Spectroscopic, kinetic and mechanistic studies of the influence of ligand and substrate concentration on the activation by peroxides of Cu^{I}–thiolate and other Cu^{I} complexes

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Both free-radical and non-radical routes for peroxide oxidation of Cu^{I} can be identified for copper complexes obtained by the reduction of Cu^{II} by thiols, including glutathione. Copper(I)–thiolate complexes are obtained, except in the presence of *e.g.* 1,10-phenanthroline, and these undergo ready reaction with H_2O_2 and ^{t}BuOOH. EPR spin-trapping studies establish a free-radical reaction mechanism (to give ^{t}BuO$^{\bullet}$) with the latter, and the formation of HO$^{\bullet}$ from the former occurs only at low concentrations of copper. Kinetic studies (using UV-vis and EPR spectroscopies), together with NMR analysis, lead to the proposal that Cu^{I} aggregates react *via* non-radical pathways in contrast to monomeric Cu^{I}.

Introduction

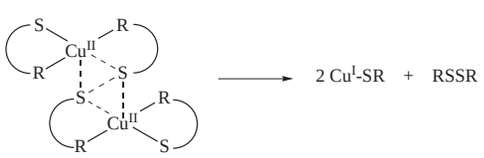
The copper-catalysed oxidation of thiols in the presence of peroxides, whereby copper mediates the overall one-electron transfer from thiol to peroxide, is believed to proceed *via* a complex mechanism, critically affected by a number of factors (including structure of the thiol, nature of the ligands and solvent, and the pH); Scheme 1 represents an overview of the process. As

$$Cu^{II} + RSH \longrightarrow Cu^{I} + {}^{1}/_{2}\,RSSR \quad (1)$$

$$Cu^{I} + H_2O_2 \longrightarrow Cu^{II} + OH^{\bullet} + OH^{-} \quad (2)$$

Scheme 1

shown by us and others,[1–3] the thiol complexes to Cu^{II} prior to electron transfer; the lifetime of this complex (sometimes EPR-detectable) depends on the steric and electronic properties of the ligands.[3] There is no clear evidence for thiyl-radical formation at this stage in the thiols so far studied (for example, such radicals cannot be intercepted *via* spin-trapping experiments[3]), and the kinetic results suggest that dimerisation of the copper–thiolate complex allows concerted reduction of copper(II) and disulfide formation (see Scheme 2). Stoichiometric studies



Scheme 2

establish that an extra equivalent of thiol is required to stabilise the Cu^{I} formed, at least in aqueous solution and in the absence of chloride ions.[3]

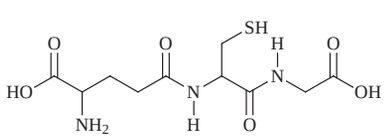
The possible formation of oxygen-centred radicals[4–6] (or even Cu^{III}[7,8] or copper-peroxo complexes[9,10]) *via* the re-oxidation of Cu^{I} by peroxides leads to concern over the toxicological significance of the copper–thiol reactions. For example, the neurological disorder Wilson's disease is believed[11] to result from excessive levels of copper in the body (possibly leading to oxidative stress), and the thiol penicillamine is employed in its treatment to complex Cu^{II}. *In vivo* studies[12] have clearly established that intracellular reduction of copper complexes (prior to transport of Cu^{I} to copper-containing proteins, *e.g.* metallothionein, superoxide dismutase) is achieved by thiols, especially glutathione; the reoxidation of Cu^{I} may well then be potentially toxic—especially given the finding that some copper complexes are only effective activators of hydrogen peroxide in the presence of cysteine and other thiols.[6,13,14] Copper-catalysed damage to DNA has been reported for a number of systems[15,16] and it may be especially relevant that copper 1,10-phenanthroline is known to be an effective DNA nuclease (ref. 17 and references therein).

The aim of the research described here (and in a preliminary account[18]) has been to obtain detailed mechanistic and kinetic information about the two key steps in Scheme 1, and especially to explore the effect of ligands, with emphasis on those of biological relevance. We have employed EPR spectroscopy, both directly (to detect Cu^{II}) and with spin-trapping techniques (to detect RO$^{\bullet}$ and RS$^{\bullet}$), and UV-visible spectroscopy to monitor the rates of appropriate reactions. NMR spectroscopy has also been employed in an attempt to characterise Cu^{I}–thiol complexes.

Results and discussion

a) Reduction of Cu^{II} by glutathione [GSH (1)] and other thiols

(i) NMR, UV-visible and stoichiometric studies. In initial



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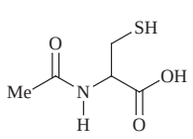

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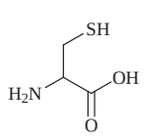

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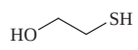

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experiments we monitored the disappearance of the Cu^{II} signal in the EPR spectrum upon addition of a solution of GSH in the pH range 4–7 (see also ref. 18); this typically involved experiments with 10^{-2} mol dm^{-3} Cu^{II} (as copper sulfate) and GSH in the range 4×10^{-3}–2×10^{-2} mol dm^{-3}. The stoichiometry for complete removal of the copper(II) signal was found to be 1

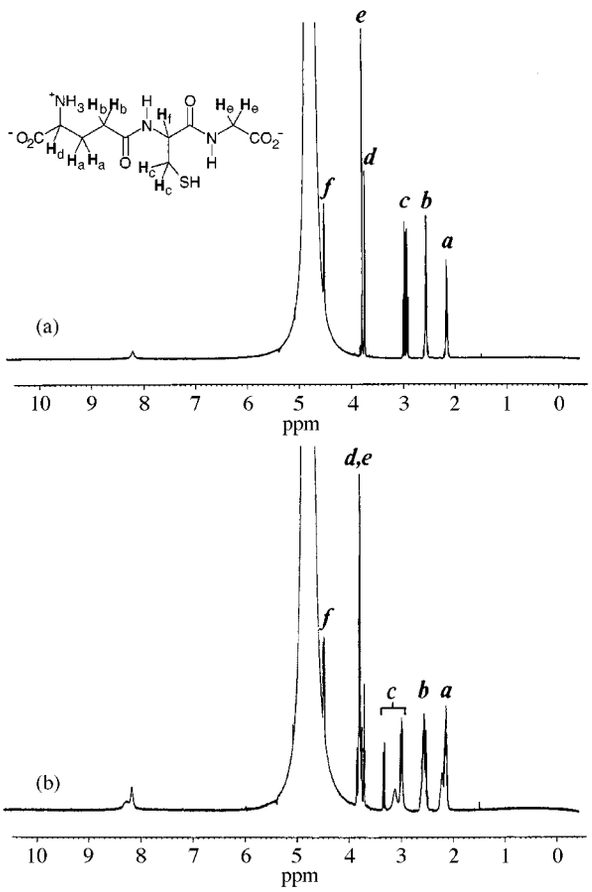


Fig. 1 a) ^{1}H NMR spectrum (500 MHz) of glutathione (0.1 mol dm^{-3}) in D_2O–H_2O at pH 7.5; b) ^{1}H NMR spectrum (500 MHz) of the copper(I)–glutathione complex and GSSG obtained from the reaction between GSH (0.1 mol dm^{-3}) and $CuSO_4$ (3×10^{-2} mol dm^{-3}) at pH 7.5.

Cu^{II}:2 GSH, consistent with the mechanism described above. We next utilized NMR spectroscopy to compare the product of this reaction with that reported by Sadler[19] and co-workers who obtained ^{1}H and ^{13}C NMR spectra of a Cu^I–glutathione complex prepared by reacting GSH directly with copper(I) (as CuCl). GSH (10^{-1} mol dm^{-3}) and $CuSO_4 \cdot 5H_2O$ (3×10^{-2} mol dm^{-3}, added as a solid) were mixed under nitrogen, at pH 7.5 in H_2O–D_2O (1:1); the well-resolved ^{1}H-NMR spectrum of the clear solution obtained (which itself suggests that Cu^{II} has been reduced to Cu^I), shown and assigned in Fig. 1, is closely similar to that recorded by Sadler and is characteristic of the Cu^I–SG complex (the other peaks are from GSSG). The most informative resonances lie in the range δ 2.8–3.3 and correspond to the cys-H_β protons in the tripeptide. The structure of the complex, proposed on the basis of extended X-ray absorption fine structure (EXAFS) studies,[19] is believed to be polymeric with the thiolate sulfur atoms triply bridging between Cu^I ions. The broad resonances observable at δ 3.0 and 3.1 ppm are not present in either simple thiol or disulfide systems and are interpreted to be from a copper(I)–glutathione complex; the two sharp doublets of doublets at δ 3.0 and 3.35 ppm are attributed to the disulfide. Integration of these resonances indicates that 0.5 equivalents of disulfide are generated relative to the copper(II) added [reaction (6), see later]. The precise shape and position of the broad absorptions are to some extent dependent on the concentration of copper(II) and thiol and it is believed that glutathione exchanges rapidly between a free and a complexed [to copper(I)] form.

Broadly similar observations were made employing *N*-acetylcysteine (**2**), cysteine (**3**) and 2-mercaptoethanol (**4**). For each substrate, the thiol and appropriate disulfide peaks can be clearly distinguished in the absence of copper(II). For solutions

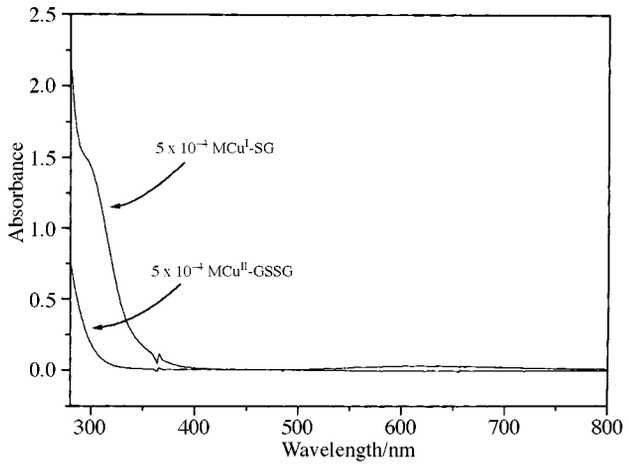


Fig. 2 UV-vis spectra obtained from the reaction between GSH (2×10^{-3} mol dm^{-3}) and $CuSO_4$ (5×10^{-4} mol dm^{-3}) and with subsequent addition of H_2O_2 (5×10^{-3} mol dm^{-3}); absorptions from Cu^I–SG and Cu^{II}–GSSG are indicated.

of the thiol, in the presence of copper(II), broadened signals are observed which, to some extent, are dependent on the concentration of copper(II) and thiol (as above); these are assigned to the protons in the complexed thiol, which itself undergoes exchange with free thiol. The signals observed in the cysteine and 2-mercaptoethanol systems are somewhat sharper, which may reflect a more rapid rate of thiol exchange. The exchange phenomena suggested by the NMR have not been studied further at this stage.

Fig. 2 shows the UV spectrum of the solution obtained by mixing GSH and $CuSO_4$ in deaerated phosphate buffer at pH 7.4 (for concentrations see figure legend); the absorption at 300 nm, attributed to the Cu^I–SG complex (see *e.g.* ref. 20), appeared unaltered as [GSH] was increased to 5×10^{-3} mol dm^{-3}. Addition of H_2O_2 (5×10^{-3} mol dm^{-3}) was found to regenerate copper(II), detectable as the Cu^{II}–GSSG complex (λ_{max} = 625 nm), with its characteristic EPR signal.[18]

(ii) Reduction of copper(II) phenanthroline. Related experiments were carried out on the copper(II) 1,10-phenanthroline complex, $Cu(phen)_2$, at least in part since this is known to be an effective nuclease for double-stranded DNA in the presence of certain combinations of reductant and oxidant;[17] it has been suggested that $Cu^I(phen)_2$ is the active species. In initial experiments, we followed the decrease in the intensity of the copper(II) EPR signal upon adding increasing amounts of thiol ($\leqslant 2 \times 10^{-2}$ mol dm^{-3}) to $Cu(phen)_2$ (10^{-2} mol dm^{-3}), in deoxygenated pH 7.4 buffered solution (10^{-1} mol dm^{-3} phosphate), in order to quantify the extent of reduction. The ratio for complete removal was found to be 1:1 for GSH, cysteine, *N*-acetylcysteine and 2-mercaptoethanol; this suggests that $Cu^I(phen)_2$ is stable as a discrete complex and does not require the extra equivalent of thiol for stabilisation.

Reduction of this complex was also investigated through UV-vis spectroscopy by mixing deoxygenated and pH 7.4 buffered (2×10^{-2} mol dm^{-3} phosphate) solutions of $Cu^{II}(phen)_2$ (10^{-3} mol dm^{-3}) and thiol (GSH, *N*-acetylcysteine, cysteine and mercaptoethanol at concentrations $\leqslant 5 \times 10^{-3}$ mol dm^{-3}). In all cases (see *e.g.* Fig. 3), absorptions at *ca.* 400 and 550 nm were immediately apparent and these were observed to increase in intensity with increases in [RSH] for copper:thiol ratios of up to 1:1. This indicates the formation of $Cu^I(phen)_2$, and further evidence for this interpretation is provided by the observation of identical behaviour when ascorbate (10^{-3} mol dm^{-3}) is employed as reductant. The 400 nm and 550 nm absorptions are known to be very much dependent on the concentration of $Cu^I(phen)_2$ present in solution since extensive dimerisation/aggregation of the complex occurs at concen-

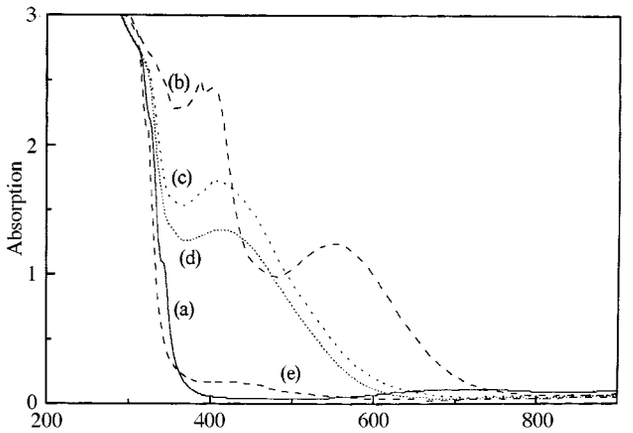


Fig. 3 UV-vis spectra obtained upon mixing $Cu^{II}(phen)_2$ (10^{-3} mol dm^{-3}) with GSH at a) 0 mol dm^{-3}; b) 10^{-3} mol dm^{-3}; c) 2×10^{-3} mol dm^{-3} and d) 5×10^{-3} mol dm^{-3}. Spectrum e) results from mixing with cysteine at 5×10^{-3} mol dm^{-3}.

trations any greater than micromolar (at these low concentrations the 400 nm absorption shifts to 430 nm and the 550 nm absorption is negligible). It was not possible to relate quantitatively successive additions of thiol to increases in the $Cu^{I}(phen)_2$ absorptions since Beer–Lambert's law is not obeyed; however, it appears that reduction of $Cu^{II}(phen)_2$ requires one equivalent of thiol to form $Cu^{I}(phen)_2$.

In all cases, however, upon addition of a further equivalent of the respective thiol, the spectra obtained exhibited only a single absorption at *ca.* 410 nm which gradually decreased in intensity with successive additions of thiol. This was most clearly evident in cysteine systems for which a 5-fold excess suppressed the spectrum almost entirely; with mercaptoethanol present in a 4-fold excess, precipitation of a yellow–brown solid occurred. These observations suggest that $Cu^{I}(phen)_2$ in the presence of excess thiol does not exist as a discrete $Cu^{I}(phen)_2$ species but rather as a mixed complex of the form $Cu^{I}(phen)$–SG. From the reported values for the association constants of copper(I) with phen and GSH,† we can also deduce that, at pH 7.4, RSH preferentially ligates copper(I) over the second phenanthroline ligand and hence when present in a one-fold excess the copper is likely to be present as $Cu^{I}(phen)$–SR. Indeed, a mixture of $Cu^{II}(phen)$ and GSH in a ratio of 1:2 resulted in an absorption at the same wavelength and extinction coefficient (results not shown). As the thiol concentration is further increased, the remaining phenanthroline ligand will also be displaced and the copper(I)–SR species should be increasingly favoured; this is indicated by the decrease in the absorption at 410 nm. For cysteine this is a particularly favourable process, presumably because of its ability to act as a bidentate ligand (through sulfur and nitrogen).

We also recorded the ^{1}H-NMR spectrum of GSH (10^{-1} mol dm^{-3}) in the presence of $Cu^{II}(phen)_2$ (0.03 mol dm^{-3}), as described above. The spectrum obtained indicated the formation of disulfide, while the remaining GSH was free, *i.e.* completely dissociated from the copper(I); the phenanthroline proton resonances (*ca.* δ 7–7.5, significantly lower than expected for free phenanthroline) were very broad. We believe that at this concentration of $Cu^{I}(phen)_2$, aggregation is favoured over thiol complexation (the observation of a purple precipitate forming with time is further evidence to support this conclusion).

† The association constants for copper(I) with phenanthroline[21] and glutathione[22] are:

$$Cu^{I} + phen \rightleftharpoons Cu^{I}(phen) \quad K = 10^{10.3} \qquad (3)$$

$$Cu^{I}(phen) + phen \rightleftharpoons Cu^{I}(phen)_2 \quad K = 10^{5.5} \qquad (4)$$

$$Cu^{I} + GS^{-} \rightleftharpoons Cu^{I}(SG) \quad K = 10^{24.9} \qquad (5)$$

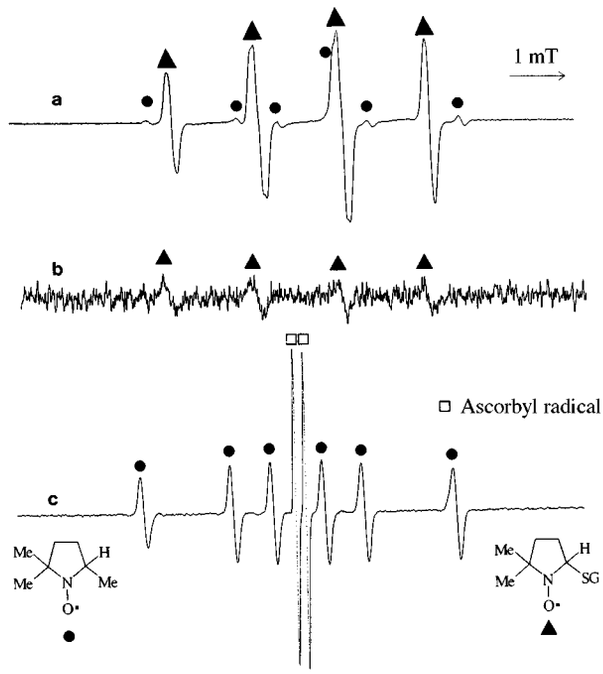


Fig. 4 a) EPR spectrum of the radical adducts from $Me^{\bullet}$ (●) and $GS^{\bullet}$ (▲) with DMPO obtained upon mixing $CuSO_4$ (2.5×10^{-4} mol dm^{-3}) with GSH (10^{-2} mol dm^{-3}), ^{t}BuOOH (10^{-2} mol dm^{-3}) and DMPO (10^{-2} mol dm^{-3}); b) as for 4a but with H_2O_2 instead of ^{t}BuOOH. c) EPR spectrum of the radical adduct of $Me^{\bullet}$ (from $HO^{\bullet}$ and added DMSO) with DMPO upon mixing $CuSO_4$ (10^{-3} mol dm^{-3}) with ascorbate (2×10^{-3} mol dm^{-3}), H_2O_2 (2×10^{-3} mol dm^{-3}), DMPO (10^{-2} mol dm^{-3}) and DMSO (1.0 mol dm^{-3}). The ascorbyl radical signals are also indicated.

b) Reoxidation of Cu^{I} and Cu^{I}–thiol complexes. Effect of ligand, thiol and peroxide

i) EPR results: GSH experiments with copper(II) sulfate. We first employed the EPR spin-trapping technique, as described earlier,[3,18] in order to provide evidence as to whether or not radicals are generated during the reoxidation of Cu^{I} [see *e.g.* reactions (6)–(9)]; experiments were conducted for potentially catalytic and stoichiometric quantities of copper.

$$Cu^{II} + 2\,GSH \longrightarrow Cu^{I}\text{-}SG + {}^1/_2\,GSSG + 2\,H^{+} \qquad (6)$$

$$Cu^{I}\text{-}SR + {}^t\!BuOOH \longrightarrow Cu^{II}\text{-}SR + OH^{-} + {}^t\!BuO^{\bullet} \qquad (7)$$

$${}^t\!BuO^{\bullet} \longrightarrow Me^{\bullet} + Me_2CO \qquad (8)$$

$${}^t\!BuO^{\bullet} + RSH \longrightarrow {}^t\!BuOH + RS^{\bullet} \qquad (9)$$

Scheme 3

Initial experiments, which illustrate the approach, involved adding copper as copper(II) sulfate (to give a final concentration of 2.5×10^{-4} mol dm^{-3}) to pH 7.4 buffered solutions (0.1 mol dm^{-3} phosphate) containing the spin-trap 5,5-dimethyl-3,4-dihydropyrroline *N*-oxide, DMPO, (10^{-2} mol dm^{-3}), GSH (in the range $1.0–5.0 \times 10^{-2}$ mol dm^{-3}) and either H_2O_2 or ^{t}BuOOH (2.0×10^{-2} mol dm^{-3}). Under these conditions we expect Cu^{II} to be reduced to Cu^{I} by thiol, followed by reoxidation by the peroxide. With ^{t}BuOOH as oxidant, strong EPR signals (see *e.g.* Fig. 4) were obtained from aminoxyls which are assigned to the adducts of $Me^{\bullet}$ and $GS^{\bullet}$ with DMPO (with parameters of $a_N = 1.64$ mT, $a_H = 2.35$ mT and $a_N = 1.52$ mT, $a_H = 1.63$ mT, $a_{2H} = 0.06$ mT respectively). Signals for the $GS^{\bullet}$ adduct increased in intensity at the expense of the former as the concentration of GSH increased.

These results are consistent with the effective one-electron oxidation of Cu^{I} by ^{t}BuOOH to give Cu^{II} and ^{t}BuO$^{\bullet}$ which then fragments rapidly to $Me^{\bullet}$; the thiyl radical presumably arises

from oxidation of thiol by ^{t}BuO$^{\bullet}$ (although abstraction by Me$^{\bullet}$ cannot be ruled out) as shown in Scheme 3.

When these experiments were repeated with H_2O_2 (2.0×10^{-2} mol dm^{-3}) very weak signals were obtained (even with a receiver gain increased by a factor of *ca.* 20) indicating that only trace quantities of HO$^{\bullet}$ (and hence GS$^{\bullet}$) had been generated. The signals were unaltered when excess DMSO (1.0 mol dm^{-3}) was also present: no Me$^{\bullet}$ was trapped which would be expected if significant quantities of HO$^{\bullet}$ were to be formed [reaction (10)].[3,18] These experiments were repeated with comparable

concentrations of GSH (1–2×10^{-3} mol dm^{-3}) and Cu^{II} (10^{-3} mol dm^{-3}) with both peroxides (2×10^{-3} mol dm^{-3}). Once again a dominant Me$^{\bullet}$ adduct was observed with ^{t}BuOOH, after a gradual build up over *ca.* 6 minutes. However, no signals were observed in the H_2O_2 reaction, in the presence or absence of DMSO.

In marked contrast, the reaction of Cu^{II} (10^{-3} mol dm^{-3}), H_2O_2 (2×10^{-3} mol dm^{-3}) and ascorbate (2×10^{-3} mol dm^{-3}) as reductant in the presence of DMPO (10^{-2} mol dm^{-3}), resulted in very strong signals from the methyl adduct, indicating that in this system the Cu^{I}–ascorbate–H_2O_2 couple is an effective source of HO$^{\bullet}$. With ^{t}BuOOH, strong signals from the methyl-radical adduct were observed, characteristic of effective formation of ^{t}BuO$^{\bullet}$, which then fragments rapidly.

ii) EPR results with other ligands and thiols. A similar investigation was conducted on the reaction of GSH and copper(II) complexes and both ^{t}BuOOH and H_2O_2 with the ligands 1,10-phenanthroline, 2,2′-bipyridine and ethylenediamine.

With ^{t}BuOOH and the higher concentrations of GSH (see above) both Me$^{\bullet}$ and GS$^{\bullet}$ were trapped, the latter more so at higher GSH [indicating the occurrence of reactions (7)–(9) above]. In the case of Cu(phen)$_2$ and Cu(bipy)$_2$, strong signals from the same adducts were observed but decayed with time (2 min). With lower concentrations of GSH, and equivalent concentrations of copper, signals from Me$^{\bullet}$ were obtained, especially prominent from Cu(bipy)$_2$ for which the DMPO adduct of ^{t}BuO$^{\bullet}$ was also seen (with $a_N = 1.48$ mT, $a_H = 1.66$ mT). With H_2O_2 only very weak signals were obtained in the high [GSH] experiments except for those with Cu(bipy)$_2$ and GSH (10^{-2} mol dm^{-3}) which gave relatively intense GS$^{\bullet}$ signals, as did those with Cu(phen)$_2$ and GSH (*ca.* 3×10^{-2} mol dm^{-3}) which also upon addition of DMSO (1.0 mol dm^{-3}) gave Me$^{\bullet}$, evidently *via* HO$^{\bullet}$. With lower concentrations of GSH (typically 10^{-3} mol dm^{-3}) no signals were observed with Cu(en)$_2$; with Cu(phen)$_2$ and Cu(bipy)$_2$, HO$^{\bullet}$ (and Me$^{\bullet}$ with DMSO) was clearly detected. In the presence of ascorbate as reductant, Me$^{\bullet}$ was trapped from ^{t}BuOOH with Cu(phen)$_2$ and Cu(bipy)$_2$ but not Cu(en)$_2$ (it is presumably not reduced to Cu^{I}); with H_2O_2 strong HO$^{\bullet}$ signals (and Me$^{\bullet}$ with DMSO) were obtained.

In summary, spin-trapping experiments show that the Cu^{I} species generated in the reaction between GSH and the Cu^{II} complexes, activates ^{t}BuOOH in a one-electron transfer process to yield ^{t}BuO$^{\bullet}$, which subsequently fragments to yield Me$^{\bullet}$ or reacts with GSH, when in excess, to give the thiyl radical. Similar Fenton-type behaviour is also observed when ascorbate is employed as the reductant in both ^{t}BuOOH and H_2O_2 systems. Differences between the copper complexes are apparent however, when H_2O_2 and GSH are employed and are dependent on the thiol concentration and ligand. With Cu(phen)$_2$ and Cu(bipy)$_2$, H_2O_2 appears to react in a Fenton-type reaction when GSH is present at lower concentrations (~10^{-2} mol dm^{-3}) but when in excess, and with $CuSO_4$ and Cu(en)$_2$, the mechanism is predominantly non-radical. This non-Fenton mechanism thus appears to operate when the copper is present predominately as Cu^{I}–SG.

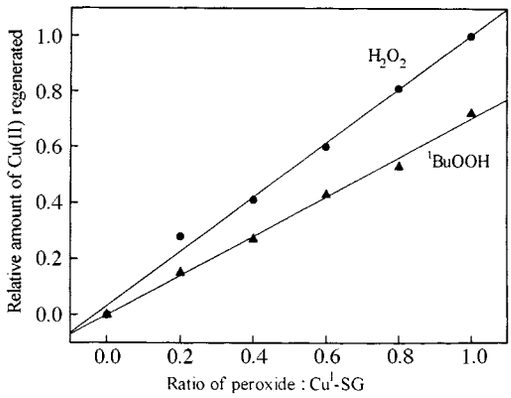


Fig. 5 Variation with concentration of added peroxide of the absorbance at λ 625 nm for the Cu^{II}–GSSG complex [formed from Cu^{I}–SG, 10^{-2} mol dm^{-3}; see text].

iii) Stoichiometry of reoxidation of Cu^{I}–SG, determined by UV-vis measurements. The stoichiometry of the reoxidation of the Cu^{I}–SG species by H_2O_2 and ^{t}BuOOH was explored by UV-vis spectroscopy. A solution of 10^{-2} mol dm^{-3} Cu^{I}–SG [prepared by mixing GSH (2×10^{-2} mol dm^{-3}) with $CuSO_4$ (10^{-2} mol dm^{-3}) under constant degassing with nitrogen] was mixed with increasing concentrations of peroxide ($\leqslant 2 \times 10^{-2}$ mol dm^{-3}) in 10^{-1} mol dm^{-3} pH 7.4 phosphate buffer under nitrogen and left to react for 10 minutes. The UV-vis spectrum was recorded for each sample and the absorbance at $\lambda = 625$ nm, attributed to the Cu^{II}–GSSG complex, was monitored; the dependence of absorbance on peroxide concentration was then plotted (see Fig. 5). The gradients of these plots equate to the stoichiometry of reoxidation for each peroxide; the ratio of [Cu^{I}–SG]/[peroxide] is 0.95 (±0.04) for H_2O_2 but only 0.70 (±0.02) for ^{t}BuOOH.

The procedure was repeated again in the presence of 1.0 mol dm^{-3} sodium formate which is a known radical scavenger [reactions (11) and (12)]. The stoichiometric ratio for complete

$$HO^{\bullet} + HCO_2^{-} \longrightarrow CO_2^{-\bullet} + H_2O \qquad (11)$$

$$CO_2^{-\bullet} + Cu^{II} \longrightarrow Cu^{I} + CO_2 \qquad (12)$$

reduction was essentially unaltered for H_2O_2 (0.98 ± 0.03) but fell further still for ^{t}BuOOH (0.47 ± 0.01).

Both peroxides have 2 oxidizing equivalents and hence the stoichiometric ratio for reoxidation might be expected to be unity. This is clearly so for H_2O_2 but with ^{t}BuOOH it is significantly lower. We believe this is due to the fact that whilst ^{t}BuO$^{\bullet}$ can oxidize either Cu^{I} or GS^{-}, the methyl radical, formed following fragmentation, is less effective; the presence of formate provides a further opportunity for scavenging of ^{t}BuO$^{\bullet}$ and the stoichiometric ratio falls further. In H_2O_2 systems however, the ratio is unaltered which strongly suggests that no free hydroxyl radical (or oxidizing equivalent) is generated in the mechanism of reoxidation.

c) Kinetic studies of the reoxidation of Cu(I)–SG by peroxides

i) EPR measurements of the generation of Cu(II) in H_2O_2 and ^{t}BuOOH reactions. In initial kinetic studies on reoxidation of Cu^{I}–SG we employed EPR spectroscopy to monitor the reappearance of copper(II), specifically as the Cu^{II}–GSSG complex (Fig. 6a). Deoxygenated solutions of $CuSO_4$ (5×10^{-3} mol dm^{-3})/GSH (2×10^{-2} mol dm^{-3}) and peroxide (5×10^{-2}–10^{-1} mol dm^{-3}), buffered at pH 7.4 (10^{-1} mol dm^{-3} phosphate), were mixed in a stopped-flow system and the resulting data from each run (see *e.g.* Fig. 6b) were found to be pseudo-first order in

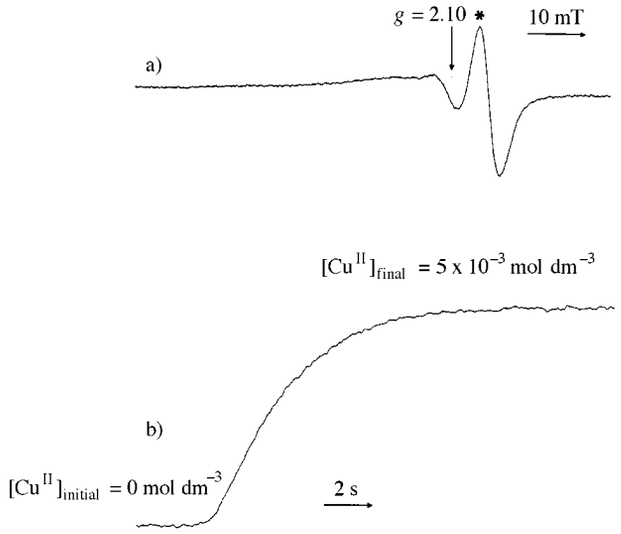
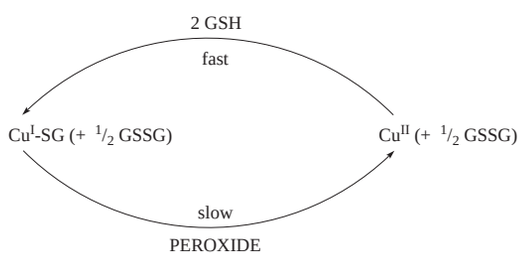


Fig. 6 a) EPR spectrum of Cu^{II}–GSSG (5×10^{-3} mol dm^{-3}); b) EPR kinetic monitoring of the formation of Cu^{II}–GSSG (specifically, the peak indicated * in Fig. 6a after mixing $CuSO_4$ (5×10^{-3} mol dm^{-3})–GSH (5×10^{-3} mol dm^{-2}) with H_2O_2 (7×10^{-2} mol dm^{-3}).

copper. The presence of an induction period was apparent and is explained by the occurrence of the reactions as in Scheme 4;



Scheme 4

the copper is present in the cuprous form until all excess GSH has been consumed and then reaction with peroxide will result in the permanent regeneration of copper(II).

The pseudo-first order rate constants, k_{obs} (s^{-1}), then yielded second-order rate constants for the reaction between Cu^{I}–SG and peroxide under these conditions and at 17.5 °C (290.5 K) as follows: k (H_2O_2) = 4.42 ± 0.12 dm^3 mol^{-1} s^{-1} and k (^{t}BuOOH) = 1.38 ± 0.06 dm^3 mol^{-1} s^{-1}.

To rule out specific effects of the buffer the reoxidation with H_2O_2 was repeated in pH 7.4 tris buffer [tris(hydroxymethyl)-aminomethane, 0.05 mol dm^{-3}] and a similar rate constant was determined (k = 3.69 ± 0.16 dm^3 mol^{-1} s^{-1} at 13 °C). Since tris at this concentration would be expected to scavenge $HO^{\bullet}$, this finding adds further evidence to the proposal that free $HO^{\bullet}$ is not liberated in the reaction under these conditions. Experiments with aerated solutions gave similar results for H_2O_2 and ^{t}BuOOH and we conclude that oxygen does not affect the reaction under these conditions.

ii) UV-vis study of the reaction between Cu(I)–SG and H_2O_2 or ^{t}BuOOH. The reoxidation over a range of temperatures was studied by monitoring the decay of the Cu^{I}–SG species at 300 nm. A solution of $CuSO_4$ (5×10^{-4} mol dm^{-3})–GSH (2×10^{-3} mol dm^{-3}) was prepared in deoxygenated pH 7.4 buffer (5×10^{-2} mol dm^{-3} phosphate) and transferred to the stopped-flow apparatus in an air-tight syringe. Kinetic runs were commenced upon mixing with a buffered solution of either H_2O_2 or ^{t}BuOOH (5×10^{-3}–1.75×10^{-2} mol dm^{-3}); each run was repeated at least twice and at 5 different temperatures. Specific effects of the buffer were ruled out by repeating the experiments at 25 °C in 0.05 mol dm^{-3} HEPES buffer (*N*′-[2-hydroxyethyl]-piperazine-*N*-ethanesulfonic acid).

Table 1 Rate constants for the reactions between Cu^{I}–SG (5×10^{-4} mol dm^{-3}) and a) H_2O_2 b) ^{t}BuOOH (both 4.5×10^{-3}–1.7×10^{-2} mol dm^{-3})

a)		b)	
T/°C	$k(H_2O_2)$/ dm^3 mol^{-1} s^{-1}	T/°C	$k(^t$BuOOH)/ dm^3 mol^{-1} s^{-1}
20.0	8.389 ± 0.442	19.0	3.554 ± 0.175
25.0	9.378 ± 0.121	25.2	4.490 ± 0.236
29.7	10.429 ± 0.160	29.1	5.162 ± 0.199
34.6	11.667 ± 0.372	34.6	6.023 ± 0.233
39.8	12.096 ± 0.261	39.8	6.384 ± 0.205

Table 2 Summary of the activation parameters and rate constants obtained for the reaction between Cu^{I}–SG (5×10^{-4} mol dm^{-3}) and peroxides (4.5×10^{-3}–1.7×10^{-2} mol dm^{-3})

	Cu^{I}–SG/H_2O_2	Cu^{I}–SG/^{t}BuOOH
E_a/kJ mol^{-1}	14.7 ± 1.3	21.9 ± 2.1
A/dm^3 mol^{-1} s^{-1}	(3.6 ± 1.8) × 10^3	(30.7 ± 26.1) × 10^3
$\Delta H^{\ddagger}$/kJ mol^{-1}	12.2 ± 1.3	19.4 ± 2.2
$\Delta S^{\ddagger}$/J K^{-1} mol^{-1}	−185 ± 4	−167 ± 7
$k_{298\ K}$/dm^3 mol^{-1} s^{-1}	9.4	4.5

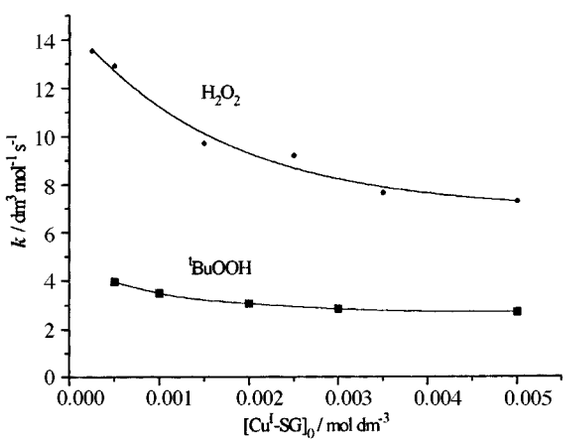


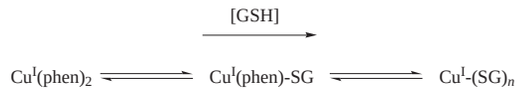
Fig. 7 Dependence of the second order rate constants, k, on copper concentration in the reaction between Cu^{I}–SG and peroxides at 298 K.

Pseudo-first order kinetic behaviour (in copper concentration) was observed and analysis leads to the second-order rate constants for the reaction of Cu^{I}–SG with H_2O_2 and ^{t}BuOOH, under these conditions, as shown in Tables 1a and 1b. The activation parameters calculated through Arrhenius and Eyring analysis are given in Table 2. The discrepancy observed between the rates obtained in this analysis and those in the EPR study is believed to reflect the difference in concentrations of copper employed (see below).

iii) UV-vis determination of the rate of reoxidation of Cu(I)–SG by peroxide at various copper concentrations. The UV-vis method described in the previous section was next employed to monitor the kinetics of the reoxidation of Cu^{I}–SG at different copper concentrations at pH 7.4. Buffered solutions (10^{-1} mol dm^{-3} phosphate) of GSH (2×10^{-2} mol dm^{-3}) and peroxide (5×10^{-2}–9×10^{-2} mol dm^{-3}) were mixed in the presence of $CuSO_4$ (2.5×10^{-4}–5×10^{-3} mol dm^{-3}) and the decay of the Cu^{I}–SG species monitored. Second-order rate constants for the reaction with H_2O_2 and ^{t}BuOOH, determined as previously, were indeed found to vary with copper concentration (see Fig. 7); the increase in rate as the concentration of copper is lowered can be explained in terms of the extent to which the Cu^{I}–SG species is aggregated—see next section).

d) Summary and discussion

These results show that Cu^{II} is readily reduced by GSH (and other thiols) at near neutral pH to yield a Cu^{I} species and the corresponding disulfide. Two equivalents of GSH are required to perform this reduction (one equivalent for reduction and the other to stabilize the resultant Cu^{I}) except in systems where other ligands are present which will sufficiently stabilize Cu^{I} alone, *e.g.* phenanthroline. However, in the presence of excess GSH a distribution of Cu^{I} species will exist in solution (see Scheme 5).



Scheme 5

The Cu^{I}–SG complex generated in $CuSO_4$–GSH systems has been shown to be readily oxidized by both H_2O_2 and ^{t}BuOOH in a reaction which is found to be first order in both copper and peroxide. The rate constants for this reoxidation are relatively low when compared with reaction rate constants for $Cu^{I}_{(aq)}$ with H_2O_2 and ^{t}BuOOH of *ca.* 4×10^3 and 5×10^3 dm^3 mol^{-1} s^{-1}, respectively.[9] The reoxidation of Cu^{I} when complexed to a stoichiometric quantity of GSH thus retards the rate considerably, which presumably reflects the stabilisation glutathione affords copper(I) (reaction with H_2O_2 in the presence of a large excess of chloride ions proceeds with a rate[3] of 15.0 dm^3 mol^{-1} s^{-1}).

The activation parameters obtained for the reoxidation reaction at 5×10^{-3} mol dm^{-3} copper exhibit very small pre-exponential constants and large, negative $\Delta S^{\ddagger}$ values which suggest that it is the entropic component of the activation energies which underlies the slow rates observed. We believe that the unfavourable entropy changes may result from Cu^{I}–SG aggregation at this concentration (see ref. 19 and references therein), which serves to shield Cu^{I} units within the aggregate and also to hinder the approach of the peroxide.

This is further substantiated by our findings that the reoxidation rate constant is dependent upon the concentration of copper present; presumably the Cu^{I}–SG species becomes less aggregated at lower concentrations and a higher reoxidation rate constant results (see Fig. 7).

Spin-trapping results further indicate that the mechanism of reoxidation may be predominantly radical or non-radical in nature (*i.e.* Fenton-like or not) depending crucially on the system employed. With ^{t}BuOOH, Fenton-type activation is observed with several copper(II) complexes and with both GSH and ascorbate as reductants. Similar behaviour is observed with H_2O_2 except in systems with Cu^{I} present as a thiolate complex, *i.e.* as the Cu^{I}–SG species, where a non-radical pathway predominates.

However, when the Cu^{I}–SG species is less aggregated (at low concentration) it might be anticipated that the mechanism will involve a Fenton-type reaction, with the release of free radicals. To test this hypothesis we performed a number of spin-trapping experiments, employing DMPO as before, at various concentrations of copper. Peroxide (H_2O_2 or ^{t}BuOOH, 2×10^{-2} mol dm^{-3}), GSH (2×10^{-2} mol dm^{-3}) and DMPO (10^{-2} mol dm^{-3}) in pH 7.4 buffered solution (5×10^{-2} mol dm^{-3} phosphate) were mixed with various concentrations of $CuSO_4$ (10^{-5}–10^{-2} mol dm^{-3}) to initiate the reaction. As can be seen from the spectra (Fig. 8), with ^{t}BuOOH, a high radical yield of Me$^{\bullet}$ at high copper concentration is observed, but as [Cu] is reduced, the GS$^{\bullet}$ species predominates (this presumably reflects the scavenging ability of GSH when in excess). With H_2O_2, no radical adduct signals are observed at the higher copper concentrations but as this is lowered there is the weak but definite appearance of EPR signals which indicate the formation and trapping of GS$^{\bullet}$. Therefore, at these lower concentrations of copper, where

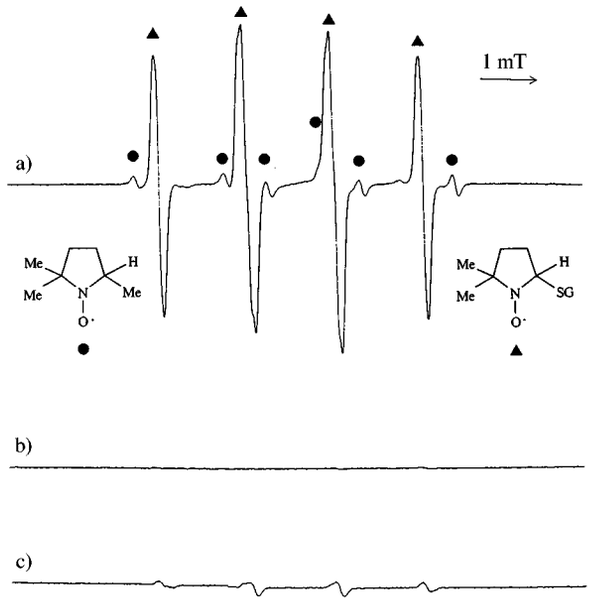
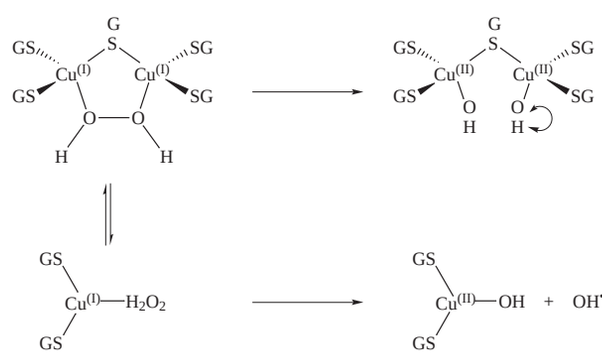


Fig. 8 EPR spectra of the radical adducts of DMPO obtained from the reaction between Cu^{I}–SG and peroxide in the presence of DMPO (10^{-2} mol dm^{-3}) and GSH (2×10^{-2} mol dm^{-3}); a) 2×10^{-2} mol dm^{-3} ^{t}BuOOH–10^{-3} mol dm^{-3} Cu^{I}–SG; b) 2×10^{-2} mol dm^{-3} H_2O_2–10^{-3} mol dm^{-3} Cu^{I}–SG, and c) 2×10^{-2} mol dm^{-3} H_2O_2–10^{-4} mol dm^{-3} Cu^{I}–SG.

we expect the Cu^{I}–SG to be less aggregated, Fenton-type activation of H_2O_2 apparently occurs with the release of free hydroxyl radicals.

The redox potentials at pH 7 (E^0) for the one- and two-electron oxidations of H_2O_2 are 0.46 and 1.32 V respectively,[23] whereas the corresponding values for ^{t}BuOOH have been estimated as 1.9 and 1.7 V;[24] thus a two-electron oxidation step is thermodynamically preferred for H_2O_2 whereas the reverse is true for ^{t}BuOOH. It is hence understandable that in Cu^{I}–SG/peroxide systems ^{t}BuOOH is activated *via* a one-electron reaction (yielding ^{t}BuO$^{\bullet}$), as found here, whereas H_2O_2 proceeds by a two-electron transfer where this can easily be achieved, *i.e.* when the Cu^{I}–SG species is aggregated (see Scheme 6). When



Scheme 6

this is not possible, at low concentrations of Cu^{I}–SG, a one-electron mechanism operates.

Experimental

EPR spectra were recorded on either a JEOL JES-RE1X or a Bruker ESP300. Splitting constants were determined to within ±0.01 mT using the spectrometer field scan and *g*-values (for the copper species) to within ±0.005. UV-vis spectra were measured on either a Hewlett Packard 8452A fitted with a stopped-flow unit or a Hitachi U-3000. ^{1}H-NMR spectra were obtained on a Bruker AMX500 and were referenced by the HOD signal at δ 4.8. pH measurements were made using simple indicator paper to within ±0.5.

All chemicals were obtained from either Aldrich or Sigma and were used as supplied except for DMPO which was further purified by stirring with activated charcoal for 30 minutes and then filtering. Peroxide concentrations were accurately determined by iodometric titration. Complexes of copper(II) were simply prepared by mixing the relative amounts of $CuSO_4$ and ligand—a slight excess of ligand over copper was used to ensure total complexation, *i.e.* $Cu^{II}(phen)_2$ solutions contained copper : phen in the ratio 1 : 2.2.

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