Revisiting the pro-oxidant activity of copper: interplay of ascorbate, cysteine and glutathione

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Abstract

Copper (Cu) is essential for most organisms, but it can be poisonous in excess, through mechanisms such as protein aggregation, trans-metallation and oxidative stress. Latter could implicate the formation of potentially harmful Reactive Oxygen Species (ROS: $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$) via the redox cycling between Cu(II)/Cu(I) states in the presence of dioxygen and physiological reducing agents such as ascorbate (AscH), cysteine (Cys) and the tripeptide glutathione (GSH). Although the reactivity of Cu with these reductants has been previously investigated, the reactions taking place in a more physiologically-relevant mixture of these biomolecules are not known. Hence, we report here on the reactivity of Cu with binary and ternary mixtures of AscH, Cys and GSH. By measuring ascorbate and thiol oxidation, as well as $HO^{\bullet}$ formation, we show that Cu reacts preferentially with GSH and Cys, halting AscH oxidation and also $HO^{\bullet}$ release. This could be explained by the formation of Cu-thiolate clusters with both GSH and, as we first demonstrate here, Cys. Moreover, we observed a remarkable acceleration of Cu-catalysed GSH oxidation in the presence of Cys. We provide evidence that both thiol-disulfide exchange and the generated H_2O_2 contribute to this effect. Based on these findings, we speculate that Cu-induced oxidative stress may be mainly driven by GSH depletion and/or protein disulfide formation rather than by $HO^{\bullet}$ and envision a synergistic effect of Cys on Cu toxicity.

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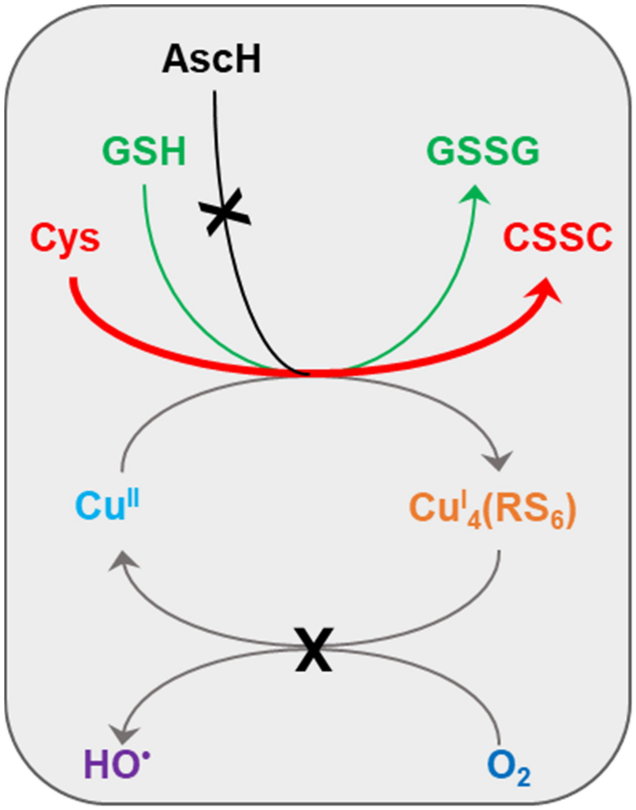
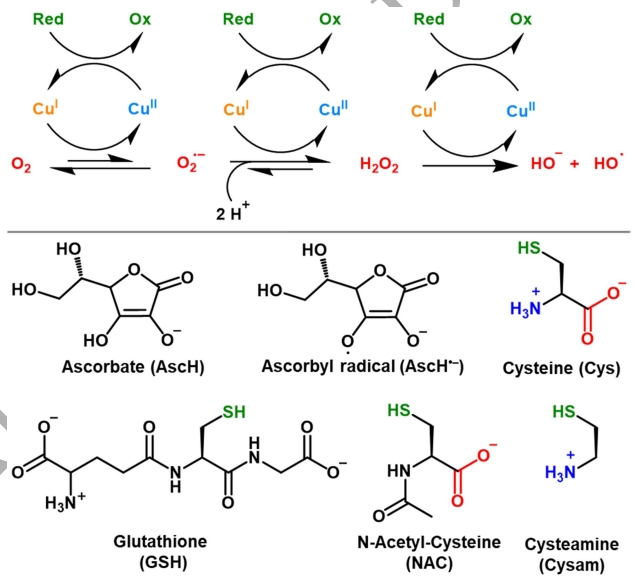
Graphical Abstract



Introduction

Copper (Cu) is involved in fundamental biochemical processes (e.g. cellular respiration) and hence is an essential element for most organisms. In humans, Cu mostly serves as a redox cofactor of enzymes catalysing the activation of oxygen (e.g. oxidases and monooxygenases) via its cycling between Cu^{I} and Cu^{II} redox states.[1] To prevent undesired Cu redox activity outside the active sites of Cu-enzymes, a set of extracellular carriers, membrane transporter and cytosolic Cu-chaperones ensure a safe Cu transport in the body. Such transporters stabilize Cu in one of the possible redox states, notably Cu^{II} in the oxidizing extracellular milieu and Cu^{I} in the reducing intracellular environment.[2] Nevertheless, their "Cu-buffering" capacity can be overcome in the case of Cu overload, eventually resulting in Cu toxicity. Along with the recently identified trans-metallation of Fe-S clusters and protein aggregation,[3–7] the aerobic redox chemistry of labile (i.e. loosely-bound) Cu, which can lead to the formation of potentially harmful Reactive Oxygen Species (ROS, such as $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$, Scheme 1) and oxidative stress, has been commonly considered accountable for Cu toxicity.[8] Among ROS, $HO^{\bullet}$ radical is considered to be one of the most dangerous, due to its higher intrinsic reactivity and to the absence of specific scavenging systems, which instead exist for $O_2^{\bullet-}$ (i.e. superoxide dismutase, SOD) and H_2O_2 (catalase).[9] Cu-catalysed ROS generation can be fuelled by different physiological reducing agents (Scheme 1), such as ascorbate (AscH), glutathione (GSH) and cysteine (Cys).



Scheme 1. *Top*) Mechanism of Cu-catalysed ROS production in the presence of dioxygen and a reducing agent (Red), which is converted to its oxidized form (Ox). *Bottom)* Possible reducing agents in cells are Ascorbate (AscH), Glutathione (GSH) and Cysteine (Cys), which are converted into Ascorbyl radical ($AscH^{\bullet-}$), Glutathione Disulfide (GSSG) and Cystine (CSSC), respectively. N-acetyl-Cysteine (NAC) and cysteamine (Cysam) are Cys analogues.

AscH is present up to millimolar concentration in cells and is very competent in inducing Cu-catalysed ROS formation in the test tube.[10,11]
GSH is the most abundant thiol in cells (1-10 mM), where it helps keep Cu in the reduced Cu^{I} state.[12] In the test tube, GSH binds Cu^{I} in relatively redox-stable Cu-thiolate clusters, and in cells, it binds labile Cu in the case of overload.[13–15] In contrast, cysteine (Cys, Scheme 1), which reacts with Cu faster than GSH,[16] is found at much lower concentrations (30-250 μM) and becomes toxic at higher levels.[17]
Although the behaviour of Cu with each of the above-mentioned reductants has been investigated, little is known about the reactions taking place in a more physiologically-relevant mixture of these biomolecules. Therefore, we explored the reactivity of Cu with binary and ternary mixtures of the above-mentioned biomolecules, with the aim to speculate about the possible mechanisms behind Cu-induced oxidative stress.

Results and discussion

Impact of thiols on Cu-catalysed ascorbate oxidation and hydroxyl radical generation

First, we assessed the effect of thiols on Cu-catalysed aerobic ascorbate oxidation, which can be monitored photometrically via the decrease of its characteristic absorption at 265 nm. Unless otherwise stated, reactions were performed in open microplates, ensuring the availability of dioxygen (dissolved concentration of about 270 μM). In the absence of thiols, the addition of Cu^{II} promptly triggered the aerobic oxidation of AscH (Fig. 1), which is complete within ~50 min. When GSH was present (at a concentration equal to AscH, 100 μM) before Cu addition, the onset of AscH oxidation appeared to be remarkably delayed (Fig. 1, green). We supposed, in agreement with the literature,[18] that during such a lag phase Cu catalysed the aerobic oxidation of GSH to GSSG. Indeed, the quantification of GSH through the classical DTNB assay (also known as Ellmann's test) showed that (in the absence of AscH) Cu^{II} was able to catalyse the aerobic oxidation of GSH in a timespan (~180 min) similar to the lag phase observed in the ascorbate oxidation (Fig S1, green).[†] Cys also delayed the onset of Cu-catalysed AscH oxidation, despite to a much lesser extent (~ 5 min) compared to GSH, which is coherent with the very much faster Cu^{II}-catalysed oxidation of Cys compared to GSH (Fig. 1 and S1, red).[16] Actually, such an inhibitory effect of thiols on Cu-catalysed ascorbate oxidation has been long known,[18–22] and can be interpreted considering that Cu^{II} is first reduced and coordinated by the thiols, where it is stabilized in the Cu^{I} state, and catalyses their oxidation to disulfide by O_2, while it catalyses the oxidation of AscH only once little or no reduced thiols are present. Interestingly, when both Cys and GSH were present, the lag phase in AscH oxidation was much shorter than in the absence of Cys (Fig. 1, blue), suggesting that Cys accelerates GSH oxidation, as we also

[†] The results show that GSH oxidation to GSSG is independent of the presence of AscH, indicating that AscH is not catalysing GSH oxidation. Indeed, the steadiness of AscH could have been only apparent, and notably due to a fast reduction of the ascorbyl radical ($AscH^{\bullet-}$) by GSH.[47] This appears to be at most negligible in light of the very similar kinetics of thiols oxidation in the absence and in the presence of ascorbate.

observed in the absence of AscH (Fig. S1, blue). Remarkably, such acceleration is also observed with 10-fold less Cys (100 μM) than GSH (1 mM), which are in the range of physiological concentrations (Fig. S2).

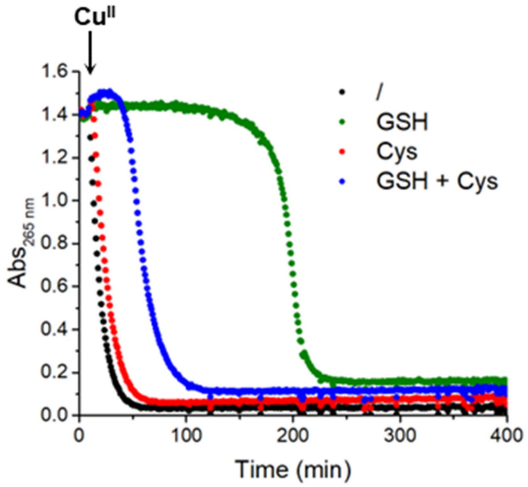


Figure 1. Effect of thiols on Cu^{II}-catalysed oxidation of AscH. Ascorbate oxidation in the presence of Cu^{II} only (black), Cu^{II} and GSH (green), Cu^{II} and Cys (red), Cu^{II}, GSH and Cys (blue); conditions: [AscH] = 100 μM, [Cu^{II}] = 10 μM, [GSH] = 100 μM, [Cys] = 100 μM, phosphate buffer 50 mM pH 7.4.

Since Cu-catalysed aerobic oxidation of ascorbate and thiols is normally accompanied by the formation of ROS (Scheme 1), we also followed the $HO^{\bullet}$ production using CCA (Coumarin-Carboxylic Acid), which forms the fluorescent 7-hydroxy-CCA (λ_{ex} = 390 nm, λ_{em} = 452 nm) upon reaction with $HO^{\bullet}$. Interestingly, no $HO^{\bullet}$ was detected as long as reduced thiols were present (Fig. S3A). Noteworthy, the time delay observed for the onset of $HO^{\bullet}$ production fits that of the beginning of AscH oxidation. Moreover, although no $HO^{\bullet}$ was detected in the presence of Cys only (Fig. S3B), the latter was oxidised too fast to significantly delay the $HO^{\bullet}$ production in the presence of AscH. Of note, the measurement of TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) EPR signal, which is quenched by reaction with $HO^{\bullet}$, also showed that no significant amount of $HO^{\bullet}$ is produced by Cu^{II} in the presence of thiols (Fig. S3C).

Spectroscopic evidence for the formation of Cu-Cys clusters

In order to explain why the Cu-catalysed GSH oxidation by O_2 does not generate $HO^{\bullet}$ radicals, it has been suggested that GSH-bound Cu^{I} reduces H_2O_2 to H_2O, rather than $HO^{\bullet}$.[23] This process is indeed plausible, especially in light of the currently-known formation of multinuclear $Cu_x(GS)_y$ clusters,[13] where the proximity of several Cu ions could favour the occurrence of a two-electron reduction. Since Cu^{II} did not produce $HO^{\bullet}$ in the presence of Cys, we assessed whether Cu-thiolate clusters are also formed with Cys by means of X-ray absorption spectroscopy (XAS) and low-temperature luminescence.

Both the Cu K-edge XANES (X-ray Absorption Near Edge Spectroscopy) and EXAFS (Extended X-ray Absorption Fine Structure) spectra (Fig. 2) of Cu-Cys and Cu-GSH samples are indistinguishable within the experimental error. Therefore, the Cu coordination in Cu-Cys is

substantially identical to that in Cu-GSH, which forms mostly $Cu_4(GS)_6$ clusters.[13] Indeed, the EXAFS data can be fitted by a model Cu_4S_6 cluster,[24,25] in which each Cu atom is surrounded by 3 S atoms at about 2.3 Å and a "disordered" shell (i.e. with relatively high values of σ^2, the mean square deviation of the distance) composed of 3 Cu atoms at about 2.8 Å (Fig. S4 and Table S1), although models with a different number of Cu scatterers cannot be ruled out solely based on EXAFS data.

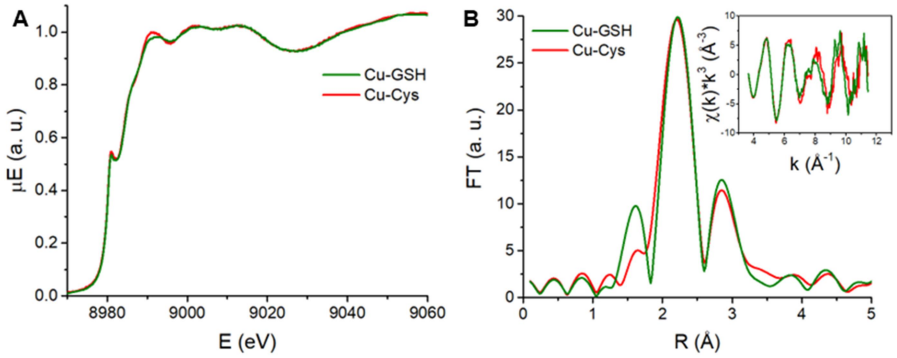


Figure 2. XANES spectra (A) and Fourier Transforms (B) of the EXAFS (inset) spectra of Cu-GSH (green) and Cu-Cys (red) complexes. Conditions: [Cu^{II}] = 1 mM, [GSH] = 10 mM, [Cys] = 10 mM, phosphate buffer 200 mM pH 7.4.

In light of the known luminescent emission of $Cu_4(GS)_6$ and Cu-metallothioneins clusters,[13,26] we also recorded low-temperature (77 K) luminescence spectra of Cu-Cys and Cu-GSH. Interestingly, upon excitation at 310 nm, Cu-Cys exhibited a luminescent emission band at 418 nm, similar but slightly blue-shifted compared to the emission of Cu-GSH at 423 nm (Figure 3A), attributed to tetranuclear $Cu_4(GS)_6$. Likewise, the excitation spectrum (λ_{em} = 310 nm) of Cu-Cys appears to be blue-shifted compared to that of Cu-GSH (Figure 3B), in agreement with the absorption spectra reported in the literature.[13,27] This further confirms that, similarly to GSH, Cu and Cys form tetranuclear $Cu_4(Cys)_6$ clusters. In addition, we measured the luminescence of a mixture of Cu, GSH and Cys, which showed emission and excitation spectra very similar to those of Cu-GSH clusters, suggesting that GSH has a higher affinity for Cu than Cys. Notwithstanding, the accelerating effect of Cys on GSH oxidation suggests that a minor portion of Cu-Cys or mixed Cu-GSH/Cys clusters exist.

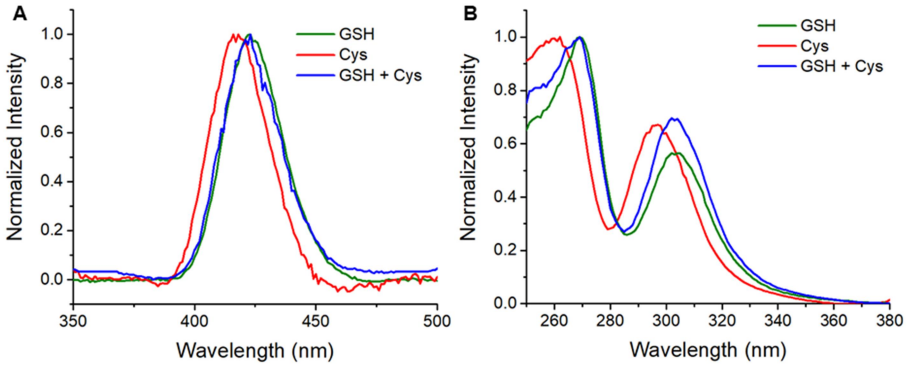


Figure 3. Low-temperature (77 K) luminescence emission (A) and excitation (B) spectra of Cu-thiols mixtures (GSH, green; Cys, red; GSH and Cys, blue). Conditions: [Cu^{II}] = 100 µM, [GSH] = 1 mM, [Cys] = 1 mM, phosphate buffer 50 mM pH 7.4.

Insights into the mechanism of Cys-accelerated GSH oxidation

Previous reports have shown the acceleration of GSH oxidation by Cys in the absence of Cu or the presence of Cu,Zn-SOD, and the thiol-disulfide exchange reaction between cysteine disulfide, CSSC, and GSH (Eq. 1) has been postulated as the mechanism.[28–30]

$$2\ GSH + CSSC \rightarrow 2\ Cys + GSSG \qquad \text{Eq. 1}$$

To assess such a hypothesis, we compared the kinetics of GSH oxidation by Cu^{II} in the presence of Cys and CSSC using the lag phase in AscH oxidation assay as a convenient read-out for the thiol oxidation kinetics. Indeed, if CSSC accelerates GSH oxidation simply via thiol-disulfide exchange, a shorter lag phase (of less than ~5 min in our conditions, i.e. the time needed to completely oxidise Cys to CSSC) would be expected when directly adding the corresponding amount (0.5 equivalent, i.e. 50 µM) of CSSC, rather than Cys, to the mixture containing AscH, GSH and Cu^{II}. Actually, although CSSC accelerated GSH oxidation, the lag phase proved to be longer when 50 µM CSSC, rather than 100 µM Cys, was added (Fig. 4, orange), suggesting that the catalytic effect of Cys on GSH oxidation is not merely driven by the thiol-disulfide exchange between CSSC and GSH. Considering that H_2O_2 is also formed along Cys oxidation to CSSC,[31] we explored the possibility that H_2O_2 also contributes to the acceleration of GSH oxidation. Indeed, the addition of 50 µM H_2O_2 together with 50 µM CSSC (Fig. 4, gold), decreased the lag phase more than CSSC alone, and notably to a very similar extent compared to 100 µM Cys (Fig. 4, blue). Therefore, along with the thiol-disulfide exchange previously suggested, the H_2O_2 produced during Cys oxidation by Cu^{II} seems to contribute to the acceleration of GSH oxidation. Interestingly, the Cu/Cys-catalysed aerobic GSH oxidation could contribute to Cys toxicity and also exacerbate the toxicity of excess Cu in cells.

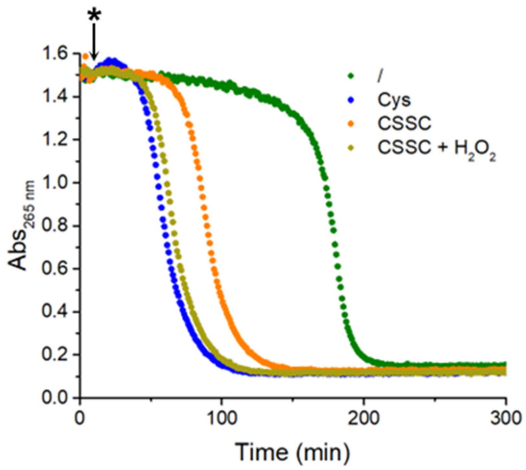


Figure 4. Effect of CSSC and H_2O_2 on the Cu^{II}-catalysed oxidation of AscH in the presence of GSH. Conditions: [AscH] = 100 µM, [Cu^{II}] = 10 µM, [GSH] = 100 µM, [Cys] = 100 µM, [CSSC] = 50 µM, [H_2O_2] = 50 µM, phosphate buffer 50 mM pH 7.4. First, AscH and GSH were pre-mixed; then, at the time point indicated by *, the following compounds were added: Cu^{II} (green), Cu^{II} and Cys (blue), Cu^{II} and CSSC (orange), Cu^{II}, CSSC and H_2O_2 (gold).

To better understand the faster oxidation of Cys compared to GSH and its accelerating effect, we also examined the reactivity of its derivatives N-Acetyl-Cysteine (NAC, Scheme 1), which is commonly used as an antioxidant,[32] and cysteamine (Cysam, Scheme 1), a simple aminothiol. Thus, we assessed the effect of NAC and Cysam on the GSH oxidation in the presence of Cu^{II} using the AscH oxidation assay (Fig. 5 and S5). Cu^{II}-catalysed aerobic NAC oxidation also appeared to be faster than GSH oxidation (Fig. 5, magenta), but to a much lower extent than for Cys. Instead, Cysam reacted, at least, as fast as Cys (Fig. S5). However, contrary to Cys and Cysam (Fig. S5), NAC had little (if at all) impact on the rate of GSH oxidation (Fig. 5, violet). Such faster oxidation of Cys, Cysam and NAC compared to GSH could be explained by their ability to chelate Cu^{II} in a bidentate fashion via the thiol group together with the amino (Cys and Cysam) or carboxylate (NAC) moiety (Scheme 1). Indeed, since the re-oxidation of Cu^{I} to Cu^{II} is considered the rate-limiting step of the Cu-catalysed thiol oxidation,[16] the stabilization of the Cu^{II} state by Cys, Cysam and NAC can fasten the reaction. Besides, the negligible effect of NAC on the rate of GSH oxidation can be attributed to its higher pK_a (9.5) compared to Cys (8.3) and Cysam (8.2), which implies a much slower thiol-disulfide exchange.[33,34]

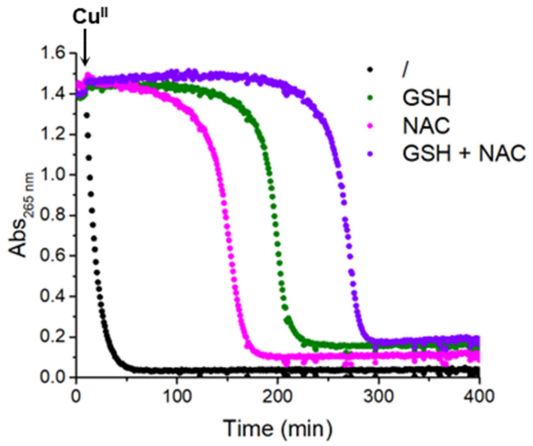


Figure 5. Effect of NAC on GSH oxidation. Ascorbate oxidation in the presence of Cu^{II} (black), Cu^{II} and GSH (green), Cu^{II} and NAC (magenta) or Cu^{II}, GSH and NAC (violet); conditions: [AscH] = 100 μM, [Cu^{II}] = 10 μM, [GSH] = 100 μM, [NAC] = 100 μM, phosphate buffer 50 mM pH 7.4.

Conclusions

Excess labile copper is generally considered to be toxic owing, among other mechanisms, to the formation of ROS through O_2 reduction by Cu^{I} ions. Hence, Cu-catalysed ROS production depends primarily on dioxygen (whose level in cells can vary quite a lot, from normoxic, to hypoxic such as in cancer, down to zero in cells living anaerobically) and reducing agents. In cells, the most relevant Cu reductants are ascorbate, GSH or Cys. In this study, we confirm that in a mixture of such biomolecules, oxidation of the two thiols occurs before that of ascorbate. Interestingly, the spectrophotometric measurement of ascorbate concentration at 265 nm results to be a convenient *in-situ* readout of thiol oxidation. Importantly, and in line with the literature,[23] Cu-catalysed aerobic GSH/Cys oxidation is not accompanied by the release of harmful $HO^{\bullet}$ radicals, which are instead detected during Cu-catalysed aerobic ascorbate oxidation. It is worth noting that this behaviour is not an intrinsic feature of thiols, as $HO^{\bullet}$ can be detected when thiol oxidation is catalysed by Cu-complexes.[35] Hence, we speculate that such reactivity of thiols arises from the formation of Cu-thiolate clusters. Indeed, we showed that Cys also forms tetranuclear Cu-S clusters similar to the already known $Cu_4(GS)_6$.[13] Interestingly, the proximity of several Cu ions in a multinuclear cluster could favour the two-electron reduction of H_2O_2 to H_2O that was suggested by previous studies.[23] Alternatively, $HO^{\bullet}$ radicals could be formed by Cu-GSH/Cys but react with the neighbouring thiols of the cluster, preventing their release and detection by external probes (CCA or TEMPOL in this study). Hence, the potential role of Cu-thiolate clusters in preventing $HO^{\bullet}$ formation or release is worthy of further experimental and computational investigations. Besides, in analogy with previous reports,[28–30] we observed that Cys, as well as Cysam but not NAC, accelerates Cu-catalysed GSH oxidation, although Cu-GSH is the predominant species even in the presence of equimolar Cys. We also showed that GSH oxidation is accelerated even at physiologically relevant sub-

stoichiometric Cys:GSH ratio of 1:10. Moreover, not only thiol-disulfide exchange but also oxidation via H_2O_2 contribute to the Cu-catalysed acceleration of GSH oxidation by Cys.

From a biological perspective, our findings suggest that, in the case of Cu overload, oxidative stress arises, in the first instance, from aberrant disulfide, notably GSSG, formation, rather than $HO^{\bullet}$ production. It is indeed well established that excess Cu promotes GSH depletion, altering the GSH/GSSG balance.[15,36–38] Although this impairs cellular redox homeostasis, excess Cu buffering by GSH helps to protect other potential Cu targets, such as protein thiols. Indeed, upon GSH depletion, Cys-containing proteins with high Cu-affinity (e.g. those containing the CXXC motif)[39] could undergo Cu-catalysed oxidation resulting in loss of function. Moreover, as recently demonstrated, Cu can also target protein thiols in compartments such as bacterial periplasm, where GSH concentration is lower.[40,41] Furthermore, in light of the recent discovery of lipoylated proteins as preferential targets of Cu toxicity,[6] the ability of Cu to catalyse lipoic acid (a dithiol) oxidation despite the presence of excess GSH remains to be assessed. Whether Cu-catalysed protein disulfide formation is accompanied by $HO^{\bullet}$ formation is another important aspect that warrants future studies. Interestingly, the ability of Cu to oxidize and hence deplete Cys faster than GSH, could be one of the reasons why, during evolution, an N-protected thiol like GSH, rather than an aminothiol like Cys, was selected as the most abundant intracellular thiol: GSH represents a more resilient Cu buffering system than Cys, ensuring longer protection and survival in the case of Cu stress. Finally, it is noteworthy that, as it has been shown for Fe,[42] high Cys levels may exacerbate the poisonous effects of excess Cu ions on cellular redox homeostasis, and hence the increase of Cys levels can be envisioned as a strategy to enhance the cytotoxic activity of Cu for therapeutic purposes.

Acknowledgements

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Materials and methods

Stock solutions

Commercially available chemicals were used without further purification. All stock solutions were prepared in ultrapure water (ρ = 18.2 M$\Omega \cdot cm^{-1}$). Cu^{II} stock solution was prepared by dissolving $CuCl_2 \cdot 2H_2O$ salt and its concentration was assessed by UV-vis absorption at 780 nm (ε_{780} = 12 $M^{-1}cm^{-1}$). A stock solution of the phosphate buffer (pH 7.4) was prepared by mixing KH_2PO_4 and K_2HPO_4 and adjusting the pH with a concentrated solution of NaOH. Solutions of sodium ascorbate, GSH, Cys and NAC were freshly prepared before the experiments. Cysteamine stock solution was prepared as follows: cysteamine hydrochloride powder was flushed with N_2, then dissolved in 1 mM HCl thoroughly flushed with N_2 and stored at -20 °C. The concentration of cysteamine was determined via the DTNB assay (see below).

Ascorbate oxidation assay

Ascorbate oxidation was monitored by absorption at 265 nm on a CLARIOstar (BMG Labtech) plate reader inside an open 96-well microplate (Greiner) or in a closed cuvette using an Agilent Cary 60 spectrophotometer (Fig. S2). After mixing AscH (100 µM) and thiols (100 µM each) in phosphate buffer (50 mM, pH 7.4), the signal was monitored for about 10 min to assure no ascorbate auto-oxidation was taking place. Then, Cu^{II} (10 µM) was added and the reaction was monitored over time.

DTNB assay

Thiols oxidation was measured upon the reaction of reduced thiols with the Ellmann's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), monitoring the formation of the TNB^{2-} product by absorption at 412 nm on a CLARIOstar plate reader inside a 96-well microplate (Greiner). After mixing Cu^{II} (10 µM) and thiols (100 µM each) in phosphate buffer (50 mM, pH 7.4), aliquots (25-50 µl) were taken at several time points and transferred to the assay mixture (final volume 100 µl) containing 100 µM DTNB and 1 mM EDTA in 50 mM TRIS buffer pH 8.2 (final concentration of thiol being 50 µM). Thiol concentration was calculated using ε_{412} = 14150 $M^{-1}cm^{-1}$.

CCA assay

The formation of 7-hydroxy-coumarin-3-carboxylic acid (7-OH-CCA) was monitored by fluorescence emission at 452 nm upon excitation at 390 nm on a CLARIOstar plate reader inside a 96-well microplate (Greiner). Cu^{II} (10 µM) was added to a solution containing CCA (500 µM) and AscH, thiols or their mixture (each at 100 µM) in phosphate buffer (50 mM, pH 7.4) and the reaction was monitored over time.

EPR spin scavenging

EPR spin scavenging experiments were performed at room temperature (T = 295 ± 1K) using an EMX-plus (Bruker Biospin GmbH, Germany) X-band EPR spectrometer equipped with a high sensitivity resonator (4119HS-W1, Bruker). Samples were introduced into glass capillaries (Hirschmann, 25 µL) sealed at both ends and rapidly transferred into the EPR cavity for measurement. The principal experimental parameters were microwave frequency of ~9.8 GHz, microwave power of ~4.5 mW, modulation amplitude 1 G, time constant of ~5 ms, conversion time of ~12.5 ms. A scan (sweeping time of ~10 s) was then acquired every 17 s to obtain the kinetics of TEMPOL reduction over time. All spectra were best simulated and the resulting simulations were doubly integrated to relatively quantify the concentration of remaining TEMPOL (I/I_0 = I(t)/I(t=0)). Data analysis and simulations based on experimental data were performed using Xenon (Bruker Biospin GmbH, Germany) and lab-made routines based on EasySpin Toolbox under Matlab (Mathworks) environment.[43]

XAS

XAS data at the Cu K-edge were acquired at the BM30 beamline of the European Synchrotron Radiation Facility (ESRF - Grenoble, France). The beamline energy was calibrated using a metallic Cu foil by setting the position of the absorption edge (defined as the first maximum of the first derivative curve) to 8979 eV. Spectra were recorded in fluorescence mode using a 13-element solid-state Ge detector. In order to minimize X-ray-induced damage, the samples were kept at 10 K in a He cryostat throughout the measurements. The ATHENA software[44] was used to normalize XANES data and to extract the EXAFS signal, which was obtained by cubic splines interpolation as implemented in the AUTOBKG algorithm.[45] The quantitative analysis of the EXAFS spectra was performed using the EXCURV98 code.[46]

Low-temperature luminescence

Low-temperature luminescence spectra were recorded using a FluoroMax Plus spectrofluorometer (Horiba Scientific) equipped with a cylindrical quartz dewar filled with liquid nitrogen (at 77 K). 500 µl samples were transferred to quartz tubes with 4 mm inner diameter and freeze-quenched into liquid nitrogen before their introduction in the dewar.

Data Availability

Data available on request.

References

1. Festa RA, Thiele DJ. Copper: An essential metal in biology. *Curr Biol* 2011;**21**:R877–83.
2. Rubino JT, Franz KJ. Coordination chemistry of copper proteins: How nature handles a toxic cargo for essential function. *J Inorg Biochem* 2012;**107**:129–43.
3. Macomber L, Imlay JA. The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proc Natl Acad Sci U S A* 2009;**106**:8344–9.
4. Wiebelhaus N, Zaengle-Barone JM, Hwang KK, Franz KJ, Fitzgerald, MC. Protein Folding Stability Changes across the Proteome Reveal Targets of Cu Toxicity in E. coli. *ACS Chem Biol* 2021;**16**:214–24.
5. Robison ATR, Sturrock GR, Zaengle-Barone JM, Wiebelhaus N, Dharani A, Williams IG, Fitzgerald MC, Franz KJ. Analysis of copper-induced protein precipitation across the E. coli proteome. *Metallomics* 2023;**15**, DOI: 10.1093/mtomcs/mfac098.
6. Tsvetkov P, Coy S, Petrova B, Dreishpoon M, Verma A, Abdusamad M, Rossen J, Joesch-Cohen L, Humeidi R, Spangler RD, Eaton JK, Frenkel E, Kocak M, Corsello SM, Lutsenko S, Kanarek N, Santagata S, Golub TR. Copper induces cell death by targeting lipoylated TCA cycle proteins. *Science* 2022;**375**:1254–61.
7. Zuily L, Lahrach N, Fassler R, Genest O, Faller P, Sénèque O, Denis Y, Castanié-Cornet MP, Genevaux P, Jakob U, Reichmann D, Giudici-Orticoni MT, Ilbert, M. Copper Induces Protein Aggregation, a Toxic Process Compensated by Molecular Chaperones. *MBio* 2022;**13**, DOI: 10.1128/mbio.03251-21.
8. Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. *Toxicology* 2011;**283**:65–87.
9. He L, He T, Farrar S, Ji L, Liu T, Ma X. Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species. *Cell Physiol Biochem* 2017;**44**:532–53.
10. Rice ME. Ascorbate regulation and its neuroprotective role in the brain. *Trends Neurosci* 2000;**23**:209–16.
11. Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: Combinations to avoid. *Radiat Res* 1996;**145**:532–41.
12. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 2001;**30**:1191–212.
13. Morgan MT, Nguyen LAH, Hancock HL, Fahrni CJ. Glutathione limits aquacopper(I) to sub-femtomolar concentrations through cooperative assembly of a tetranuclear cluster. *J Biol Chem* 2017;**292**:21558–67.
14. Morgan MT, Bourassa D, Harankhedkar S, McCallum AM, Zlatic SA, Calvo JS, Meloni G, Faundez V, Fahrni CJ. Ratiometric two-photon microscopy reveals attomolar copper buffering in normal and Menkes mutant cells. *Proc Natl Acad Sci* 2019;**116**:12167–72.
15. Stewart LJ, Ong CLY, Zhang MM, Brouwer S, McIntyre L, Davies MR, Walker MJ, McEwan AG, Waldron KJ, Djoko KY. Role of Glutathione in Buffering Excess Intracellular Copper in Streptococcus pyogenes. *MBio* 2020;**11**:1–19.
16. Smith RC, Reed VD, Hill WE. Oxidation of thiols by copper(II). *Phosphorus Sulfur Silicon Relat Elem* 1994;**90**:147–54.
17. Osman LP, Mitchell SC, Waring RH. Cysteine, its Metabolism and Toxicity. *Sulfur reports* 1997;**20**:155–72.
18. Carrasco-Pozo C, Aliaga ME, Olea-Azar C, Speisky H. Double edge redox-implications for

the interaction between endogenous thiols and copper ions: In vitro studies. *Bioorg Med Chem* 2008;**16**:9795–803.
19. Frederick Gowland Hopkins B, James Morgan E. Some relations between ascorbic acid and glutathione. *Biochem J* 1936;**30**:1446.
20. Gould IA. Protective Influence of Glutathione on Copper-Induced Oxidation of Ascorbic Acid in Milk. *J Dairy Sci* 1940;**23**:991–6.
21. Ionescu JG, Poljsak B. Metal ions mediated pro-oxidative reactions with vitamin c: Possible implications for treatment of different malignancies. *Cancer Prev Res Perspect* 2011;**3**:149–82.
22. Du YT, Long Y, Tang W, Liu XF, Dai F, Zhou B. Prooxidative inhibition against NF-κB-mediated inflammation by pharmacological vitamin C. *Free Radic Biol Med* 2022;**180**:85–94.
23. Spear N, Aust SD. Hydroxylation of deoxyguanosine in DNA by copper and thiols. *Arch Biochem Biophys* 1995;**317**:142–8.
24. Dance IG, Bowmaker GA, Clark GR, Seadon JK. The formation and crystal and molecular structures of hexa(μ-organothiolato)tetracuprate(I) cage dianions: bis-(tetramethylammonium)hexa-(μ-methanethiolato)tetracuprate(I) and two polymorphs of bis(tetramethylammonium)hexa-(μ-benzenethiolato)-tetracuprate(I). *Polyhedron* 1983;**2**:1031–43.
25. Pickering IJ, George GN, Dameron CT, Kurz B, Winge DR, Dance IG. X-ray Absorption Spectroscopy of Cuprous-Thiolate Clusters in Proteins and Model Systems. *J Am Chem Soc* 1993;**115**:9498–505.
26. Pountney DL, Schauwecker I, Zarn J, Vašák M. Formation of Mammalian Cu8-Metallothionein in Vitro: Evidence for the Existence of Two Cu(I)4-Thiolate Clusters. *Biochemistry* 1994;**33**:9699–705.
27. Pecci L, Montefoschi G, Musci G, Cavallini D. Novel findings on the copper catalysed oxidation of cysteine. *Amino Acids* 1997;**13**:355–67.
28. Winterbourn CC, Peskin A V., Parsons-Mair HN. Thiol oxidase activity of copper,zinc superoxide dismutase. *J Biol Chem* 2002;**277**:1906–11.
29. Bakavayev S, Chetrit N, Zvagelsky T, Mansour R, Vyazmensky M, Barak Z, Israelson A, Engel S. Cu/Zn-superoxide dismutase and wild-type like fALS SOD1 mutants produce cytotoxic quantities of H_2O_2 via cysteine-dependent redox short-circuit. *Sci Rep* 2019;**9**:1–13.
30. Hogg N. The effect of cyst(e)ine on the auto-oxidation of homocysteine. *Free Radic Biol Med* 1999;**27**:28–33.
31. Kachur A V., Koch CJ, Biaglow JE. Mechanism of copper-catalyzed autoxidation of cysteine. *Free Radic Res* 1999;**31**:23–34.
32. Zhitkovich A. N-Acetylcysteine: Antioxidant, Aldehyde Scavenger, and More. *Chem Res Toxicol* 2019;**32**:1318–9.
33. Whitesides GM, Lilburn JE, Szajewski RP. Rates of Thiol-Disulfide Interchange Reactions between Mono and Dithiols and Ellman's Reagent. *J Org Chem* 1977;**42**:332–8.
34. Nagy P. Kinetics and mechanisms of thiol-disulfide exchange covering direct substitution and thiol oxidation-mediated pathways. *Antioxidants Redox Signal* 2013;**18**:1623–41.
35. Falcone E, Ritacca AG, Hager S, Schueffl H, Vileno B, El Khoury Y, Hellwig P, Kowol CR, Heffeter P, Sicilia E, Faller P. Copper-catalysed glutathione oxidation is accelerated by the anticancer thiosemicarbazone Dp44mT and further boosted at lower pH. *J Am Chem Soc* 2022;**144**:14758–14768.
36. Stoiber TL, Shafer MM, Perkins DAK, Hemming JDC, Armstrong DE. Analysis of glutathione endpoints for measuring copper stress in Chlamydomonas reinhardtii. *Environ*

Toxicol Chem 2007;**26**:1563.
37. Hultberg M, Isaksson A, Andersson A, Hultberg B. Traces of copper ions deplete glutathione in human hepatoma cell cultures with low cysteine content. *Chem Biol Interact* 2007;**167**:56–62.
38. García-Fernández AJ, Bayoumi AE, Pérez-Pertejo Y, Motas M, Reguera RM, Ordóñez C, Balaña-Fouce R, Ordóñez D. Alterations of the glutathione-redox balance induced by metals in CHO-K1 cells. *Comp Biochem Physiol C Toxicol Pharmacol* 2002;**132**:365–73.
39. Brose J, La Fontaine S, Wedd AG, Xiao Z. Redox sulfur chemistry of the copper chaperone Atox1 is regulated by the enzyme glutaredoxin 1, the reduction potential of the glutathione couple GSSG/2GSH and the availability of Cu(i). *Metallomics* 2014;**6**:793–808.
40. Eben SS, Imlay JA. Excess copper catalyzes protein disulfide bond formation in the bacterial periplasm but not in the cytoplasm. *Mol Microbiol* 2023, DOI: 10.1111/mmi.15032.
41. Hiniker A, Collet J-F, Bardwell JCA. Copper Stress Causes an in Vivo Requirement for the Escherichia coli Disulfide Isomerase DsbC. *J Biol Chem* 2005;**280**:33785–91.
42. Park S, Imlay JA. High levels of intracellular cysteine promote oxidative DNA damage by driving the Fenton reaction. *J Bacteriol* 2003;**185**:1942–50.
43. Stoll S, Schweiger A. EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J Magn Reson* 2006;**178**:42–55.
44. Ravel B, Newville M. ATHENA, ARTEMIS, HEPHAESTUS: Data analysis for X-ray absorption spectroscopy using IFEFFIT. *J Synchrotron Radiat* 2005;**12**:537–41.
45. Newville M, Liviņ P, Yacoby Y, Rehr JJ, Stern EA. Near-edge x-ray-absorption fine structure of Pb: A comparison of theory and experiment. *Phys Rev B* 1993;**47**:14126–31.
46. Binsted N, Gurman SJ, Campbell JW. Daresbury Laboratory {EXCURV}98 Program. *CLRC Daresbury Lab Warringt* 1998.
47. Winkler BS, Orselli SM, Rex TS. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. *Free Radic Biol Med* 1994;**17**:333–49.