochrome *c* reduction assay

The superoxide-dependent reduction of cytochrome *c* was assessed as described before[42] by monitoring the increase in OD_{550nm} that followed the addition of Cyt *c* (50 μM) to cuvettes containing a pre-incubated (15-min, 22 °C) mixture of GSH (30 μM) plus Cu^{2+} (10 μM). Controls of reduction were carried out using a mixture of xanthine/xanthine oxidase (0.5 mM, 40 mU/mL). The influence SOD on the reduction of Cyt *c* was assessed using either a pre-incubated mixture of the complex (Cu^{2+} 10 μM/GSH 30 μM) with the enzyme (50 U/mL during 60 min, 22 °C) or a non-pre-incubated mixture of the complex plus the enzyme added directly (50 and 150 U/mL). Neither GSH (30 μM) nor Cu^{2+} (10 μM), each added alone, had an effect on Cyt *c* reduction.

5.8. Data expression and analysis

Data points in figures represent the means of at least three-independent experiments, each conducted in quadruplicate. The SD of such data is not included as this generally represented less than 10% of the means. When evaluated, statistical significance between points was assessed using the Student's *t*-test. Differences at $p < 0.05$ were considered to be significant. GraphPad Prism 4 was used as statistical software.

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