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Superoxide-dependent reduction of free Fe^{3+} and release of Fe^{2+} from ferritin by the physiologically-occurring Cu(I)–glutathione complex

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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 radicals ($O_2^{\cdot-}$). Based on such ability, we addressed the potential of this complex to generate the redox-active Fe^{2+} species, during its interaction with free Fe^{3+} and with ferritin-bound iron. Results show that: (i) the complex reduces free Fe^{3+} through a reaction that totally depends on its $O_2^{\cdot-}$-generating capacity; (ii) during its interaction with ferritin, the complex reduces and subsequently releases iron through a largely (77%) SOD-inhibitable reaction; the remaining fraction is accounted for by a direct effect of GSH molecules contained within the complex. The $O_2^{\cdot-}$-dependent iron-releasing efficiency of the complex was half that of its iron-reducing efficiency; (iii) the ability of the complex to release ferritin-bound iron was increased, concentration-dependently, by the addition of GSH and totally prevented by SOD; (iv) in the presence of added H_2O_2, the Fe^{2+} ions generated through (i) or (ii) were able to catalyze the generation of hydroxyl radicals. Thus, the present study demonstrates the ability of the Cu(I)–$[GSH]_2$ complex to generate the redox-active Fe^{2+} species and suggest that by favouring the occurrence of superoxide-driven Fenton reactions, its pro-oxidant potential could be increased beyond its initial $O_2^{\cdot-}$-generating capacity.

1. Introduction

Iron is an essential element for all living cells. It plays a vital role as component of heme-containing proteins, and as a metal cofactor of non-heme iron-containing enzymes.[1,2] Despite its essential nature, iron can be potentially toxic when present as 'free ion'.[3] Its toxicity may arise from its capacity to participate, via the Fenton reaction (1), as a catalyst in the formation of hydroxyl radical, a species capable of inducing the oxidation of lipids, proteins and DNA.[3–5]

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^{\cdot} + HO^{-} \quad (k = 70\ M^{-1}\ s^{-1})^{6} \qquad (1)$$

As a form to secure its availability and to protect cells from undergoing the toxic effects of free iron ions, the metal is bound to various peptides and proteins, among which ferritin is recognized as the single most important iron-storing protein.[1,4,7] In fact, ferritin has been estimated to be able to sequester up to 4500 iron atoms per molecule; storage of such atoms occurs under the form of a ferrihydrite mineral core.[8,9] Although much is known about the ability of ferritin to recycle and make readily available iron for cellular needs,[7,10] relatively little is known about the physiological mechanisms underlying the release of iron from this protein. Some studies indicate that iron would be released from ferritin mainly by a mechanism that involves its lysosomal,[11,12] and proteosomal degradation.[13] It has been suggested however that the release of iron achieved after proteolytic degradation can be studied only under complex artificial conditions.[14]

In the absence of reducing agents, the iron bound to the ferritin core is stable, it does not exchange among molecules but is susceptible to be slowly released by Fe(III) chelators such as desferroxamine.[15] Release of iron from ferritin has been also reported to follow the exposure of ferritin to physiologically-occurring reducing agents such as flavins,[16] some thiols,[16,17] and ascorbate.[18,19] In addition to such agents, superoxide anions ($O_2^{\cdot-}$), which are continually generated within cells,[20] have been shown to release iron from ferritin. Thus, $O_2^{\cdot-}$ generated in vitro by PMA-stimulated polymorphonuclear leukocytes,[21] by a xanthine/xanthine oxidase system,[22,23] or by di(4-carboxibenzil)hyponitrito (SOTS-1),[24] have been shown to effectively reduce and release iron from ferritin. Recently, our laboratory reported,[25] that Cu(I)–$[GSH]_2$, a complex known to occur physiologically and to accumulate in copper-exposed cells,[26,27] is able to function as a continuous source of $O_2^{\cdot-}$. The latter poses the complex as a potential intracellular

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mechanism to modify the functioning of various superoxide-susceptible biological targets. Considering the previously reported capacity of $O_2^{\cdot-}$ to induce the release of iron from ferritin,[21–24] in the present study we addressed in vitro the potential of the Cu(I)–$[GSH]_2$ complex to reduce free Fe^{3+} and to release Fe^{2+} from ferritin. In addition, the possibility that the Fe^{2+} ions expected to be generated from the reduction of Fe^{3+} and/or released from ferritin could catalyse the generation of hydroxyl radicals was evaluated. Recently, we observed that the capacity of the Cu(I)–$[GSH]_2$ complex to generate $O_2^{\cdot-}$ can be substantially increased in the presence of a molar excess of GSH.[25] Prompted by the latter observation, we also investigated the extent to which GSH molecules, added in near-physiological concentrations, could affect the herein postulated ability of the Cu(I)–$[GSH]_2$ complex to induce the reduction and release of iron from ferritin.

2. Results

2.1. Superoxide-dependent Cyt *c* reduction by the Cu(I)–$[GSH]_2$ complex

Prior to addressing the effect of the interaction between iron (both, free and ferritin-bound) and the complex, we evaluated whether in the range of concentrations used in the present study, a direct relationship exists between the concentration of the Cu(I)–$[GSH]_2$ complex and the magnitude at which the $O_2^{\cdot-}$ are generated. To assess the latter, Cyt *c*, which undergoes reduction by $O_2^{\cdot-}$ according to a 1:1 molar reaction,[28] was used as a superoxide-sensitive probe. To ascertain the participation of $O_2^{\cdot-}$, these experiments were carried out both, in absence and presence of SOD. As depicted in Figure 1a, at concentrations of the complex Cu(I)–$[GSH]_2$ equal to or lower than 10 μM, Cyt *c* was reduced in a concentration-dependent near linear manner. Assuming that 1 mol of complex generates 1 mol of $O_2^{\cdot-}$,[29] the reduction of Cyt *c* by low concentrations of the complex appears to be a very efficient process (Fig. 1a). An apparently lower Cyt *c*-reducing efficiency is observed, however, for concentrations of the complex greater than 10 μM. No increment in Cyt *c* reduction was evident for the 20–30 μM range of concentration. Regardless of the concentration of the complex, Cyt *c* reduction was substantially but not totally inhibited by SOD (250 U/well). Further additions of SOD (up to 500 U/well) did not result in greater degrees of inhibition (not shown). Since the fraction of Cyt *c* that was not susceptible to inhibition by SOD seemed to depend on the concentration of the complex (Fig. 1a), we investigated whether GSH molecules were, as such, able to reduce Cyt *c*, accounting for the SOD-insensitive reduction. As shown in Figure 1b, around 10% of the Cyt *c* that was reduced by the complex (10 μM) was not susceptible to inhibition by SOD. When GSH was added at 20 μM, a concentration which equals that of the GSH molecules present in a 10 μM concentration of the Cu(I)–$[GSH]_2$ complex, the magnitude of the reduction induced by the thiol was identical to that induced by the mixture of 10 μM Cu(I)–$[GSH]_2$ plus SOD (Fig. 1b). The effect of adding GSH alone was not affected by the addition of SOD.

2.2. Reduction of free-Fe^{3+} by the Cu(I)–$[GSH]_2$ complex

In view of the ability of Cu(I)–$[GSH]_2$ to generate $O_2^{\cdot-}$, we investigated the capacity of such complex to reduce free Fe^{3+} ions. The latter was assessed using TPTZ and BPS as Fe^{2+}-chelating agents (Table 1), and was expressed as ΔOD at 593 and 530 nm, and their corresponding μM concentration values, respectively. The ability of the complex to reduce Fe^{3+} was compared to that of ascorbate and GSH. Using the extinction coefficients of the Fe(II)-complexes for TPTZ and BPS (Section 5), we estimated that ascorbate (250 μM) reduced Fe^{3+} by near 90%. Comparatively, using the same chelating agents, a 10 μM concentration of the complex was found to reduce Fe^{3+} by 81% (TPTZ) and 83% (BPS). Over 90% of such reductions were SOD-inhibitable (Table 1). GSH (100 μM) totally failed to reduce Fe^{3+} (not shown).

In addition, Table 1 provides data on the degree at which each chelating agent, TPTZ and BPS, might bind Cu^{2+} or Cu^{2+} plus ascorbate (as a form of generating Cu^{+} ions). Comparatively, the reaction between such ions and BPS gave place only to a negligible increase in the OD at which Fe^{3+} reduction was evaluated. Thus, BPS was used thereafter to further characterize the ability of the Cu(I)–$[GSH]_2$ complex to reduce iron (both, in its free and ferritin-bound forms).

Figure 2 depicts the effect of increasing concentrations of the Cu(I)–$[GSH]_2$ complex on the reduction of free Fe^{3+}. While the reduction of free-Fe^{3+} increased linearly as the concentrations of the complex increased from 5 to 10 μM, concentrations of the latter greater than 10 μM (and up to 30 μM) were associated with

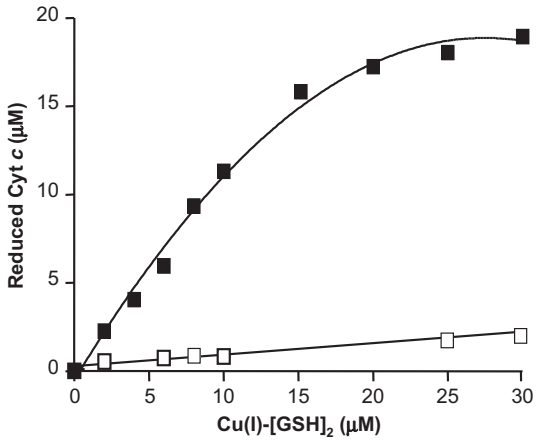

Figure 1a. Effect of increasing concentrations of Cu(I)–$[GSH]_2$ on the reduction of cytochrome *c*. Cyt *c* (50 μM) was added to solutions containing increasing concentrations of the Cu(I)–$[GSH]_2$ complex (2–30 μM) and 2 min after the OD_{550nm} was recorded. Results are expressed as concentration of reduced Cyt *c* (μM). The symbols (□) and (■) represent the reduction of Cyt *c* in the presence and absence of SOD (250 U/well), respectively.

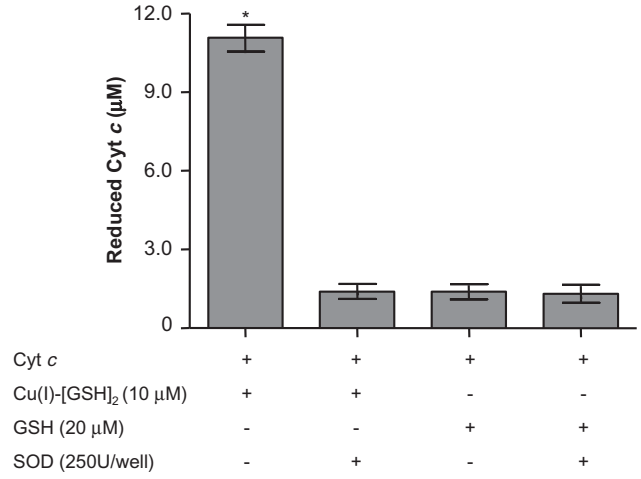

Figure 1b. Effect of GSH on the reduction of cytochrome *c*. Cyt *c* (50 μM) was added to solutions containing the Cu(I)–$[GSH]_2$ complex (10 μM) or GSH (20 μM) and 2 min after, the OD_{550nm} was recorded. Results are expressed as concentration of reduced Cyt *c* (μM). The superoxide dependence was evaluated in the presence of SOD (250 U/well). The asterisk indicates that such value is statistically different, at the level of a p <0.001, from the other values.

Table 1
Reduction of Fe^{3+} induced by Cu(I)–$[GSH]_2$: comparative suitability of using TPTZ or BPS as Fe(II)-chelating agents

Conditions	$\Delta OD_{593\ nm}$	Conditions	$\Delta OD_{530\ nm}$
TPTZ + Fe(III) + AA	0.229 [10.13 μM]	BPS + Fe(III) + AA	0.201 [9.08 μM]
TPTZ + Fe(III)	0.026 [1.15 μM]	BPS + Fe(III)	0.008 [0.36 μM]
TPTZ + Fe(III) + Cu(I)–$[GSH]_2$	0.207 [9.15 μM]	BPS + Fe(III) + Cu(I)–$[GSH]_2$	0.192 [8.67 μM]
TPTZ + Fe(III) + Cu(I)–$[GSH]_2$ + SOD	0.016 [0.71 μM]	BPS + Fe(III) + Cu(I)–$[GSH]_2$ + SOD	0.015 [0.68 μM]
TPTZ + Cu(II)	0.030 [1.33 μM]	BPS + Cu(II)	0.001 [0.005 μM]
TPTZ + Cu(II) + AA	0.028 [1.24 μM]	BPS + Cu(II) + AA	0.009 [0.41 μM]

BPS (1 mM) or TPTZ (1 mM) were added to solutions containing a fixed concentration of $FeCl_3$ (10 μM). The experiments were carried out in presence of the Cu(I)–$[GSH]_2$ complex (10 μM) or AA (250 μM). SOD was added to demonstrate the superoxide-dependence (250 U/mL). Results are expressed as ΔOD_{530nm} for BPS and ΔOD_{593nm} for TPTZ and their corresponding μM concentration values (square brackets).

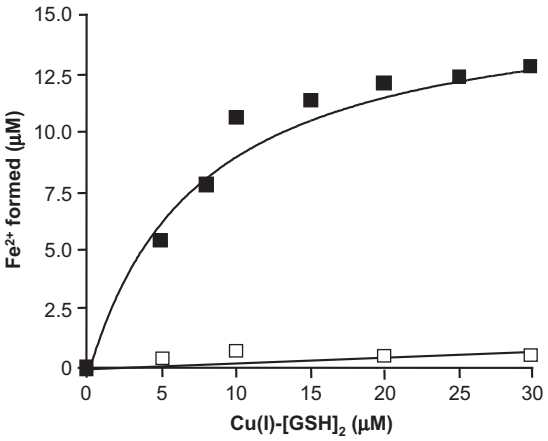

Figure 2. Effect of increasing concentrations of Cu(I)–$[GSH]_2$ on the reduction of free Fe^{3+}. BPS (1 mM) was added to solutions containing $FeCl_3$ (50 μM) and increasing concentrations of the Cu(I)–$[GSH]_2$ complex (5–30 μM). After 15 min, the OD_{530nm} was recorded. Results are expressed as concentration of Fe^{2+} formed (μM), using an ϵ value of 22.14 $mM^{-1}\ cm^{-1}$ for the Fe^{2+}–BPS complex. The symbols (□) and (■) represent the reduction of free Fe^{3+} in the presence and absence of SOD (250 U/well), respectively.

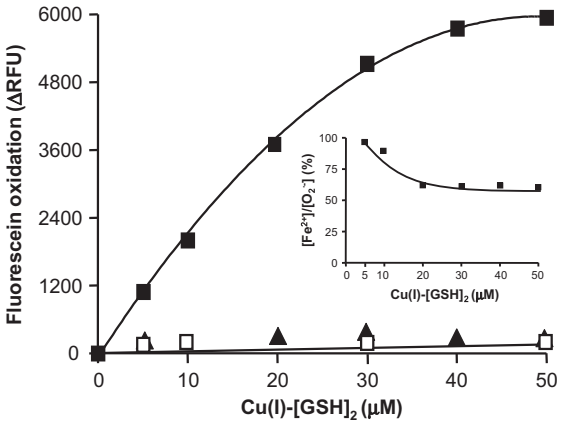

Figure 3. Effect of increasing concentrations of Cu(I)–$[GSH]_2$ on the iron-dependent oxidation of fluorescein: FL (70 nM) was added to solutions containing $FeCl_3$ (50 μM), H_2O_2 (500 μM) and increasing concentrations of the Cu(I)–$[GSH]_2$ complex (5–50 μM). Results obtained 60 min after are expressed as delta relative fluorescence units ($\Delta RFU = RFU_{FL/mixture} - RFU_{FL\ alone}$). The symbols (□) and (■) represent the oxidation of FL in the presence and absence of mannitol (10 mM), respectively, and (▲) represents the results from experiments carried out in the absence of H_2O_2 and mannitol. Insert to the figure shows the efficiency (expressed as percentages) of increasing concentrations of the Cu(I)–$[GSH]_2$ complex (5–50 μM) to reduce free Fe^{3+} ions. Efficiency was defined as the product of 100 by the ratio between the concentration of Fe^{2+} reduced by the complex (Fig. 2) and the concentration of $O_2^{\cdot-}$ estimated from data obtained in the Cyt *c*-reduction experiments (depicted in Fig. 1a).

proportionally a lower reduction efficiency. In fact, a 3-fold increase in the concentration of the complex (from 10 to 30 μM) resulted in no more than a 16% increase in the level of Fe^{3+} reduction (Fig. 2).

2.3. Generation of hydroxyl radicals following the reduction of free-Fe^{3+} by the Cu(I)–$[GSH]_2$ complex

Based on the ability of the complex to reduce free Fe^{3+} ions, we evaluated the possibility that the Fe^{2+} ions thus formed could generate hydroxyl radicals. The latter was assessed adding free Fe^{3+} ions and hydrogen peroxide to the complex, and by measuring the decrease in fluorescence that results from the oxidation of fluorescein (FL). In the absence of hydrogen peroxide, no oxidation of FL took place (Fig. 3). However, in the presence of the peroxide (which as such induced no oxidation; not shown), the complex was able to induce FL oxidation in a near concentration dependent-manner. The efficiency of the complex to induce the iron-mediated oxidation of FL was markedly lower within the range of 30–50 μM. FL oxidation was totally blocked by mannitol, a recognized hydroxyl-radical scavenger.[30]

Insert to Figure 3 depicts the relationship between increasing concentrations of the Cu(I)–$[GSH]_2$ complex (5–50 μM) and its efficiency to reduce free Fe^{3+}. Assuming that 1 mol of Fe^{3+} is reduced by 1 mol of $O_2^{\cdot-}$ (generated by the complex), and basing the estimates of $O_2^{\cdot-}$ generation on data obtained from the Cyt *c* reduction assay (Fig. 1a), a maximal iron-reducing efficiency, of near 96%, was observed for a 5 μM concentration of the complex. For concentrations equal to or greater than 20 μM the efficiency dropped to 60% and remained as such up to 50 μM.

2.4. Reduction and release of iron from ferritin by the Cu(I)–$[GSH]_2$ complex

Considering the above-shown superoxide-mediated ability of Cu(I)–$[GSH]_2$ to reduce free-Fe^{3+} ions, we evaluated the ability of such complex to induce the reduction and release of Fe^{2+} from ferritin. Figure 4a shows that the interaction between the complex (10 μM) and ferritin (0.4 μM) resulted in a time-dependent increase in the amount of Fe^{2+} (BPS-assayable) released into the media.

Controls conducted with BPS (in absence of the complex) show that, under the conditions of the assay (e.g., incubation at 37 °C during 400 min), ferritin exhibits—in the absence of the complex—a basal Fe^{2+} release of near 30%. A similar basal release value has been previously described.[23] On the other hand, as shown in Figure 4b, ascorbic acid (250 μM), previously reported as able to induce the release of Fe^{2+} from ferritin in a superoxide-independent form,[19] led to a substantial release of (BPS-assayable) iron from the protein. Noteworthy, the iron-releasing capacity of the complex was near half that of ascorbic acid, despite having added the former at a concentration 25-fold lower.

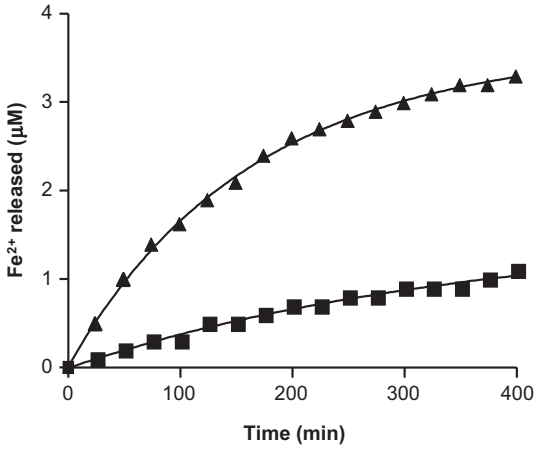

Figure 4a. Reduction and release of iron from ferritin by Cu(I)–$[GSH]_2$: Time-course studies. BPS (1 mM) was added to solutions containing ferritin (0.4 μM) in the presence (▲) or absence (■) of the Cu(I)–$[GSH]_2$ complex (10 μM). The increase in OD_{530nm} was recorded during 400 min. Results are expressed as concentration (μM) of Fe^{2+} released from ferritin, as described in Section 5.

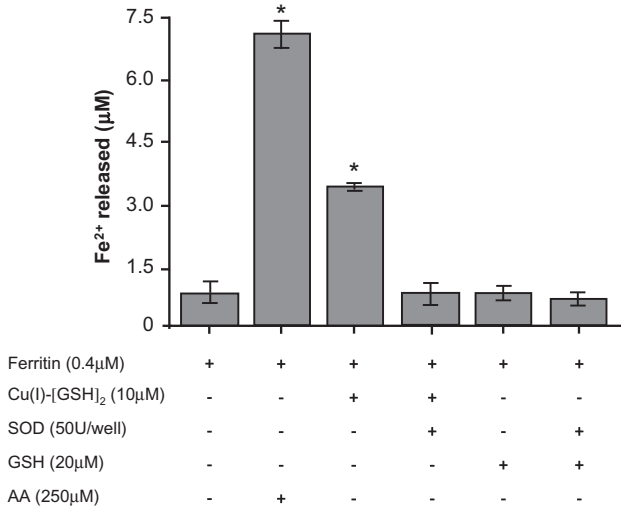

Figure 4b. Effect of ascorbic acid and GSH on the reduction and release of iron from ferritin. BPS (1 mM) was added to solutions containing ferritin (0.4 μM) plus the Cu(I)–$[GSH]_2$ complex (10 μM), AA (250 μM) or GSH (20 μM). The superoxide dependence was evaluated in the presence of SOD (50 U/well). Results are expressed as the concentration (μM) of Fe^{2+} released from ferritin, as described in Section 5. The asterisk on the bars indicate that such values are statistically different, at the level of a p <0.001, from all other values.

The addition of SOD (50 U/well) to a ferritin plus Cu(I)–$[GSH]_2$ mixture led to a marked inhibition (close to 77%) of the total Fe^{2+} released by the complex. Higher concentrations of SOD (up to 250 U/well) did not result in greater inhibitions, suggesting that approximately 77% (71.7 Fe atoms/ferritin molecule) of the total releasable Fe^{2+} was released by the $O_2^{\cdot-}$ generated by the complex. Aiming to evaluate whether the GSH molecules present in the 10 μM Cu(I)–$[GSH]_2$ complex could account for the 23% remaining total Fe^{2+} releasable fraction, a 20 μM concentration of GSH, equivalent to that present in the complex, was evaluated for its ability to release Fe^{2+}. As seen in Figure 4b, the addition of GSH to ferritin led to the release of an identical amount of Fe^{2+} as that released by the complex in presence of SOD. The amount of iron released from ferritin, attributed to a direct reducing effect of GSH, was estimated as 21.6 Fe atoms/ferritin molecule (which corresponds to the above referred 23% value). Figure 5a depicts the existence of a direct

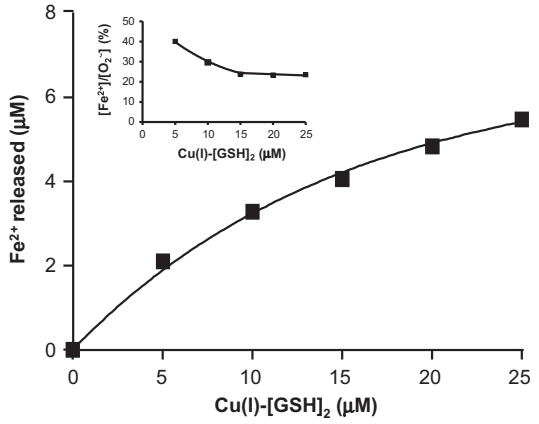

Figure 5a. Reduction and release of iron from ferritin by Cu(I)–$[GSH]_2$: concentration-dependence studies. BPS (1 mM) was added to solutions containing ferritin (0.4 μM) and increasing concentrations of the Cu(I)–$[GSH]_2$ complex (5–25 μM). The increase in OD_{530nm} was recorded after 400 min. Results are expressed as concentration (μM) of Fe^{2+} released from ferritin, as described in Section 5. Insert to the figure shows the efficiency (expressed as percentages) of increasing concentrations of the Cu(I)–$[GSH]_2$ complex (5–25 μM) to release Fe^{2+} from ferritin. Efficiency was defined as the product of 100 by the ratio between the concentration of Fe^{2+} released from ferritin by the complex (Fig. 5a) and the concentration of $O_2^{\cdot-}$ estimated from data obtained in Cyt *c*-reduction experiments (depicted Fig. 1a).

relationship between increasing concentrations of the Cu(I)–$[GSH]_2$ complex (5–25 μM) and the amount of Fe^{2+} released by ferritin. The efficiencies of Fe^{2+} release from ferritin by the complex are shown in insert to Figure 5a. A maximal efficiency of near 40% was observed between 5 and 10 μM of the complex. Concentrations greater than 15 μM were associated with a minimal efficiency of 24%.

2.5. Generation of hydroxyl radicals following the reduction and release of iron from ferritin by the Cu(I)–$[GSH]_2$ complex

Based on the above-described capacity of the complex to reduce and release iron from ferritin, we investigated whether the ultrafiltrate (e.g., non-ferritin containing fraction) was able to catalyze the generation of hydroxyl radicals. The formation of such species was evaluated through the FL oxidation assay. As shown in Figure 5b,

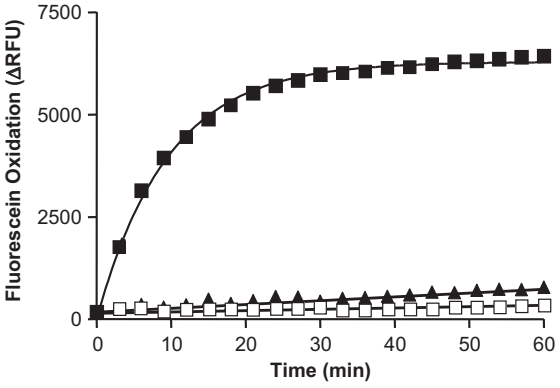

Figure 5b. Fluorescein oxidation resulting from the interaction between Cu(I)–$[GSH]_2$ and ferritin: time-course studies. FL (70 nM) and AA (250 μM) were added to solutions containing an 'ultrafiltrate' resulting from the incubation of ferritin with the complex (as described in Section 5). Results obtained from monitoring during 60 min are expressed as delta relative fluorescence units ($\Delta RFU = RFU_{FL/mixture} - RFU_{FL\ alone}$). The symbols (□) and (■) represent the oxidation of FL induced by the 'ultrafiltrate' in the presence and absence of mannitol (10 mM), respectively. The symbol (▲) represents the ultrafiltrate in presence of added H_2O_2 (500 μM) and in the absence of AA.

the ultrafiltrate was unable to induce the oxidation of FL, whether in absence or presence of added H_2O_2 (500 μM). FL oxidation became, however, clearly manifest when immediately after its preparation, the ultrafiltrate was added hydrogen peroxide (500 μM) and ascorbate (250 μM). The addition of ascorbate was aimed to prevent re-oxidation of the iron released during the incubation of ferritin with the Cu(I)–$[GSH]_2$ complex. FL oxidation induced by the latter was almost totally blocked by mannitol (10 mM). No oxidation of FL was seen when the ultrafiltrate was added ascorbate alone (not shown).

2.6. Effect of the addition of an excess of GSH on the reduction and release of iron from ferritin induced by the Cu(I)–$[GSH]_2$ complex

GSH concentration within cells is expected to largely surpass the concentration of copper available to be complexed by the tripeptide. The latter may be of particular interest since the ability of the Cu(I)–$[GSH]_2$ to generate $O_2^{\cdot-}$ was recently found to be largely exacerbated by the addition of a molar excess of GSH.[25] Based of these observations, we conducted experiments to investigate whether the previously seen ability of the complex to induce the reduction and release of iron from ferritin can also be increased by adding GSH in-excess. As shown in Figure 6, the addition of increasing concentrations of GSH (50–500 μM) to a mixture containing ferritin (0.4 μM) and the complex (10 μM) led (after 15 min) to a substantial increment in the amount of Fe^{2+} released into the media. When GSH was added at a 50 μM concentration, namely, at a concentration 2.5-fold higher than that of GSH molecules present in the complex, the iron-releasing effect of the complex was increased by 200%. This effect was concentration-dependent and reached almost 400% for a 500 μM GSH concentration. When these experiments were carried out in the presence of SOD (from 50 to 500 U/well), the magnitude of the iron released by the GSH plus complex mixtures was largely but not totally prevented. As depicted in Figure 6, the SOD-insensitive fraction of iron released from ferritin was almost identical to that induced by the sole and direct addition of GSH to such protein. The iron-releasing effect of GSH was concentration-dependent (shown in the same figure) but unaffected by SOD (not shown).

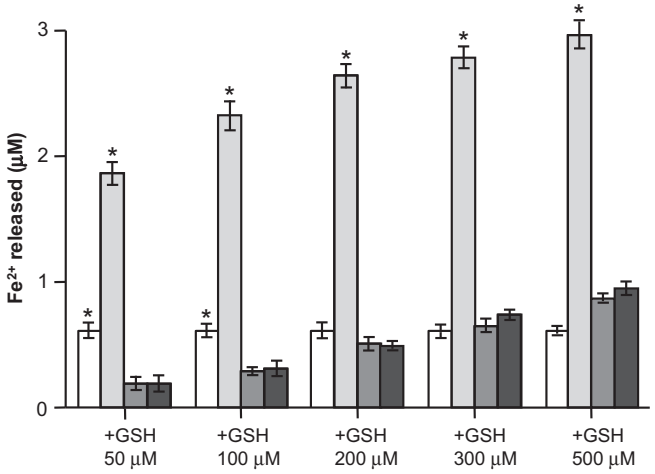

Figure 6. Effect of GSH on the reduction and release of iron from ferritin by Cu(I)–$[GSH]_2$. BPS (1 mM) was added to solutions containing ferritin (0.4 μM), the Cu(I)–$[GSH]_2$ complex (10 μM) and increasing concentrations of GSH (50–500 μM). The increase in OD_{530nm} was recorded after 15 min. Results are expressed as concentration of Fe^{2+} released (μM) as described in Material and methods section. For each GSH concentration: white bars, light grey bars, grey bars and dark grey bars represent solutions containing the complex (10 μM) alone, the complex plus GSH, the complex plus GSH and SOD or, GSH alone, respectively. For each GSH concentration, the presence of an asterisk on a given bar indicates that such value is statistically different, at the level of a p <0.001, from the mean of any other adjacent bar.

3. Discussion

Superoxide anions are involved in the development of several pathobiological processes.[31,32] Their potential to induce deleterious modifications to biological targets can be exacerbated in the presence of iron since, besides converting free Fe^{3+} into the redox-active Fe^{2+} species, superoxide is also able to induce the release of Fe^{2+} from ferritin.[21–24] Prompted by the reported ability of Cu(I)–$[GSH]_2$ to generate $O_2^{\cdot-}$,[29] we addressed here the redox consequences that arise from the interaction between such complex and free and ferritin-bound iron. Results confirm the capacity of Cu(I)–$[GSH]_2$ to generate $O_2^{\cdot-}$ and demonstrate that, throughout a reaction mediated by such radicals, the complex is able to reduce free Fe^{3+} and to release Fe^{2+} from ferritin-bound iron. The iron-releasing ability of the complex largely depended (by near 77%) on its capacity to generate $O_2^{\cdot-}$. The remaining fraction, not susceptible to inhibition by SOD, could be attributed to a direct action of the GSH contained within the complex since free GSH molecules were also found to be able to induce such release. Interestingly, the release induced by GSH molecules alone occurred via a superoxide-independent mechanism, and took place in a magnitude that equated the molar presence of the tripeptide within the complex. As reported previously by other investigators,[18,19] we observed that ascorbate is also able to induce iron release from ferritin. Although the molecular size of GSH and ascorbate could allow both reducing molecules to enter the ferritin cavity,[16,33] their participation in the physiological mechanisms of iron release from ferritin[16,18,19] still remains to be established. Comparatively, the ability of a 10 μM concentration of the complex to release iron from ferritin was over 4-fold higher than that of a 20 μM GSH concentration and half that of a 25-fold higher concentration of ascorbate. The ability of the Cu(I)–$[GSH]_2$ complex to induce iron release from ferritin through a $O_2^{\cdot-}$ dependent reaction is in line with previous work in which other chemical and/or biological sources of $O_2^{\cdot-}$ were employed.[21–24] Although the Cu(I)–$[GSH]_2$ complex has been reported to occur physiologically,[26,27] its iron-reducing and iron-releasing capacity might also be of potential toxicological interest since, in the case of copper overloaded cells, up to 60% of the metal entering such cells can be recovered in association with the tripeptide.[26,27] Considering the latter, we focused further our study in evaluating whether, as result of its interaction with free and ferritin-bound iron, the Cu(I)–$[GSH]_2$ complex could enhance its ability to generate other reactive oxygen species. Thus, upon establishing its ability to reduce free iron, we showed that in the presence of added hydrogen peroxide, the complex promotes the formation of hydroxyl radicals.

Compared to an efficiency of 96% for the reduction of free Fe^{3+}, the efficiency of the complex to release Fe^{2+} from ferritin was only 40%. Such difference may be explained on the basis that while the rate constant for the reduction of Fe^{3+} by $O_2^{\cdot-}$ ($10^8\ M^{-1}\ s^{-1}$)[34] is three orders of magnitude greater than that for the dismutation of $O_2^{\cdot-}$ ($2 \times 10^5\ M^{-1}\ s^{-1}$),[34] the difference between the rate constant for the reaction between $O_2^{\cdot-}$ and ferritin ($10^6\ M^{-1}\ s^{-1}$)[35] and that for the dismutation is only one order magnitude. The 40% value estimated by us as maximal iron-releasing efficiency of the complex (seen at 5 μM) is almost identical to that reported using activated polymorphonuclear leukocytes (also a 40% maximal for a 5 μM superoxide production)[21] and comparable to that using SOTS-1 (near 45% maximal for a 10 μM superoxide production)[24] as sources of $O_2^{\cdot-}$. The iron-releasing efficiency of the Cu(I)–$[GSH]_2$ complex depended non-linearly on the amount of $O_2^{\cdot-}$ estimated to be formed by it. Thus, compared to high levels

of $O_2^{\cdot-}$ production, low levels of the latter were associated with relatively higher levels of iron release. The latter, evidenced by a drop in the iron-releasing efficiency from 40% (5 μM) to near 20% (15 μM), confirms similar observations by Biemond et al.[21] and Paul.[24] Recently, we observed that in the absence of Fe^{3+}, increasing concentrations of the complex (8–50 μM) lead—as result of a spontaneous dismutation of superoxide—to the accumulation of increasing concentrations of hydrogen peroxide in the media.[25,36] Thus, it is likely that an increased dismutation of $O_2^{\cdot-}$ may underlie the lower iron-reducing and iron-releasing efficiencies seen at the higher concentrations of the complex. Alternatively, the drop in efficiency could be explained if an increased superoxide-mediated re-oxidation of Fe^{2+} has taken place, as previously proposed[24] when using SOTS-1. The latter contention is supported by the fact that the rate constant for the reaction between Fe^{2+} and $O_2^{\cdot-}$ ($1 \times 10^7\ M^{-1}\ s^{-1}$)[34] is two orders of magnitude higher than that for the dismutation. Finally, the possibility can not be discarded that within the ferritin molecule some sites may interact non-specifically with high (but not with low) concentrations of $O_2^{\cdot-}$, in a reaction which leads to no further release of Fe^{2+}. The relatively higher iron-releasing efficiency of low concentrations of the complex might be of biological relevance since within cells, rather than high, low concentrations of it could be expected to occur. Interestingly, the amount of iron released by the complex was drastically incremented (up to fivefold for 500 μM) by the addition of concentrations of GSH that could be found within cells. The increment in iron release induced by such addition, shown to be largely prevented by the addition of SOD, is in line with the recently reported ability of GSH to regenerate the Cu(I)–$[GSH]_2$ complex and its superoxide-forming capacity from Cu(II)–GSSG.[25] The latter complex is swiftly formed upon removal of the $O_2^{\cdot-}$ generated within a Cu(I)–$[GSH]_2$-containing solution.[25] Thus, the iron-releasing enhancing effect of GSH could result from its ability to regenerate the Cu(I)–$[GSH]_2$ complex and thereby, to sustain a greater flow of $O_2^{\cdot-}$.

Previous studies have shown that, as such, the Cu(I)–$[GSH]_2$ complex is unable to catalyze hydroxyl radical formation, even in the presence of hydrogen peroxide.[29] Likewise, the Cu(II)–GSSG complex, expected to be formed during the removal of the $O_2^{\cdot-}$ generated by Cu(I)–$[GSH]_2$,[29] is also unable to catalyze hydroxyl radical formation (data not shown). The present study demonstrates, however, that when faced to the possibility of interacting with ferritin, the ability of the Cu(I)–$[GSH]_2$ complex to generate $O_2^{\cdot-}$ can translate into an ability to form hydroxyl radicals, thus, exacerbating its pro-oxidant potential.

Some authors have suggested that ferritin may play a role as antioxidant.[37,38] The latter contention, however, relates to its capacity to sequester iron under a non-redox active form, preventing it from occurring freely to catalyze free-radical formation. Our demonstration that the Cu(I)–$[GSH]_2$ complex is able to release iron from ferritin and that—in the presence of hydrogen peroxide—the latter results in the formation of hydroxyl radicals, is in line with the previous observation that such radicals are also generated when ferritin is exposed to a mixture of hydrogen peroxide plus ascorbate (as an iron-releasing agent).[39] More recently, Rousseau and Puntarulo[40] reported that the addition of ferritin and ascorbate to rat liver homogenates leads to an increment in the labile pool of iron and to the generation of hydroxyl radicals. Like Fe^{2+}, copper ions have also the potential to catalyze hydroxyl radical formation.[3] To participate in such reaction however, copper ions need to occur in their free and reduced state.[41] Based on the here-shown ability of the Cu(I)–$[GSH]_2$ complex to generate the redox-active Fe^{2+} species, we postulate that copper ions, regardless of whether they occur freely or bound to GSH, might still be able to promote hydroxyl radical formation. Such pro-oxidant potential of the Cu(I)–$[GSH]_2$ complex may underlie the known oxidative stress and cytotoxicity associated to the over-exposure of cells,[42] rodents[43] or humans[44] to copper.

4. Conclusion

Results demonstrate the potential of the Cu(I)–$[GSH]_2$ complex to: (i) reduce free iron and (ii) release Fe^{2+} from ferritin through a reaction that was largely superoxide-dependent. The release of redox-active Fe^{2+} from ferritin was concentration-dependently exacerbated by the subsequent addition of near-physiological concentrations of GSH, and totally inhibited by SOD. In the presence of added H_2O_2, the Fe^{2+} ions generated through (i) or (ii) were shown to catalyze the generation of hydroxyl radicals. These results suggest that, by favouring the occurrence of superoxide-driven Fenton reactions, the recently established pro-oxidant potential of the complex could be increased beyond its initial $O_2^{\cdot-}$-generating capacity.[25,29]

5. Material and methods

5.1. Chemicals and reagents

Cupric chloride ($CuCl_2{\cdot}2H_2O$), reduced glutathione (GSH), oxidized glutathione (GSSG), ascorbic acid, ferric chloride ($FeCl_3{\cdot}6H_2O$), 2,4,6-Tris(2-pyridyl)-*s*-triazine, bathophenanthrolinedisulphonic acid disodium salt, fluorescein sodium salt (FL), hydrogen peroxide, mannitol, Sephadex G-25, ethylenediaminetetraacetic acid (EDTA), sodium acetate, sulfuric acid, bovine serum albumin, ferritin (Type I, from horse spleen), cytochrome *c* (Cyt *c*; from bovine heart), superoxide dismutase (SOD; EC 1.15.1.1 from bovine erythrocytes) and catalase (CAT; EC 1.11.1.6 from bovine liver) were all purchased from Sigma–Aldrich. Unless indicated otherwise, all solutions were prepared in Chelex-100-treated sodium phosphate (20 mM; pH 7.4), except for the ferritin experiments, where phosphate buffer saline (50 mM NaCl; pH 7.4) and Tris–HCl buffer (20 mM; pH 7.4) were used.

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

The Cu(I)–$[GSH]_2$ complex was prepared as previously reported,[29] mixing $CuCl_2$ and GSH in a 1:3 molar ratio. 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 complex was prepared always freshly and used within 5 min.

5.3. Assessment of the generation of superoxide anions

The generation of $O_2^{\cdot-}$ by the Cu(I)–$[GSH]_2$ complex was assessed through the Cyt *c* reduction assay, as previously described.[45] Cyt *c* reduction experiments were carried out monitoring the OD_{550nm} in a 96-well plate, using a Multi-Mode Microplate Reader (Synergy™ HT). In brief, Cyt *c* (50 μM) was added to wells containing the Cu(I)–$[GSH]_2$ complex (2–30 μM), and after 2 min the optical density was recorded (at 30 °C). SOD (250 U/well) was used as control to imply superoxide-dependent effects. The concentration of reduced Cyt *c* was estimated using a ϵ value of 21.1 $mM^{-1}\ cm^{-1}$.[46]

5.4. Measurement of free iron reduction

The reduction of free-Fe^{3+} was assessed using two different colorimetric Fe^{2+}-chelating assays (both run at 37 °C). As Fe^{2+}-chelating agents, 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) and bathophenanthrolinedisulphonic acid disodium salt (BPS) were used. The formation

of TPTZ-Fe^{2+},[47] in a solution containing acetate buffer (300 mM; pH 3.6), TPTZ (1 mM), Cu(I)–$[GSH]_2$ complex (10 μM) and $FeCl_3$ (10 μM), was monitored at 593 nm (ϵ = 22.6 mM^{-1} cm^{-1}).[48] The formation of the BPS–Fe^{2+} complex was monitored at 530 nm (ϵ = 22.14 mM^{-1} cm^{-1}),[49] in a reaction mixture containing phosphate buffer (50 mM; pH 7.4), BPS (1 mM), Cu(I)–$[GSH]_2$ complex (5–30 μM) and $FeCl_3$ (10 μM in Table 1; 50 μM in Fig. 2). In this latter assay, iron precipitation was minimized adding it directly, from a $FeCl_3$ solution prepared freshly in Chelex-100-treated deionised/distilled water, into the reaction mixture. In fact, based on the ϵ values for the corresponding TPTZ–Fe^{2+} and BPS–Fe^{2+} complexes, we estimated that the concentration of Fe^{2+} was, in both cases, identical when ascorbate was used as reductant.

5.5. Assessment of the capacity of the Cu(I)–$[GSH]_2$ complex to generate hydroxyl radicals in presence of free Fe^{3+} ions

Fluorescein (FL) was used as a probe to assess the formation of hydroxyl radicals.[50] The capacity of the Cu(I)–$[GSH]_2$ complex to generate the latter species from Fe^{3+} ions was assessed both, in the presence and absence of hydrogen peroxide (H_2O_2). FL oxidation was monitored fluorimetrically (Ex_{485nm}; Em_{520nm}) at 37 °C (during 60 min) in a 96-well plate and using a Synergy™ HT multilector. Fluorescein was added as a freshly prepared solution (70 nM) to wells containing $FeCl_3$ (50 μM), Cu(I)–$[GSH]_2$ complex (10 μM) and H_2O_2 (500 μM). Mannitol (10 mM), a known hydroxyl radical scavenger,[30] was used as control.

5.6. Preparation of ferritin

To remove traces of adventitious iron, commercial ferritin was incubated in phosphate-buffered saline (PBS), containing EDTA (10 mM) for 1 h at 4 °C. After incubation, the solution was applied to a Sephadex G-25 column (1.0 × 20 cm) previously equilibrated with PBS (10 mM). Ferritin-containing fractions were collected and concentrated by centrifugation (3000*g*, 4 °C during 90 min),[51] using a Centrifugal Filter Device (Amicon Ultra-4) Ultracel 3k®. After these treatments, ferritin was re-dissolved in Tris–HCl buffer (20 mM), stored at 4 °C and used not after a week of storage. The protein content of ferritin was determined by the method of Bradford,[52] using bovine serum albumin as standard.

5.7. Measurement of ferritin iron content

The amount of ferritin-bound iron was determined after removing the protein shell from the core with sulfuric acid, quantifying the total amount of iron present by means of the colorimetric bathophenantroline (BPS) assay. Ferritin was incubated during 30 min with sulfuric acid (2 mM) and ascorbic acid (AA; 1 mM) to denature the protein and reduce the Fe^{3+} present in the mineral core, respectively. Sodium acetate (2.5 mM) was added to bring the pH to 7.4 and BPS (1 mM) was added to complex the free Fe^{2+}. The calculations to determine the total iron per molecule of ferritin were done as previously reported.[53] Based on such form of calculation a value of 907.3 atoms of Fe(III)/molecule ferritin was estimated for the (after purification) ferritin preparation used in the present study.

5.8. Measurement of ferritin iron release

Ferritin (0.4 μM) was incubated at 37 °C in Tris–HCl buffer (20 mM) with BPS (1 mM) and increasing concentrations of the Cu(I)–$[GSH]_2$ complex (5–25 μM). In those experiments in which the effect of an excess of GSH on the ability of the complex to induce the reduction and release of iron from ferritin was studied, the protein was incubated with BPS (1 mM), a fixed concentration of Cu(I)–$[GSH]_2$ (10 μM) and increasing concentrations of GSH (50–500 μM). It is important to note that BPS was used as chelating agent since it allows assaying Fe^{2+} at neutral pH (non denaturing conditions); in contrast to the acidic pH required when TPTZ is used. Formation of the Fe^{2+}–BPS complex was continuously monitored at 530 nm, using a Multi-Mode Microplate Reader. The concentration of Fe^{2+} released from ferritin was estimated using a ϵ value of 22.14 mM^{-1} cm^{-1}.[49]

5.9. Assessment of the capacity of a Cu(I)–$[GSH]_2$-treated ferritin preparation to generate hydroxyl radicals

The capacity of a Cu(I)–$[GSH]_2$-treated ferritin preparation to generate hydroxyl radicals, was evaluated both, in the presence and absence of hydrogen peroxide, through the use of fluorescein. Ferritin (0.4 μM) was initially incubated (1 h at 37 °C) with the Cu(I)–$[GSH]_2$ complex (10 μM) in phosphate buffer. To avoid any possible interference in the quantification of the FL decay, ferritin was removed from the above solution by ultra-filtration using Amicon Ultra Centrifugal Filter tubes (Millipore, Billerica, MA 01821, USA) with a molecular weight cutoff of 3000 Da. The resultant 'ultrafiltered' solution, which contained the iron released from ferritin and the Cu(I)–$[GSH]_2$ initially added complex, was used to measure the generation of hydroxyl radicals. To prevent any possible re-oxidation of the iron expected to be released from the Cu(I)–$[GSH]_2$-treated ferritin, the formation of hydroxyl radicals was assessed adding ascorbate (250 μM) and fluorescein (70 nM) to the ultrafiltrate. Such assessment was carried out in presence and absence of H_2O_2 (500 μM). FL oxidation was monitored fluorimetrically, at 37 °C during 60 min.

5.10. Data expression and analysis

Data points represent the means of at least three independently run experiments, each conducted in triplicate. For the sake of simplicity and since the standard deviation values represented less than 10% of the means, these were omitted from all point-graphs (i.e., Figs. 1a, 2, 3, 4a, 5a and 5b). In the case of Figures 1b, 4b and 6 (plotted as bar graphs), however, since some of the means exhibited standard deviations greater than 10%, the latter were included. When evaluated, the statistical significance of the difference between bars was assessed using the Student's *t* test. GraphPad Prism 4 was used as statistical software.

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