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Redox-implications associated with the formation of complexes between copper ions and reduced or oxidized glutathione

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ABSTRACT

Binding of copper by reduced glutathione (GSH) is generally seen as a mechanism to lower, if not abolish, the otherwise high electrophilicity and redox activity of its free ions. In recent years, however, this concept has been contradicted by new evidence revealing that, rather than stabilizing free copper ions, its binding to GSH leads to the formation of a Cu(I)-$[GSH]_2$ complex capable of reducing molecular oxygen into superoxide. It is now understood that, under conditions leading to the removal of such radicals, the Cu(I)-$[GSH]_2$ complex is readily oxidized into Cu(II)-GSSG. Interestingly, in the presence of a GSH excess, the latter complex is able to regenerate the superoxide-generating capacity of the complex it originated from, opening the possibility that a GSH-dependent interplay exists between the reduced and the oxidized glutathione forms of these copper-complexes. Furthermore, recent evidence obtained from experiments conducted in non-cellular systems and intact mitochondria indicates that the Cu(II)-GSSG complex is also able to function in a catalytic manner as an efficient superoxide dismutating- and catalase-like molecule. Here we review and discuss the most relevant chemical and biological evidence on the formation of the Cu(I)-$[GSH]_2$ and Cu(II)-GSSG complexes and on the potential redox implications associated with their intracellular occurrence.

1. Introduction

1.1. Copper and reactive oxygen species

Copper is an essential element to all living organisms [1–3]. After iron and zinc, copper is the third most common trace element in the human body. In most biological milieus, copper occurs as Cu^+ (reduced or cuprous) and Cu^{2+} (oxidized or cupric) ions. Its oxidation state depends on the nature and the redox character of the molecules that it is able to interact with [4,5]. The ability of copper to alternate between its reduced and oxidized states allows it to serve as an electron donor/acceptor cofactor for many redox enzymes, among which superoxide dismutase, cytochrome c oxidase, lysyl oxidase, ceruloplasmin and tyrosinase are included [3,4,6–8].

Under physiological conditions, copper status is controlled by homeostatic mechanisms which secure its adequate intracellular occurrence, trafficking (e.g. chaperon), storage (e.g. metallothionein) and export (e.g. ceruloplasmin) [9,10]. However, under certain environmental- (e.g. over-exposure to copper) or genetically-defined conditions (e.g. Wilson's disease), such mechanisms can fail or be overridden, leading to the accumulation of potentially toxic concentrations of copper [11–14]. Secondary to the intracellular accumulation of copper, its toxicity is likely to arise from the occurrence of trace amounts of free copper ions, as the latter species have a well-established potential to induce damage to biological targets [15]. The following mechanisms are generally assumed to be the major modes through which free copper ions are deleterious to cells: (*i*) by their direct binding to nucleophilic domains of essential macromolecules (i.e. binding to some amino acid residues in proteins and to bases in nucleic acids) [15–17], and/or (*ii*) by catalyzing redox-reactions which lead to the formation of reactive oxygen species (ROS). In addition, it has been proposed that copper ions can also induce toxicity via a mechanism that involves their ability to displace iron from iron-sulfur clusters [18]. Although all these mechanisms are likely to occur in most forms of copper-induced cell damage, the ability of Cu^+ ion to catalyze the formation of hydroxyl radical ($HO^\bullet$), during its reaction with hydrogen peroxide (Rx. 1), and to generate superoxide anion ($O_2^{\bullet-}$), during its reaction with molecular oxygen (Rx. 2), appears to be the most toxicity-relevant mechanism.

$$Cu^+ + H_2O_2 \longrightarrow Cu^{2+} + HO^\bullet + HO^- \quad \text{(Rx.1)}$$

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$$Cu^{+} + O_2 \rightleftharpoons Cu^{2+} + O_2^{\bullet -} \quad \text{(Rx.2)}$$

As the single most powerful oxidizing species generated by living cells, the hydroxyl radical is capable of reacting with practically every known biological molecule [19]. Following the interaction of $HO^{\bullet}$ with lipids, proteins or nucleic acids, these molecules undergo an oxidative deterioration which often leads to the loss of their biological functions [20]. After exposing various cell types or animals to a copper overload, there is an increase in the rate of copper-dependent hydroxyl radical formation and in the degree of oxidation of a diversity of target molecules [21]. These oxidative events are generally regarded as crucial, as they can lead to cell death and/or to the development of copper-related diseases, such as Indian child cirrhosis and Wilson disease, in which a strong association between intra-hepatic copper levels and massive oxidative injury (especially within its mitochondria) has been well established [22]. Copper excess is also associated with certain forms of cancer, cardiomyopathies, Alzheimer's and Parkinson's disease [23,24].

Although reaction 1 (Rx. 1) is more often known to be catalyzed by Fe^{2+} ions (Fenton reaction), the reaction rate constant for the decomposition of hydrogen peroxide into hydroxyl radicals catalyzed by Cu^{+} ions is considerably higher, $4.7 \times 10^3\ M^{-1}\ s^{-1}$ [25] versus $70\ M^{-1}\ s^{-1}$ [26]. In order to take place, reactions 1 and 2 require copper to occur as Cu^{+}, namely, in its redox-active easily oxidizable form.

The probability that free copper ions occur intracellularly as Cu^{+} is given by the dominating presence of copper-reducing molecules within cells [27], like ascorbate, reduced glutathione and other endogenous thiols (Fig. 1).

Relative to hydroxyl radicals, superoxide anions are known to be notably less reactive (reported rate constant values for superoxide are in the range of 10^5 to $10^6\ M^{-1}\ s^{-1}$ while for hydroxyl radicals are near to $10^9\ M^{-1}\ s^{-1}$) [19]. Nonetheless, under conditions leading to their continuous formation, $O_2^{\bullet -}$ have also the potential to affect a number of vital components. Examples of molecules susceptible to undergo $O_2^{\bullet -}$-induced oxidative damage are: some transport proteins (e.g. LDL, transferrin, hemoglobin) and storing proteins (e.g. ferritin, metallothionein) [28], certain enzymes (e.g. aconitase and isocitric dehydrogenase) [29,30], several thioaminoacids (e.g. cysteine and homocysteine) and neurotransmitters (e.g. epinephrine [31] and serotonin [32]). Superoxide anions can also reductively interact with the transition metals copper and iron. Interestingly, the rate constant for the reduction of Cu^{2+} ions by $O_2^{\bullet -}$ is one order of magnitude higher than that for Fe^{3+} ($5 - 8 \times 10^9\ M^{-1}\ s^{-1}$ versus $1.5 \times 10^8\ M^{-1}\ s^{-1}$ [25]). Thus, in the presence of superoxide anion, Cu^{2+}, is more likely than Fe^{3+} to undergo reduction and subsequently catalyze the decomposition of hydrogen peroxide into $HO^{\bullet}$ radicals (Rx. 1) and the reduction of oxygen into superoxide (Rx. 2). Within cells, superoxide radicals are normally removed by a dismutation reaction [33], which intracellularly, is catalyzed by superoxide dismutase (SOD with a reaction rate constant of $2.5 \times 10^9\ M^{-1}\ s^{-1}$ [34]):

$$2O_2^{\bullet -} + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2. \quad \text{(Rx. 3)}$$

In the absence of SOD, the auto-dismutation rate constant ($5.4 \times 10^5\ s^{-1}\ M^{-1}$) is four orders of magnitude lower than that for the reaction catalyzed by the enzyme and also four-fold lower than that for the reaction between superoxide and cupric ions [25]. Thus, the reduction of Cu^{2+} ions by superoxide is feasible even in the presence of SOD.

1.2. Thiols in copper homeostasis and toxicity

Under physiological conditions most of the copper present within cells, if not all [35], is likely to occur, not as free ions, but coordinated with a variety of endogenous ligands. Such ligands, which occur primarily in free and protein-contained amino acids, feature in their structures mostly nitrogen (e.g. Cu^{2+} binding to the imidazole of histidine), sulfhydryl (e.g. Cu^{+} binding to the thioether of methionine or to the thiolate of cysteine) and/or oxygen (e.g. with Cu^{+} binding to the carboxylate of glutamate or aspartate) [36–38]. These ligands bind copper ions in a limited and controlled manner and are mostly part of proteins that

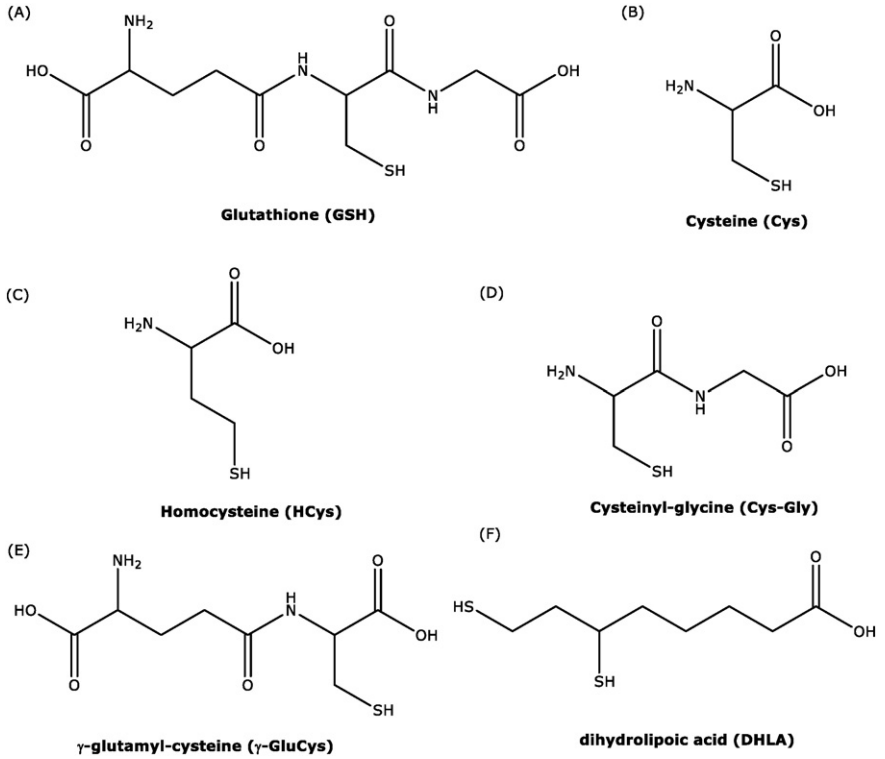

Fig. 1. Chemical structures of endogenous thiols: (A) glutathione, (B) cysteine, (C) homocysteine, (D) cysteinyl-glycine, (E) γ-glutamyl-cysteine and (F) dihydrolipoic acid.

either utilize copper as a cofactor (to carry out a specific function) or are involved in its trafficking, storage, and transport. The copper chaperones ATOX1, SCS and COX17 are worth mentioning since these thiol-rich copper ligand proteins are likely to be the main homeostatic mechanism by which, upon entering the mammalian cell, copper traverses the cytosol, enters the mitochondria, and is delivered into SOD1, copper-transporting ATPases and the trans-Golgi network [9,10]. In contrast to the normal copper exposure scenario, when the mechanisms of copper homeostasis are surpassed, the intracellular concentration of free copper ions rises, leading to a non-selective binding which often results in alterations in the structure and function of the affected molecules [39]. Within the frame of the possible metabolic and toxicological consequences associated with the indiscriminate binding of free copper ions, particular emphasis has been place on the high copper-binding ability shown by several endogenous thiols, both of protein and non-protein origin [12,40]. Examples of non-protein thiols capable of binding copper are the amino acids cysteine [41,42] and homocysteine [43] (which bind Cu^{2+} and Cu^{+}), the dipeptides cysteinyl-glycine and gamma-glutamyl-cysteine (bind Cu^{2+} and Cu^{+}; [44]) and the non-aminoacid related molecule, dihydrolipoic acid [45]. Despite occurring at relatively low intracellular concentrations, the capacity of these thio-molecules to bind copper ions sets them as molecules with a potential to lowering the electrophilic reactivity of free copper ions. Although it seems reasonable to assume that binding of copper ions to these ligands should prevent the electrophilicity of the former species, such a concept remains to be systematically assessed. Another important feature to consider in the binding of free copper ions is how the ligands influence the redox properties of the metal. While in the case of cuproenzymes binding of copper is critical to secure their catalytic redox properties, in the case of most copper-transporting proteins avoiding redox cycling might be fundamental to serve their function. Regarding the potential of thio-aminoacids to counteract the ROS-generating capacity of copper ions, it has been reported that while some copper complexes formed with cysteine, cysteinyl-glycine or gamma-glutamyl-cysteine are redox inactive, other copper complexes, such as those formed with homocysteine and reduced glutathione are able to form free radicals [44]. The formation of copper complexes lacking pro-oxidant activity between cysteine, cysteinyl-glycine or gamma-glutamyl-cysteine and copper is particularly interesting since, despite the ability of these thiols to reduce copper, they seem able to hold the cuprous ion under a redox-inactive form. However, it remains to be established if, under conditions in which these thiols and free copper ions coexist, namely intracellularly, their interaction leads indeed to a predominantly non-prooxidant effect.

Relative to the formerly referred non-protein thiols, a substantially greater potential to bind copper (theoretically within cells) is exhibited by the thiol-containing tripeptide, reduced glutathione (γ-L-glutamyl-cysteinyl-glycine, GSH) and by the low-molecular weight thiol-rich and high affinity copper-binding protein, metallothionein (MT). Together, these two molecules provide the highest intracellular availability of cysteine residues, representing the latter 33% of the aminoacidic composition of GSH and near 30% of MT. In the case of MT, binding of copper permits the buffering and storage of significant amounts of this metal within the copper-overloaded cells, and despite occurring with cuprous ions, the binding was shown early to render the metal in a redox inactive form [46]. Although MT occurs in concentrations considerably lower than those of GSH [12,47] the di novo synthesis of MT is known to be rapid and substantially enhanced when cells sense a significant increment in their internal copper [48]. Thus, binding of copper by MT contributes to protect hepatocytes and other cells, including duodenal and renal epithelium, against the toxicity induced by copper excess [40,49,50]. As referred below, the incorporation of copper into the MT molecule can be favored by the prior reduction and binding of copper ions to GSH, defining a close relationship between copper and GSH metabolism [51,52]. Besides preventing redox-active copper ions from occurring freely within cells, in vitro studies have demonstrated that MT exhibits a high reactivity towards hydroxyl radicals and other ROS [47,53]; the ROS-scavenging ability of MT is believed to reside on its cysteinyl thiolate groups. Interestingly, the rate constant for the reaction of hydroxyl radical with MT is about 340-fold higher than that with GSH [53]. Thus, it is believed that MTs would play an intracellularly dual protective "antioxidant" role against copper-induced oxidative damage [54].

In the case of the GSH molecule, the presence of a thiol group on its structure allows it to play a major role as antioxidant. Thus, by virtue of its thiol moiety, the tripeptide can act as an antioxidant (Fig. 2), either directly by scavenging various ROS (among which superoxide and hydroxyl radicals are included) or indirectly, by serving as a substrate for the glutathione transferase enzymes during the conjugation of potentially pro-oxidant electrophiles [55].

In addition, GSH can also act as indirect antioxidant by serving as cofactor for the glutathione peroxidase enzyme, which reduces the ROS hydrogen peroxide and lipid peroxides through a reaction in which GSH is oxidized into GSSG (oxidized glutathione). In turn, GSSG can be reduced back into GSH by the GSSG reductase enzyme in a reaction which requires NADPH (Fig. 2). Besides its primary cytosolic occurrence (which accounts for 80–85% of its whole cell content) GSH occurs also in mitochondria (10–15%) where it plays a major role in preventing the oxidative stress that otherwise arises from the intra-mitochondrially generated superoxide and superoxide-derived hydrogen peroxide. Since the GSH to GSSG ratio is a major determinant of the intracellular redox potential (being proportional to the log of [GSH]2/[GSSG]) the ability of the cell to regenerate GSH from GSSG is fundamental to conserve the redox equilibrium and thereby to regulate the redox status of a series of redox-dependent cell signaling processes [56]. In addition, when an oxidative stress ensues, in order to conserve its redox equilibrium, the cell responds by forming (reversible) mixed-disulfides between GSSG and the sulfhydryl groups of certain proteins, and/or by speeding up its secretion of GSSG into the extracellular medium. An intracellular elevation of GSSG, secondary to an oxidative consumption of GSH, is typically seen in vitro, in copper-overexposed cultured cells [12,15], and in vivo, in copper-overloaded experimental animals [57] and human individuals [22,58].

2. Formation of complexes between copper and reduced glutathione and characterization of their redox properties

Reduced as well as oxidized glutathione are known for their ability to form complexes with copper. However, due to the considerably higher intracellular concentration of GSH over GSSG, most studies have focused on the former peptide. Of particular interest is the ability of GSH to directly interact with cuprous ions in a reaction which leads

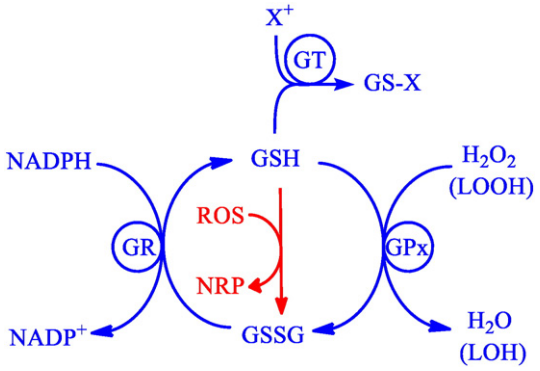

Fig. 2. Schematic representation of the main modes of antioxidant action of GSH. Indirect actions are depicted in blue color and direct actions are in red. The symbols represent: X^{+} = pro-oxidant electrophile; LOOH = lipid hydroperoxide; LOH = lipid alcohol; NRP = non-radical product; GST = glutathione transferase; GPx = glutathione peroxidase; GR = glutathione reductase.

to the formation of a stable Cu(I)- and reduced glutathione-containing complex [59–62]. Although in some reports the Cu(I) to GSH stoichiometry of the complex formed was not mentioned or clarified [60,61], other studies have addressed this point. Using ^{1}H,^{13}C-NMR and XANES (X-ray absorption near edge structure), Corazza et al. [62] observed that, irrespectively of the molar ratio of Cu^{+} and GSH added to a mixing solution (which ranged from 1:1 to 1:8), a Cu(I)-GSH and a Cu(I)-$[GSH]_2$ complex were formed, being the former the predominant species. The authors estimated that the mixture of complexes had an overall stoichiometry of approximately 1:1.2 [62]. Formerly, Osterberg et al. [59], using an Electromotive Force Titration technique, Brouwer et al. [63], using an spectrophotometric approach, and Ascone et al. [61], using X-ray absorption spectroscopy, found that when GSH is added in a molar excess equal to or greater than 3, only a copper-reduced glutathione complex with a 1:2 stoichiometry is recovered. In line with the latter results, our laboratory has consistently found that when GSH and Cu^{2+} (instead of Cu^{+}) are mixed in a molar ratio equal to or greater than 3, a Cu(I)-$[GSH]_2$ complex is formed [64–66]. In addition, Corazza et al. [62] and Brouwer et al. [63] obtained such complex by mixing Cu^{+} and GSH in a molar ratio of 1:1, or in a ratio which ranged from 1:2 to 1:8, respectively. According to Ciriolo et al. [60], it is important to note that when a cuprous ions are used, an oxygen-free atmosphere is absolutely needed to secure the coordination of the Cu(I) ions. However, by taking advantage of the ability of GSH to reduce Cu^{2+} into Cu^{+} (Rx. 4), other investigators have prepared the complex (indirectly), starting from cupric ions [59,61,64–68]. In all the latter studies, in order to obtain a Cu(I)-$[GSH]_2$ complex, the Cu^{2+} ions needed to be mixed with a molar GSH excess equal or greater than 3, assuring that at least one molar equivalent in excess be available to reduce Cu^{2+}.

$$2Cu^{2+} + 2GSH \longrightarrow 2Cu^{+} + GSSG + 2H^{+} \quad \text{(Rx.4)}$$

In Rx. 4, which depicts the equimolar reaction between Cu^{2+} and GSH, the reduction of two cupric ions leads to the formation of one GSSG molecule. Subsequent to the formation of the Cu^{+} ion, the sole presence of GSH in a molar excess equal to or greater than 2 was found to secure the formation of the Cu(I)-$[GSH]_2$ complex. As depicted in Rx. 5, the Cu^{+} ions (formed in Rx. 4) are swiftly coordinated by two GSH molecules leading to the formation of one molecule of the Cu(I)-$[GSH]_2$ complex.

$$Cu^{+} + 2GSH \longrightarrow Cu(I) - [GSH]_2 \quad \text{(Rx.5)}$$

From a kinetic point of view the formation of this complex, as studied by stopped-flow/rapid-scan spectroscopy [69], would initially take place through a fast reduction reaction where Cu^{2+} is reduced by GSH to form a Cu(II)-GS^{-} intermediate. Immediately after, this intermediate dissociates into a free Cu^{+} ion and a thiyl $GS^{\bullet}$ radical and the free cuprous ion is swiftly bound to two existing in-excess GSH molecules. However, using EPR (electron paramagnetic resonance), other investigators have failed to find evidence of the generation of a thiyl radical during the formation of the Cu(I)-$[GSH]_2$ complex [20,70–72].

Regarding the aerobic stability of the Cu(I)-$[GSH]_2$ complex, based on ^{1}H-NMR spectral data, Ciriolo et al. [60] found that the presence of oxygen makes no difference in the NMR spectra obtained when the complex was prepared in millimolar concentrations, either in air-containing or nitrogen-saturated solutions. Using EPR spectroscopy, the authors reported that after five hours of incubation, no signal associated with Cu^{2+} occur in any of the solutions. Further support for the alleged stability of the complex formed was provided by Ascone et al. [61], who reported that the XANES spectra of a solution containing the Cu(I)-$[GSH]_2$ complex remained largely unaltered after three hours when incubated in the presence of oxygen. In their stability studies, Ciriolo et al. [60] and Ascone et al. [61] used high concentrations of the complex (from 0.85-to-28 mM), precluding the possibility that significant but undetectable concentrations of the Cu(I)-$[GSH]_2$ complex could have inadvertently undergone oxidation.

Regarding the structure of the Cu(I)-$[GSH]_2$ complex, according to early studies by Osterberg et al. [59], Cu(I) binds to the cysteine of the tripeptide forming a high affinity Cu(I)-S-thiolate bond. However, based on molecular modeling studies, Ciriolo et al. [73] subsequently proposed that the GSH molecule holds the Cu(I) in a tight and packed tetracoordinate fashion in which one oxygen (from the carboxyl oxygen from glycine), two nitrogen (from the main chain of the cysteine and the gamma-glutamyl residue), and the sulfhydryl group of cysteine would coordinate the cuprous ion. Early studies by Osterberg et al. [59], reported a stability constant of 38.8 ($\log \beta$) for the Cu(I)-$[GSH]_2$ complex. Recently, however, using experimental conditions similar to those of Osterberg et al. [59], Walsh and Ahner [74] estimated a constant of 35.5. As shown in Table 1, the stability constant estimated by the latter investigators for the Cu(I)-$[GSH]_2$ complex is comparable to that reported by themselves for the complexes formed between cuprous ions and cysteine or the cysteine-containing dipeptides arginine–cysteine and glutamine–cysteine.

Moreover, the stability constant for the Cu(I)-$[GSH]_2$ complex reported by Walsh and Ahner [74] is substantially higher than that reported by other investigators for complexes formed between Cu(I) and the non-endogenous thiols bathocuproine [75], neocuproine [75] and dithiothreitol (Table 1). Interestingly, although slightly lower, the stability constant of the Cu(I)-$[GSH]_2$ complex reported by Walsh and Ahner [74], is still comparable to that reported for the therapeutically-used Cu(I)-chelating agent, penicillamine [59].

Although all formerly-mentioned studies on the Cu(I)-$[GSH]_2$ complex were conducted in aqueous and non-cellular systems, some evidence would also exist to suggest the possible intracellular formation of this complex. Early studies conducted by Freedman et al. [76] and Freedman and Peisach [51], in a human hepatoma cell line (HAC) exposed to ^{67}Cu, showed that most of the copper taken up by these cells was recovered chromatographically, assessed by fast protein liquid chromatography (FPLC), shortly after exposure (overall within the first 30 min) and in close association (co-elution) with GSH molecules (presumably complexed with it). According to Freedman et al. [76], over 60% of the cytoplasmic copper was recovered as a GSH containing complex. The latter authors come to an identical conclusion when instead of HAC, a copper-resistant cell line (HAC6oo) was exposed to a copper overload. Subsequently, using ^{64}Cu and an intestinal epithelial cell line (Caco-2 cells), Ferruzza et al. [77] characterized the uptake and the intracellular distribution of the radiolabel (also using FPLC) at different times of uptake ('pulse') and of 'chase'. These authors observed that the majority of the copper taken up (determined by absorption spectroscopy) was eluted as a single peak and that the latter fully corresponded with that where the GSH molecules were also eluted. Although the results of the studies by Freedman et al. [76] and by Ferruzza et al. [77] are coincidental and strongly suggest that the formation of a Cu(I)-reduced glutathione complex takes place soon after copper ions enter the cells, the actual intracellular formation, occurrence and quantification of the Cu(I)-$[GSH]_2$ complex remains to be established.

Table 1
Stability constants (β) of various Cu(I)-containing complexes formed with endogenous and non-endogenous ligands. Data are presented under the form of its corresponding logarithmic values ($\log \beta$).

Ligand	$\log \beta$	Method
Glutathione	38.8	Electromotive force titration [59]
Glutathione	35.5	Fluorometric competing-ligand titration [74]
Cysteine	38.4	Fluorometric competing-ligand titration [74]
Arginine–cysteine	34.7	Fluorometric competing-ligand titration [74]
Glutamine–cysteine	35.9	Fluorometric competing-ligand titration [74]
Bathocuproine	19.9	Bjerrum method [75]
Neocuproine	19.1	Spectrophotometric competing-ligand titration [75]
Dithiothreitol	15.3	Spectrophotometric competing-ligand titration [75]
Penicillamine	39.2	Electromotive force titration [59]

Perhaps contrasting the relative absence of evidence for the intracellular occurrence of Cu(I)-$[GSH]_2$, a number of studies conducted in non-cellular systems have proposed a possible biological functions for this complex. Among the most supported proposed roles, is that the complex would play a role as a carrier of cuprous ions into certain copper-dependent enzymes and into some copper-transporting [68] and copper-storing [67,78] proteins.

Using ^{1}H, ^{13}C-NMR and EPR techniques, Ciriolo et al. [60] was first in demonstrating that the complex is able to donate Cu(I) to a Cu-free, Zn-superoxide dismutase, reconstituting a 100% of the enzyme's native activity. These authors initially suggested that a ternary complex (Cu(I)-GSH-protein) is formed, which swiftly after gives place to the reconstitution of the holoenzyme. Interestingly the Cu(I)-$[GSH]_2$ complex is able to efficiently reconstitute the enzyme at any Cu^{+} to GSH molar ratio, ranging from a 1:2 to a 1:500 [60]. In a subsequent work, using the XANES, the same laboratory [61] confirmed the ability of the complex to fully reconstitute the SOD activity and concluded that Cu(I) is rapidly transferred from the Cu(I)-GSH complex into the native copper site of the apo-protein without forming an intermediate ternary complex (Cu(I)-GSH-protein). These authors alleged that, unlike the NMR, the XANES technique allows the direct monitoring of the metal oxidation state, distinguishing between Cu(I) and Cu(II) along the transferring reaction. Finally, in a third work by the group of Ciriolo [73], doing a computer docking analysis, the possibility was proposed that a ternary complex is formed during the donation of Cu(I) from the Cu(I)-$[GSH]_2$ into the Cu-free SOD.

The proposed ability of the Cu(I)-$[GSH]_2$ complex to serve as a carrier of Cu(I) would not be limited to the SOD enzyme. In fact, studies conducted by Musci et al. [68] indicate that this complex could also function as a carrier of Cu(I) to the copper-transporting protein ceruloplasmin, as it efficiently allowed the stoichiometric transferring of Cu(I), reconstituting the structural and the functional properties of the native apo-form of ceruloplasmin. Copper was found to reinstate in the various sites in a multi-step process, with metal entry into the protein in a first phase, and a second step involving conformational changes of the protein leading to the recovery of the native structural and functional properties.

The reported ability of the Cu(I)-$[GSH]_2$ complex to function as a Cu(I) carrier comprises also the apo forms of other proteins, including the copper-storing protein metallothionein [67,76], and the copper-containing proteins, hemocyanins (a blue copper protein serving as oxygen carrier in the blood of arthropods) [63] and phytochelatins [79]. As referred before in Section 1.2, there is a close interplay between the metabolism of copper and that of GSH and MT. In vitro cellular studies conducted by Freedman & Peisach [51] and Freedman et al. [76], were first in establishing that, to be possible and efficient, the incorporation of Cu(I) into MT requires the participation of the Cu(I)-$[GSH]_2$ complex. Thus, using HAC and HAC_{600} cells to evaluate the uptake of ^{67}Cu into MT, these investigators found that Cu(I) binds earlier to GSH molecules than to MT. Interestingly, they observed that depletion of cellular GSH with buthionine sulfoximine (an inhibitor of the rate-limiting GSH-synthesizing enzyme) inhibited in both cell types by more than 50% the basal rate of incorporation of ^{67}Cu into MT. Furthermore, by means of pulse-chase experiments, Freedman & Peisach [51] provided evidence to support the assertion that chelation of copper by MTs would be a reversible process. Thus, the authors observed that along the time frame of their study, the fraction of ^{67}Cu that was already bound to MTs molecules was subsequently recovered as copper bound to GSH, and thereafter incorporated into SOD molecules. In line with the studies conducted by Freedman & Peisach [51], Steinbach & Wolterbeek [78], using ^{64}Cu, a hepatoma cell line and HPLC molecular size exclusion chromatography, estimated that the depletion of GSH in these cells by BSO treatment caused a near 70% reduction of the cytosolic ^{64}Cu bound to the MT-fraction. Direct evidence on the ability of the complex to transfer Cu(I) into MTs was obtained by Da Costa Ferreira et al. [67] using a purified MT preparation from rabbit liver. These investigators showed that compared to other Cu(I)-complexes routinely used to reconstitute Cu(I)-containing proteins (like those prepared with either thiourea or with acetonitrile) [80–84], the insertion of copper into MT was distinctly more efficient when Cu(I) was offered under the form of a Cu(I)-GSH complex. In addition, Da Costa Ferreira et al. [67] showed that the Cu(I)-$[GSH]_2$ complex was also able to displace Zn(II) and Cd(II) from the naturally occurring Zn(II)-containing MT.

In addition to its proposed Cu(I) carrier function, the sole chelation of free copper ions associated with the formation of Cu(I)-$[GSH]_2$ could serve as a mechanism to protect against the toxicological consequences associated with the ability of the otherwise free copper ions to bind non-specifically to essential biomolecules [15] and/or to catalyze ROS formation [85,86]. Regarding the former toxicity mechanism, in vitro work conducted by Spear & Aust [87] in DNA molecules exposed to copper ions, demonstrated that the oxidative damage to this macromolecule (assessed as 8-hydroxydeoxyguanosine, 8-OHdG) induced by the addition of free Cu^{2+} could be totally prevented by GSH added at a molar excess sufficient to secure the total sequestering of the metal, namely, greater than 3:1. The protection induced by GSH at a 3:1 molar ratio was maximal and did not differ from that arising from adding GSH at a 10:1 ratio. In turn, when GSH was added at a molar ratio lower than 3:1, 8-OHdG production was greater than that seen in the absence of the addition of the tripeptide. Interestingly, a GSH-dependent pro-oxidant effect of Cu^{2+} was also equally seen when instead of GSH, ascorbate or cysteine was added at a molar ratio lower than 3:1. However, unlike GSH, the two latter reductive agents rather than preventing the damage, concentration-dependently exacerbated 8-OHdG production when added in molar ratios greater than 3:1 [20,87]. On the other hand, Prutz [70] reported that a DNA-Cu(I) complex is formed when Cu^{2+} is added to isolated DNA under conditions in which the GSH to Cu^{2+} molar ratio is lower than 1. According to this author, the formation of such complex would be a primary step in the cleavage of the DNA-strand induced by GSH plus Cu^{2+} formerly reported by Reed et al. [88]. It should be noted however that under physiological conditions, and even under copper over-exposure conditions, the intracellular GSH concentrations (2–6 mM) are expected to surpass by at least three orders of magnitude those expected to occur for copper ions [4,35]. Thus, under such conditions, a complete chelation of copper ions by the large excess in GSH molecules is expected to occur, precluding the possibility that within cells the tripeptide could exert a net pro-oxidant effect.

Although the formation of the Cu(I)-GSH complex could be seen as a mechanism to minimize the potential of free copper ions to otherwise bind indiscriminately to essential macromolecules, there is actually no experimental evidence to support it. In fact, the early works conducted by Freedman et al. [76] and by Steinbach & Wolterbeek [78] in various cell types exposed to copper, rather than revealing a direct and key cytoprotective role of the Cu(I)-GSH complex, demonstrates an apparently key role of the complex in securing an efficient transfer of the metal to the diverse tested copper-storing proteins (i.e. MTs) and copper-requiring enzymes (i.e. SOD and ceruloplasmin).

In contrast with the still to be established role that the Cu(I)-$[GSH]_2$ complex could play in protecting cells against the toxicity induced by free copper ions, the GSH molecules, as such, are vastly recognized for playing a major antioxidant role within cells. This function of GSH can be exerted directly, by scavenging the ROS generated within copper-exposed cells [40], as well as indirectly, by serving as cofactor for the glutathione peroxidase-dependent reductive removal of the copper-induced increment in lipid peroxides [89]. Regarding the former role of GSH, Carrasco-Pozo et al. [44] observed that under experimental conditions which ensured that all GSH molecules were complexed to copper (presumably under the form of the Cu(I)-$[GSH]_2$ complex), a mixture of copper and GSH fully conserves the free-radical scavenging properties of the tripeptide.

Interestingly, despite containing copper as Cu(I) (namely, as a potentially redox-active form), the Cu(I)-$[GSH]_2$ complex was considered

for over two decades an effective manner of stabilizing the metal, preventing it from reacting with either molecular oxygen [59,60,62,90] or with hydrogen peroxide [90,91], to form superoxide and hydroxyl radicals, respectively. Contending this concept, however, in 2008 our laboratory provided for first time evidence that, rather than being redox-inactive, the Cu(I)-$[GSH]_2$ complex is able to continually interact with molecular oxygen in a reaction which leads to the formation of superoxide radicals [64]. Initial support for the latter emerged from the demonstration that, in a solution containing micromolar concentrations of the Cu(I)-reduced glutathione complex, the basal level of dissolved oxygen started to decay only upon addition of SOD (Fig. 3).

The SOD-dependent decay in oxygen concentration was construed as an indication that superoxide anions were being continually generated by the complex. Since SOD catalyzes the conversion of two moles of superoxide into one mole of oxygen and one mole of hydrogen peroxide (Rx. 3; $k \approx 2 \times 10^9\ M^{-1}\ s^{-1}$), the decrease in oxygen concentration observed after SOD addition was interpreted to reflect only half of the actual extent at which superoxide radicals were being formed (during the interaction between Cu(I)-$[GSH]_2$ and O_2). In accordance to Rx. 6 ($k \approx 2 \times 10^7\ M^{-1}\ s^{-1}$), in the referred studies, the addition of catalase lead to a partial reversal of the ongoing decrease in oxygen concentration induced by the prior addition of SOD (not shown in Fig. 3).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad \text{(Rx. 6)}$$

To explain why in a solution containing the complex, the basal level of molecular oxygen remains largely unaltered in the absence, but not in the presence of SOD, we proposed that SOD-removable superoxide anions are permanently generated by the complex, and that in the absence of SOD, these radicals are largely quantitatively re-oxidized into molecular oxygen Rx. 7.

$$Cu(I) - [GSH]_2 + O_2 \rightleftharpoons IOC + O_2^{\bullet -} \qquad \text{(Rx.7)}$$

We have hypothesized that in the former 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, leading to the formation of an 'intermediate oxidized form' of the complex (IOC). On the other hand, considering the reversible character of Rx. 7, we have 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 (further discussed below in Section 3). A more direct evidence on the ability of the Cu(I)-$[GSH]_2$ complex (assessed at a 25 micromolar concentration) to generate superoxide anions emerged subsequently from experiments in which dihydroethidium (DHE) was used as a superoxide probe [92]. Thus, Speisky et al. [64] confirmed that DHE oxidation was indeed caused by superoxide by showing an SOD-inhibitable sustained increment in the formation of 2-hydroxyethidium, a metabolite whose formation is generated solely upon the interaction between DHE and superoxide [93]. Additional support for the ability of the Cu(I)-reduced glutathione complex to generate superoxide anions was obtained by the demonstration that the complex is also effective in inducing the reduction of cytochrome *c*. As observed 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. In addition to the use of molecules susceptible to undergo oxidation or reduction as probes to evidence the formation of superoxide, our laboratory, using EPR, demonstrated that within a solution containing millimolar concentrations of DMPO and the Cu(I)-$[GSH]_2$ complex, a DMPO-derived spectrum whose lines are consistent with the trapping of superoxide is generated. In presence of SOD, neither the latter spectrum nor one corresponding to the eventual formation of a DMPO-hydroxyl radical derived spectrum was formed. In a subsequent study, we established [65] that the superoxide-generating capacity of the complex is concentration-dependent and that, in the absence of superoxide interceptors, the equilibrium of Rx. 7 is largely shifted towards the left. Nonetheless, in Rx. 7 a small but significant part of the superoxide radicals generated under equilibrium conditions appear to undergo autodismutation ($k \approx 2 \times 10^5\ s^{-1}\ M^{-1}$) [94], as evidenced by the slow but sustained accumulation of hydrogen peroxide that time-(0–150 min) and concentration-(4–40 micromolar)-dependently was found to take place in the media [66]. At higher concentrations of the complex, the rate of spontaneous dismutation of superoxide (i.e. hydrogen peroxide accumulation) was found to be favored and a similar effect was seen when the complex was exposed to higher temperatures (from 22° to 37 °C) [65,66].

When the redox changes that follow the removal of superoxide from a medium containing the Cu(I)-$[GSH]_2$ complex (whether spontaneous or induced by Tempol, a superoxide dismutating agent) were studied, it was found that the removal of superoxide leads to an early and time-dependent oxidative disappearance of the thiol-titratable groups of GSH, and to the disappearance of bathocuproine-assayable Cu(I) ions from the Cu(I)-$[GSH]_2$ complex [95]. By taking advantage of the ability of EDTA to remove Cu(II) from the Cu(II)-GSSG complex [95], the two former changes were associated with an increment in the number of EDTA-releasable GSSG molecules. However, since the losses in GSH were seen much earlier (and/or of a greater magnitude) than those affecting the Cu(I) metal, it was suggested that the formerly referred IOC (in Rx. 7) would be a complex containing Cu(I) and one or more GSSG molecules [95]. Interestingly, we observed that the above-referred oxidative disappearance of the thiol-titratable groups of GSH and the loss of bathocuproine-assayable Cu(I) can be totally prevented when the Cu(I)-$[GSH]_2$ complex is co-incubated with an external source of superoxide anions (xanthine/xanthine oxidase). A similar behavior was observed under conditions in which the latter complex leads to the formation of the IOC (i.e. incubation times associated with the spontaneous dismutation of the superoxide generated by the reaction between oxygen and Cu(I)-$[GSH]_2$). Such result was in line with the postulated reversibility of Rx. 7, namely, that the interaction between IOC and superoxide would displace the equilibrium of this reaction towards the left. Presumably, the regeneration of the Cu(I)-$[GSH]_2$ complex from IOC would involve the necessary use of superoxide as the reductant species.

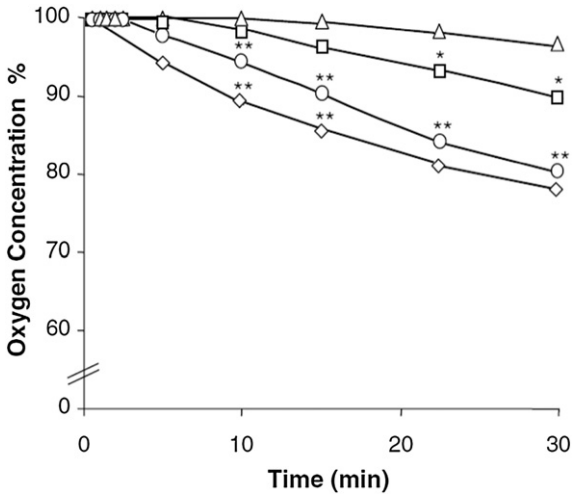

Fig. 3. Changes in oxygen concentration in a solution containing the Cu(I)-reduced glutathione complex. Oxygen concentration was continuously monitored (0–30 min) in a phosphate buffer solution (pH 7.4) containing a 15-min pre-incubated mixture of 900 μM GSH plus 300 μM Cu^{2+} both, in the absence (Δ) 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 oxygen concentration. 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 signaled value and that obtained with the closest lower SOD concentration.
Figure taken from [64].

In contrast with the above-referred reversibility of Rx. 7, it was observed that under conditions which favor an accelerated dismutation of the superoxide generated by Cu(I)-$[GSH]_2$ (namely, temperature-, SOD- or Tempol-induced), a new and stable product whose NMR spectrum was identical to that of a preformed Cu(II)-GSSG complex is formed. Removal of superoxide from Rx. 8 was proposed to drive, in a presumably irreversible manner, the oxidative conversion of IOC into Cu(II)-GSSG.

$$2IOC + 2O_2^{\bullet -} + 2H^+ \longrightarrow 2Cu(II) - GSSG + H_2O_2 + O_2 \quad (Rx.8)$$

The latter NMR results suggest that preventing the removal of the superoxide generated in Rx. 7 would be key to maintain the equilibrium of the reaction, and to secure that the postulated IOC, instead of shifting towards the formation of Cu(II)-GSSG complex (Rx. 8), would serve primarily to re-generate the Cu(I)-$[GSH]_2$ complex (Rx. 7).

As said before, under a normal biological setting, GSH concentrations are likely to surplus by three orders of magnitude the concentrations of copper expected to be found within cells [4,35]. Interestingly, the capacity of the Cu(I)-$[GSH]_2$ complex to generate superoxide was increased in a concentration-dependent manner by the addition of a (3:1 or higher) molar GSH excess [65]. Since this effect was associated with a parallel and lineal increment in the concentration of GSSG in the medium, Speisky et al. [65] proposed that the superoxide-enhancing action of GSH molecules would involve both, the displacement of GSSG molecules from the IOC formed and the simultaneous binding of GSH to the Cu(I) present in the intermediate. Reactions 7 and 9 summarize the latter concept.

$$IOC + 2GSH \rightleftharpoons Cu(I) - [GSH]_2 + GSSG \quad (Rx.9)$$

To the extent to which reactions 7 and 9 took place within a cell, the Cu(I)-$[GSH]_2$-regenerating effect of GSH (assessed through an increment in superoxide) could be seen as a mechanism to extend the pro-oxidant potential of the Cu(I)-$[GSH]_2$ complex beyond its initial concentration. Nonetheless, considering that most cells contain significant amounts of SOD and a large number of other molecules susceptible to react with superoxide (including GSH itself), it could be speculated that the Cu(I)-$[GSH]_2$ complex, rather than occurring as such would occur predominantly as its end-oxidation product Cu(II)-GSSG, after the obligatory removal of superoxide from Rx. 7. Thus, in the following section, the formation and characterization of the Cu(II)-GSSG complex in non-cellular systems, as well as the recently emerged evidence on the existence of an interplay between the latter complex and, its presumably intracellular precursor, the Cu(I)-$[GSH]_2$ complex, is presented.

3. Formation of complexes between copper and oxidized glutathione and characterization of its redox properties

As for the complexes formed with GSH, the formation and structural characterization of complexes formed between copper and oxidized glutathione (GSSG) has been addressed by using EPR, NMR and UV–vis spectroscopy techniques. The studies on the complexes formed between GSSG and copper have been largely limited to the use of cupric ions. As early shown by several laboratories [96–98], the addition of GSSG to a solution containing Cu^{2+} ions leads to the swift formation of stable Cu(II)-oxidized glutathione complexes suitable for spectroscopic analysis. Kroneck [99] was first in proposing a model for the structure of these complexes, suggesting that, at alkaline pH, a binuclear complex is formed in which each Cu(II) is bonded to five donor atoms from one GS^- moiety (two deprotonated amide nitrogen, the glutamyl carboxyl oxygen, an amine nitrogen, and the glycyl carboxyl oxygen). A last apical coordinate of each Cu(II) involves a distant sulfur of the disulfide bond. Crystals of a similar $Cu(II)_2$-GSSG complex, but with a completely different EPR spectrum, were isolated by Miyoshi et al. [96]. In subsequent studies, using X-ray crystallography, Miyoshi et al. [100] reported for this complex a slightly different square pyramidal Cu(II) site, with the near sulfur acting as the axial ligand and without any bonding to the glycine carboxylate. This structure was suggested to exist also at physiological pH, on the basis of spectroscopic evidence and model studies.

Other reports have studied the characterization of Cu(II)-GSSG complexes using NMR and EPR methods, demonstrating that at different metal to disulfide molar ratios (1:1, 1:2 and 2:1), the interaction between Cu(II) and GSSG also leads to the formation of a dimeric species where the metal is able to interact with the disulfide bridge forming Cu(II)–S–S–Cu(II) [101,102]. Regarding the stoichiometry of these complexes, Huet et al. [103] detected a variety of differently protonated complexes at low pH values, and suggested that at physiological pH, the dominant stable form was a 1:1 Cu:GSSG species [103,104], with the metal bound to the amine and carboxyl groups of the two terminal glutamate residues in a symmetrical square-planar coordination structure. Of particular interest is the study conducted by Postal et al. [97] which assessed the formation of a Cu(II)-oxidized glutathione complex, prepared in a 1:1 molar ratio (Cu(II):GSSG) in aqueous solution. EPR and visible absorption spectra of Cu(II)-GSSG solutions suggested that the stoichiometry of the Cu(II)-GSSG complexes formed at pH 6–9 was of 1:1. Postal et al. [97] also showed that the Cu(II)-GSSG complex exhibits a typical absorption band with a peak at 625 nm with an extinction coefficient of 60 M^{-1} cm^{-1}. The latter is in accordance with a previous study, which determined the absorption spectra of a Cu(II)-GSSG complex prepared in equimolar concentrations at pH 7 ($\lambda_{max.} = 620$ nm; $\varepsilon = 61\ M^{-1}\ cm^{-1}$) [102]. In 1996, Pedersen et al. [98] reported that the Cu(II) binding site in GSSG has the same ligand arrangement as in the copper complexes formed with S-methylglutathione. Thus, the binding site would be composed of the amino nitrogen and the carboxyl oxygen of two γ-glutamyl residues; there is no interaction with amide nitrogen, the sulfur bond or the glycyl carboxyl groups. At high metal to ligand ratios (namely, higher than 2) a binuclear species exists, in which each Cu(II) binds only to one γ-glutamyl residue [98]. In the same study, a Cu-GSSG stoichiometry of 1:1 and a binding site composed of two identical glutamyl residues were proposed to lead to a dimer structure where each of the two GSSG molecules provides half of the ligands for two binding sites. Using computer modeling of spectrophotometric and NMR relaxation measurements data over a wide range of pH (1–13) and metal and ligand concentrations (up to 0.1 M) Shtyrlin et al. [105] determined the formation constants and the structures of 11 Cu(II)-GSSG complexes. Among these complexes, four forms ($Cu_2(GSSG)H^{2-}_{-2}$, $Cu_2(GSSG)H^{4-}_{-4}$, $Cu_3(GSSG)_2^{2-}$ and $Cu_3(GSSG)_2H^{6-}_{-4}$) were thermodynamically characterized for first time.

Regarding the possible biological functions of the Cu(II)-oxidized glutathione complexes, Marzullo et al. [106], isolated and identified from human red blood cells exposed to copper, a "peptide-like" molecule, presumably a Cu(II)-GSSG complex, capable of acting as an inhibitor of the opiate receptor binding and of the N-methyltransferase activity. Unfortunately, no further studies on the latter or on other biological activities have been reported.

The formation of the 1:1 Cu(II)-GSSG complex could take place not only as result of the direct interaction between Cu^{2+} ions and the GSSG molecule, but also (as discussed before in Section 2) from the oxidative conversion of the Cu(I)-$[GSH]_2$ complex; that is, upon the removal of the superoxide anions generated during the interaction between Cu(I)-$[GSH]_2$ and molecular oxygen. As such, the Cu(II)-GSSG is not redox active towards molecular oxygen [65]. Interestingly, however, as reported by Aliaga et al. [107], in the presence of a threefold (or higher) GSH excess, the Cu(II)-GSSG complex can swiftly acquire the ability of its reduced precursor to generate superoxide anions (assessed as DHE oxidation). In the same study, it was shown that the "GSH-dependent acquisition" of superoxide-generating capacity of the Cu(II)-GSSG complex is accompanied by several immediate and/or subsequent spectroscopic and chemical changes which comprise: a decrease in the OD at 625 nm, formerly employed to monitor the formation of Cu(II)-GSSG [97], the appearance of Cu(I) (assessed by

the Cu(I)-bathocuproine assay), and the accumulation of GSSG molecules in the medium (assessed through the EDTA-releasable NADPH-coupled reductase assay). These changes, which pointed out to a GSH-mediated reduction of the Cu(II)-GSSG complex (Rx. 10), were complemented by NMR evidence which directly imply its conversion into the Cu(I)-$[GSH]_2$ complex. In fact, the addition of GSH to a Cu(II)-GSSG preparation led to the disappearance of its NMR spectrum, and to the concomitant appearance of an NMR pattern which is typically featured by the Cu(I)-$[GSH]_2$ complex (Rx. 10 and Rx. 11).

$$2Cu(II) - GSSG + 2GSH \longrightarrow 2Cu(I) - GSSG + GSSG + 2H^+ \quad (Rx.10)$$

$$Cu(I) - GSSG + 2GSH \longrightarrow Cu(I) - [GSH]_2 + GSSG \quad (Rx.11)$$

According to Rx. 10, the reductive effect of GSH, which is on the Cu(II), would lead to the formation of a Cu(I)-GSSG intermediate, presumably, IOC (referred to in Rx. 7). Interestingly, Aliaga et al. [107] observed that the ability of GSH to reduce Cu(II)-GSSG cannot be mimic by millimolar concentrations of ascorbate, the second most abundant cellular reductant. Based on the standard reduction potentials (E°) of GSSG/GSH, dehydroascorbate/ascorbate and Cu^{2+}/Cu^{+} (−0.26 V, 0.40 V, and 0.17 V, respectively), 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 [108]. In turn, the much negative E° value of the GSSG/GSH couple makes GSH thermodynamically capable of reducing Cu^{2+}. The lack of ability of ascorbate to reduce Cu(II)-GSSG was also shared by superoxide. Presumably, the latter species, despite having a standard reduction potential which theoretically would favor the reduction of Cu^{2+}, is unable to catalyze this reaction when the metal is bound to GSSG [107]. On the other hand, regarding the copper-binding capacity of GSH, according to Rx. 4, relative to GSSG, the tripeptide would have a greater affinity for Cu(I) [59].

Besides its potential to undergo GSH-mediated reduction into Cu(I)-$[GSH]_2$, early work conducted by Jouini et al. [104], in which a pulse radiolysis technique was used, suggested the possibility that the Cu(II)-GSSG complex exhibits a superoxide-dismutase activity. Based on Jouini's work, our laboratory investigated further the potential of Cu(II)-GSSG to directly react with the superoxide generated by a xanthine/xanthine oxidase system [109]. Notably, removal of superoxide by the Cu(II)-GSSG complex was estimated to take place at a rate constant ($k_{Cu(II)-GSSG}$) of $7.8 \times 10^6\ M^{-1}\ s^{-1}$, which highly compares with the one reported by Jouini et al. [104] ($6 \times 10^6\ M^{-1}\ s^{-1}$). Although the latter value is three orders of magnitude lower than that reported for superoxide dismutase (SOD; $2 \times 10^9\ M^{-1}\ s^{-1}$ [110]), it is totally comparable to or even greater than the rate constant values reported for several other copper-containing complexes with SOD mimetic properties; for instance, for those formed between Cu(II) and various amino acid residues and peptides [111], salicylates [112,113], semicarbazones [114–116], macrocyclic- [117] and tetradentate-Schiff-bases [118,119].

As expected for any other molecule with an SOD-like action, the study by Speisky et al. [109] found that the interaction between Cu(II)-GSSG and superoxide radicals leads to the simultaneous generation of hydrogen peroxide and oxygen molecules. As a possible mechanism for the superoxide dismutating action of the Cu(II)-GSSG complex, a redox cycling reaction has been postulated in which, initially, the Cu(II) ion undergoes a reduction by one mole of superoxide, to form one mole of molecular oxygen and one mole of a Cu(I)-RSSR complex intermediate. Subsequently, the latter species is readily oxidized by a second mole of superoxide, to regenerate the Cu(II)-RSSR complex and form one mole of hydrogen peroxide (Fig. 4).

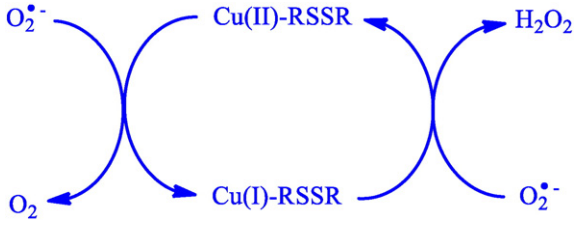

Fig. 4. Scheme representing the proposed mechanism for the dismutation of superoxide induced by the various Cu(II)-RSSR complexes.
Figure taken from [109].

Interestingly, however, in the same work, it was observed that the concentrations of hydrogen peroxide and molecular oxygen generated during the removal of superoxide were below and above those theoretically-expected, respectively. These results were finally explained by the observation that the Cu(II)-GSSG complex is able to react directly with hydrogen peroxide to generate molecular oxygen. According to Speisky et al. [109] the former results would indicate that the Cu(II)-GSSG complex would not only have a capacity to dismutate superoxide but also to simultaneously act like a catalase mimetic molecule. Noteworthy, these abilities of the Cu(II)-GSSG complex were found not to be limited to the GSSG molecule as they were also observed for complexes formed between copper(II) ions and other biologically-occurring disulfides (like cystine, homocystine and α-lipoic acid). Nonetheless, the latter complexes differ among themselves in terms of the rate constants for their superoxide-dismutating and initial rates of conversion of hydrogen peroxide into molecular oxygen. Thus, while the Cu(II)-GSSG and the Cu(II)-α-Lipoic acid complexes were the most active complexes in terms of their SOD-mimetic action, the Cu(II)-Cystine complex was the one displaying the highest catalase-like action. To explain the disproportionate ability of these complexes to convert H_2O_2, based on what was early proposed by Abd El-Motaleb & Ramadan [120] for other metal-containing complexes, our laboratory has proposed as a possible mechanism reactions 12 and 13:

$$Cu(II) - RSSR + H_2O_2 \longrightarrow Cu(I) - RSSR + HOO^{\bullet} + H^+ \quad (Rx.12)$$

$$Cu(I) - RSSR + HOO^{\bullet} + H^+ + H_2O_2 \longrightarrow Cu(II) - RSSR + 2H_2O + O_2 \quad (Rx.13)$$

The validity of the above-proposed mechanism, which assumes redox cycling reactions involving the copper ion, has not been established yet. Its assessment requires spectroscopic studies on the transient formation of the Cu(I)-RSSR intermediates (Rx. 12), the conservation of the Cu(II)-RSSR complexes, and the quantitative disappearance of H_2O_2 and recovery of molecular oxygen.

4. Potential biological implications associated with the formation of the Cu(I)-$[GSH]_2$ and the Cu(II)-GSSG complexes

The ability of Cu(I)-$[GSH]_2$ to function as a continuous source of superoxide renders this complex with a potential to modify the functioning of various superoxide-susceptible biological targets. Among these are the heme iron-containing enzymes aconitase [29], isocitrate dehydrogenase [30], NADH: ubiquinone oxidoreductase, and the iron-storing protein ferritin [28,121–123].

Prompted by the established ability of Cu(I)-$[GSH]_2$ to generate $O_2^{\bullet -}$ [64], Aliaga et al. (2011) studied the redox consequences that could arise from the interaction between this complex and ferritin. In its in vitro work, Aliaga et al. [124] demonstrated that, throughout a reaction mediated by such radicals, the complex is able to reduce the Fe^{3+} ions bound to ferritin and to release them under the form of free Fe^{2+}

ions (which were assessed as iron complexed by bathophenanthroline). The iron-releasing ability of the Cu(I)-$[GSH]_2$ complex was found to depend largely (by nearly 80%) on its capacity to generate $O_2^{\bullet-}$. The remaining fraction, found not to be susceptible to inhibition by SOD, was attributed to a direct action of the GSH contained within the complex since free GSH molecules were also found to be able to induce Fe^{2+} release, although to a much lesser extent. The maximal Fe^{3+}-reducing/Fe^{2+}-releasing efficiency of the complex (seen at 5 μM) was near 40%. Interestingly, this value compares well with those reported formerly by other studies in which the superoxide-dependent release of iron from ferritin was achieved using activated polymorphonuclear leukocytes (obtaining a 40% efficiency under experimental conditions which led to a 5 μM superoxide production) [121] or di(4-carboxybenzyl)hyponitrite (SOTS-1) (45% efficiency for a 10 μM superoxide production) [28]. Additionally, Aliaga et al. [124] demonstrated that when H_2O_2 was present in a ferritin-containing media, the ability of the Cu(I)-$[GSH]_2$ complex to generate $O_2^{\bullet-}$ translates into an ability to form hydroxyl radicals, exacerbating its superoxide-related pro-oxidant potential. In line with the latter, Rousseau & Puntarulo [125] have 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 also have the potential to catalyze hydroxyl radical formation [25]. However, to participate in such reaction (Rx. 1), copper ions need to occur in their free and reduced state [126]. In fact, as reported by us [64] and earlier by other investigators [87,90,91], the Cu(I)-$[GSH]_2$ complex is totally unable to catalyze the decomposition of H_2O_2 into hydroxyl radicals. However, based on the ability of the Cu(I)-$[GSH]_2$ complex to generate the redox-active Fe^{2+} species (either from free or ferritin-bound iron), we have proposed that in the presence of ferritin, copper ions, regardless of whether they occur freely or bound to GSH, would be able to promote hydroxyl radical formation. Considering that oxygen and ferritin molecules normally occur intracellularly, it is reasonable to assume the existence of a pro-oxidant potential for the Cu(I)-$[GSH]_2$ complex within cells. This potential would be expected to be exacerbated under conditions which favor the formation of the Cu(I)-$[GSH]_2$ complex, for instance, during or following the over-exposure of isolated cells [40], rodents [127] or humans [128] to copper.

In contrast with the pro-oxidant potential demonstrated by the Cu(I)-$[GSH]_2$ complex is the antioxidant potential of the Cu(II)-GSSG complex. As described before (Section 2), the latter complex is formed upon removal of the superoxide anions generated by Cu(I)-$[GSH]_2$. In the non-biological setting in which most studies were conducted (Rx. 7 and Rx. 8), removal of superoxide was experimentally achieved either through autodismutation or induced by SOD or Tempol. Given the presence of SOD in cytosol and mitochondria, a similar scenario could be expected to occur intracellularly. One might assume that the oxidative stress observed in copper-overloaded cells [40] reflects an unbalance in which the rate of generation of ROS surpasses the cell's capacity to remove these species. Within such context, it is not feasible to know whether a given flow of superoxide anions generated by the Cu(I)-$[GSH]_2$ complex would be sufficiently counteracted by the co-occurring superoxide-removing mechanisms. However, regardless of whether the superoxide generated by the latter complex undergo SOD-mediated dismutation or are removed as a consequence of their eventual targeting of some particularly superoxide-reacting molecules, from Rx. 8 it would seem reasonable to assume that in any of these scenarios, a Cu(II)-GSSG complex is likely to be formed. We speculate that, given the SOD- and catalase-like properties of the latter complex, its formation is likely to contribute to the overall antioxidant capacity of the cell. To gain more insights into the potential relevance of the SOD and/or catalase-like activity of the Cu(II)-RSSR complexes in biologically-relevant systems, recently Speisky and co-workers (Speisky et al. 2013) investigated both properties in a preparation of rat duodenal epithelium-isolated mitochondria. Taking advantage of the ability of non-steroidal anti-inflammatory drugs to inhibit the mitochondrial complex I [129], which translates into a significant increase of the intra-mitochondrially-generated superoxide, a series of Cu(II)-RSSR complexes were shown to be able to act as SOD mimetics, inhibiting the DHE-superoxide reaction. Among four studied complexes, the highest SOD mimetic activity was observed at very low (2–4 μM) concentrations for the complexes formed between Cu(II) and α-lipoic acid (disulfide form of dihydrolipoic acid), GSSG and homocystine; while the lowest activity was reported for Cu(II)-Cystine complex. Interestingly, the three former Cu(II)-containing complexes were found not only to totally restore the oxidative tone in superoxide-overproducing mitochondria, but also to lower the mitochondrial production of superoxide below the basal level (when tested at a 4 μM concentration). In view of the latter results, further research aimed at evaluating the potential of some of the formerly referred Cu(II)-disulfides to control the mitochondrial production of ROS under physiological and/or oxidative-stress conditions appears warranted.

5. Conclusions

The presence of free copper ions in a biological milieu is associated with their deleterious binding to essential ligands, and with the occurrence of copper-catalyzed ROS formation. In the case of GSH, in particular, binding of copper has been long seen as a possible mechanism to lower, if not abolish, the otherwise high electrophilicity and redox activity displayed by free copper ions. Recently, however, as result of a systematic research conducted primarily by our laboratory, the presumed protective role of the Cu(I)-$[GSH]_2$ complex has been opposed by evidence revealing that binding to GSH, rather than stabilizing free copper ions, translate into the formation of a significant source of superoxide radicals. Indeed, employing various experimental approaches, Speisky and collaborators [64–66] demonstrated that the Cu(I)-$[GSH]_2$ complex undergoes swift oxidation in the presence of molecular oxygen, forming superoxide radicals and a Cu(I)-GSSG complex intermediate. Furthermore, it is now understood that conditions leading to the removal of superoxide (namely, in the presence of SOD or some superoxide-reacting target molecules), allow the further oxidation of the former intermediate into the Cu(II)-GSSG complex. Interestingly, evidence also exist showing that in the presence of a molar GSH excess, the latter complex can undergo reduction to regenerate both, the reduced complex it originated from as well as its superoxide-generating capacity. Thus, the possibility existed that in the presence of high GSH concentrations (as occurring intracellularly), the Cu(I)-$[GSH]_2$ and Cu(II)-GSSG complexes be part of a GSH-dependent interplay. The latter would take place through a set of reactions in which superoxide anions would be generated, on one hand directly by the former complex, and on the other, indirectly, upon the GSH-mediated reduction of Cu(II)-GSSG. However, in view of recent evidence in favor of a ROS-removing capacity of the Cu(II)-GSSG complex, the proposed interplay would not necessarily imply that in the presence of a GSH excess both complexes would only contribute to elevate superoxide formation. In fact, as shown by Speisky et al. [109], upon its formation, the Cu(II)-GSSG complex can not only undergo GSH-mediated reduction, but also serve catalytically as an efficient superoxide dismutating- and catalase-like molecule. These ROS-removing properties of Cu(II)-GSSG were recently confirmed when added to superoxide-generating mitochondria; thus, presumably, even in the presence of intramitochondrial GSH, the oxidized complex appears to conserve its antioxidant properties. Beyond its possible relevance to enhance our understanding on the possible mechanisms underlying copper cytotoxicity, further research on the actual intracellular formation and on the redox properties of the reduced and oxidized copper-glutathione complexes seems warranted.

Abbreviations

ATX1	antioxidant protein 1
BSO	buthionine sulfoximine

CCS	copper chaperone for superoxide dismutase 1
COX17	mitochondrial copper chaperone
Cu^{+}	reduced or cuprous ions
Cu^{2+}	oxidized or cupric ions
Cys	reduced cysteine
Cys-Gly	cysteinyl-glycine
Cyt c	cytochrome *c*
DHE	dihydroethidium
DHLA	dihydrolipoic acid
DMPO	5,5-Dimethyl-1-Pyrroline-N-Oxide
EDTA	ethylene diamine tetra-acetic acid
EPR	electron paramagnetic resonance
FPLC	fast protein liquid chromatography
Fe^{2+}	reduced or ferrous ions
Fe^{3+}	oxidized or ferric ions
GSH	reduced glutathione
GSSG	oxidized glutathione
γ-GluCys	γ-glutamyl-cysteine
HCys	homocysteine
$HO^{\bullet}$	hydroxyl radical
HAC	human hepatoma cell
IOC	intermediate oxidized form of the complex
LDL	low density lipoproteins
MT	metallothionein
NADH	reduced form of nicotinamide adenine dinucleotide
NADPH	dihydronicotinamide-adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
ROS	reactive oxygen species
RSSR	disulfide(s)
SOD	superoxide dismutase
$O_2^{\bullet-}$	superoxide radical
XANES	X-ray absorption near edge structure
8-OHdG	8-hydroxydeoxyguanosine

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