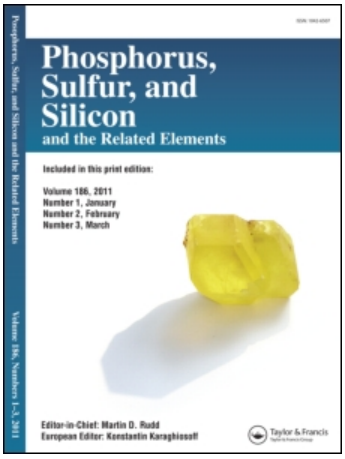

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Oxidation Of Thiols By Copper(II)

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OXIDATION OF THIOLS BY COPPER(II)

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Thiols were oxidized by Cu(II) in a reaction that had two phases. There was an immediate reaction between thiol and Cu(II) with loss of thiol, measured by its reaction with 5,5′-dithio-bis(2-nitrobenzoic acid), and formation of Cu(I), measured by its reaction with bathocuproinedisulfonic acid. With equal molar concentrations of thiols and Cu(II), only the first phase was observed and thiols were oxidized in less than 15 sec. When the concentration of Cu(II) was limiting (200 μM thiol and 10 to 160 μM Cu(II)), the second phase of the reaction was observed; this phase was much slower for glutathione (GSH) than it was for cysteine and accounts for previous reports that GSH is oxidized by Cu(II) at a slower rate than cysteine. It is proposed that Cu(II) reacts with thiols to form Cu(II)-thiol complexes, Cu(II) is reduced to Cu(I), and the thiols are oxidized to the corresponding radicals. Two of the radicals react to form a disulfide and Cu(I) reacts with a second thiol to form a Cu(I)-thiol complex in which the Cu(I) is oxidized to Cu(II) by oxygen. The rate of reoxidation of Cu(I) is dependent on the R group of the thiol and is the rate limiting step of the reaction.

Key words: Thiols; oxidation; copper(II); copper(I); copper(II)-thiol complex; copper (I)-thiol complex.

INTRODUCTION

Thiols are oxidized by Cu(II) to the corresponding disulfides at rates that vary over a wide range.[1–6] For instance, although cysteine is a component amino acid of the tripeptide glutathione (GSH), cysteine is oxidized by copper(II) much faster than GSH.[2,4–6] Although the mechanism for these oxidation reactions isn't known completely, it is generally agreed that the reactions proceed by formation of a complex between Cu(II) and thiol (reaction 1).[2]

$$Cu^{II} + RSH \rightarrow Cu^{II} - SR + H^{+} \quad (1)$$

The thiol is oxidized to the corresponding radical (·SR) and Cu(II) is reduced to Cu(I) (reaction 2).

$$Cu^{II} - SR \rightarrow Cu^{I} + {}^{\cdot}SR \quad (2)$$

Two of the radicals can react to form the corresponding disulfide (reaction 3) and the Cu(I) is oxidized to Cu(II)

$$2{}^{\cdot}SR \rightarrow RSSR \quad (3)$$

*Author to whom correspondence should be addressed.

by oxygen (reaction 4).

$$Cu^{I} + O_2 \rightarrow Cu^{II} + O_2\cdot \quad (4)$$

The differences in oxidation rates of thiols with similar pK and E'^0 values produced by Cu(II) is thought to be due to structural differences between thiols that stabilize the free radical intermediate to different extents.[1,2]

Thiols such as cysteine that were rapidly oxidized by Cu(II) were not effective in protecting oxyhemoglobin from oxidation by Cu(II), whereas thiols such as GSH that are slowly oxidized by Cu(II) protected oxyhemoglobin from oxidation.[6] The present studies were carried out to attempt to explain the mechanism responsible for these differences in rate of reaction between Cu(II) and thiols.

RESULTS

When Cu(II) was added to 200 μM GSH and the amount of thiol present assayed by the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reaction at 412 nm (A_{412}), a two stage reaction was observed. At 15 seconds after mixing the thiol and Cu(II), the color that developed in the reaction between GSH and DTNB was lowered as the concentration of Cu(II) increased from 40 μM to 160 μM (Figure 1A). With 200

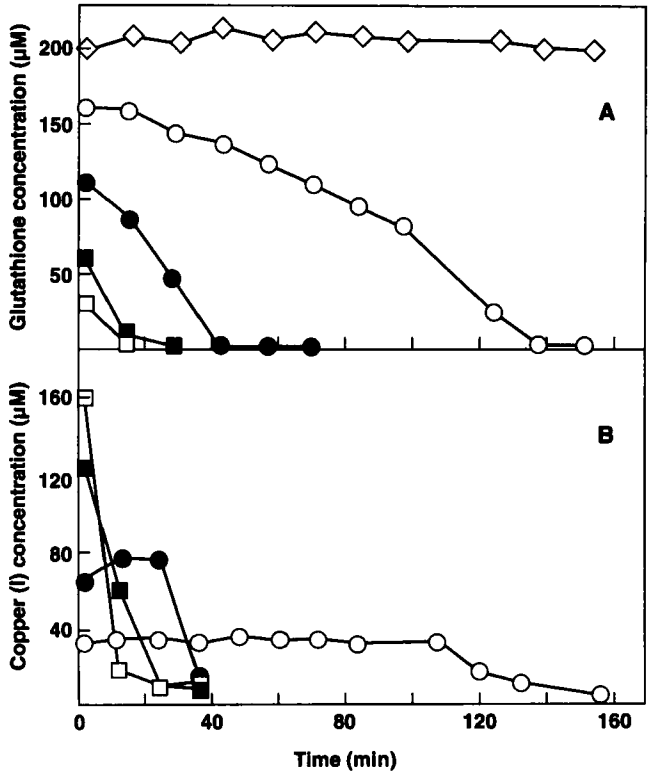

FIGURE 1 Effect of concentration of Cu(II) on oxidation of glutathione. The reaction mixture contained 200 μM glutathione, 50 mM Tris buffer, pH 7.5, and either no addition (◇), or Cu(II) at 40 μM (○), 80 μM (●), 120 μM (■), and 160 μM (□). Curves in 1A are for free sulfhydryl groups of glutathione (A_{412}) and curves in 1B are for Cu(I) (A_{485}). Curves shown are representative of three experiments.

μM Cu(II) no color developed. This initial rapid decrease in A_{412} could be the result of very rapid oxidation of GSH to oxidized glutathione (GSSG) by Cu(II); alternatively Cu(II) could alter the spectral properties of the chromophore. Cu(3,5-diisopropylsalicylate)$_2$, Cu(acetate)$_2$, and Cu(*o*-phenanthroline)$_2$ caused the disappearance of A_{412} formed when cysteine, penicillamine, or GSH reacted with DTNB.[7,8] After this initial decrease in A_{412}, there was a second slower rate of decrease in DTNB-reactive material that varied with the concentration of Cu(II) until A_{412} reached 0 (Figure 1A).

The concentration of Cu(I) in the reaction mixtures was also assayed after Cu(II) was added to GSH (Figure 1B). Within 15 sec after Cu(II) was added, all Cu(II) was converted to Cu(I) and the concentration of Cu(I) remained elevated until GSH was almost completely oxidized. The concentration of Cu(I) then decreased as it was reoxidized to Cu(II) by oxygen. These data suggested that there was an immediate oxidation of GSH by Cu(II) resulting in its reduction to Cu(I) and that copper ion was present as Cu(I) until the GSH was oxidized.

When 40 μM Cu(II) was reacted with 200 μM L-cysteine, the decrease in A_{412} was so rapid that two stages were not seen unless either the concentration of cysteine was increased or the concentration of Cu(II) was decreased. The data for 200 μM L-cysteine and 10 μM Cu(II) are given in Figure 2. As with GSH, the concentration of Cu(I) was zero before the reaction was started, immediately increased to 10 μM, remained at this concentration while the reaction was proceeding, and then decreased when the reaction was complete. The data with GSH and L-cysteine show that when the concentration of thiol is greater than that of Cu(II), GSH is oxidized at a much slower rate than L-cysteine when assayed with the DTNB reagent and that Cu ions are present as Cu(I) during the reaction. However, when

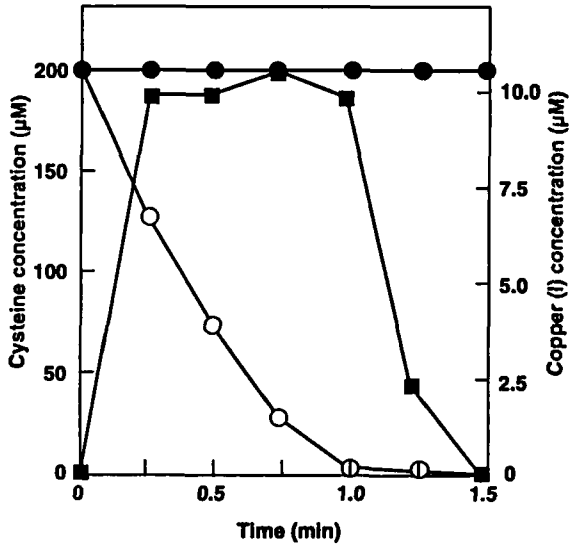

FIGURE 2 Oxidation of L-cysteine by Cu(II). The reaction mixture contained 200 μM L-cysteine, 50 mM Tris, pH 7.5, and either no addition (●, A_{412}) or 10 μM Cu(II) (○, A_{412}; ■, A_{485}). Curves shown are representative of three experiments.

the thiol and Cu(II) are present at the same concentration, both GSH and cysteine are rapidly oxidized.

Saez et al.[9] reported that cysteine was autoxidized to cystine, cysteine sulfinate, and cysteine sulfonate. With 0.1 mM cysteine, cystine accounted for about 80% of the products and the rest were the sulfinate and sulfonate. With 100 mM cysteine, cystine was the main product observed. Chansoria and Mishra[10] reported recently that cystine was the oxidation product of cysteine oxidation under anaerobic conditions in acidic medium. Chromatography of the products formed by oxidation of L-cysteine and GSH by Cu(II) in the present experiments showed that cystine and GSSG were the only products detected (Table I). No other ninhydrin-positive products were detected even when the chromatograms were loaded with ten times the amount of reaction mixture used to detect cystine.

Because compounds of Cu(II) affect the A_{412} developed in the DTNB reaction,[7,8] the oxidation of thiols by Cu(II) was also ascertained using the oxygen electrode. As observed by Tsen and Tappel,[4] the rate of oxygen uptake by solutions of thiols and Cu(II) varied widely. L-Cysteine was oxidized at a much faster rate than either N-acetyl-L-cysteine or GSH. When the ratio of thiol to Cu(II) was 2:1, the uptake of oxygen had two phases; an initial slower phase followed by a much faster phase as the thiol was oxidized (Figure 3). When the ratio of either GSH or N-acetyl-L-cysteine to Cu(II) was 1:1, the rate of oxygen uptake was much faster than with the 2:1 ratio and the rate varied with the different thiols. It was concluded from these first three experiments that the rate limiting reaction for the oxidation of thiols present in excess by Cu(II) may not be the initial reaction between thiol and

TABLE I

Chromatography of the products formed from the oxidation of L-cysteine and GSH by Cu(II)

Compound	Solvent		
	A*	B	C
L-Cysteine	0.76	0.68	
L-Cystine	0.11	0.54	
L-Cysteine sulfonic acid	0.18	0.72	
L-Cysteine sulfinic acid	0.23	0.74	
Product from oxidation of L-cysteine by Cu(II)	0.11	0.54	
GSH			0.72
GSSG			0.54
Product from oxidation of GSH by Cu(II)			0.54

* Solvent A, 95% ethanol:acetic acid:water (65:1:34); solvent B, butanol:acetone:acetic acid:water (35:35:10:20); solvent C, 95% ethanol:water (7:3).

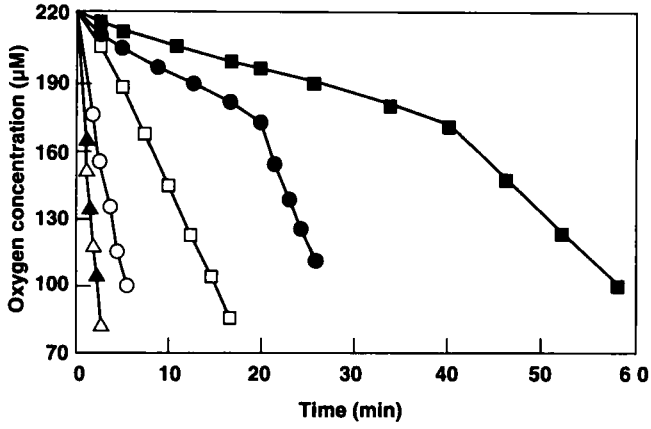

FIGURE 3 Oxygen uptake following addition of Cu(II) to thiols. In the first experiment the reaction mixture contained 100 μM Cu(II), 50 mM Tris, pH 7.5, and either 200 μM L-cysteine (▲), 200 μM N-acetyl-L-cysteine (●), or 200 μM glutathione (■). In the second experiment, the reaction mixture contained 200 μM Cu(II), 50 mM Tris, pH 7.5, and either 200 μM L-cysteine (△), 200 μM N-acetyl-L-cysteine (○), or 200 μM glutathione (□). Curves shown are representative of three experiments.

Cu(II), to form the thiol and Cu(I), but the rate of oxidation of Cu(I) by O_2 may be limiting and the ability of Cu(I) bound to thiols to react with O_2 may be dependent of the R group of the thiol.

When Cu(I) was incubated in the oxygen chamber, there was a rapid uptake of oxygen (Figure 4). The addition of thiols to the incubation mixture decreased the rate of oxygen uptake; L-cysteine, which was oxidized rapidly by Cu(II), had only a slight effect on the rate of oxygen uptake. N-Acetyl-L-cysteine and GSH, which were oxidized slowly by Cu(II), markedly decreased the rate of oxygen uptake compared to Cu(I) alone.

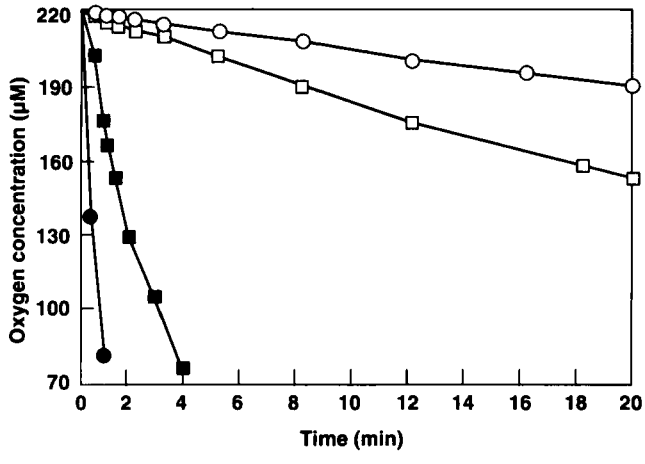

FIGURE 4 Oxygen uptake following addition of Cu(I) to thiols. The reaction mixture contained 100 μM Cu(I), 50 mM Tris, pH 7.5, and either no addition (●), 1.0 mM L-cysteine (■), 1.0 mM N-acetyl-L-cysteine (□), or 1.0 mM glutathione (○). Curves shown are representative of three experiments.

DISCUSSION

The rate of oxidation of thiols by Cu(II) varies widely. This variation in rates has been suggested to be due to structural differences between thiols that stabilize the radical intermediates (RS·) to different extents.[2] The rate of reactivity of thiols with the stable radicals 2,2-diphenyl-1-picrylhydrazyl and galvinoxyl and with ferricytochrome *c* also vary.[11-15] GSH, for instance, is less reactive than cysteine and mercaptoimidazoles with these agents.

From the results presented in this paper, we suggest that the rate determining step for the oxidation of thiols by Cu(II), when its concentration is limiting, is the rate of reoxidation of Cu(I) by O_2 and this rate is determined by the R group of the thiol, rather than rate of formation of the thiol radical. The reactions in Figure 5 show the mechanism we are proposing. First, Cu(II) reacts with a thiol to form a Cu(II)-thiol complex (reaction 1); an electron is transferred from the thiol to form Cu(I) and a thiol radical (reaction 2). Two of the thiol radicals condense to form the corresponding disulfide (reaction 3) and the copper(I) reacts with a second thiol to form a Cu(I)-thiol complex (reaction 4) that is oxidized by oxygen to regenerate Cu(II) (reaction 5 and/or 6). During this reaction the thiol may remain bound to the copper during its oxidation (reaction 5) or it may be released and then recombine with Cu(II) after it is formed (reaction 6). When thiols are not present, Cu(I) is reoxidized by oxygen (reaction 7). The rate determining step in these reactions would be the reoxidation of the Cu(I)-thiol complex and would be dependent on the R group. Once the thiol is completely oxidized the rate of the reaction will increase because there is no free thiol remaining to complex the Cu(I). However, the rate of reoxidation may not be the same for Cu(I) in all systems because disulfides such as cystine formed in the reaction may also bind to Cu(I) and affect the rate of reoxidation of Cu(I).[16] In the case of at least one thiol, GSH, its radical (GS·) also reacts with GS^- to form the radical anion, $GSSG\cdot^-$ which reacts with oxygen to form GSSG and superoxide.[17,18] The rate of reaction of the radical anion $GSSG\cdot^-$ with oxygen is close to diffusion controlled[19] and could not be the rate determining step in the oxidation of GSH reported here.

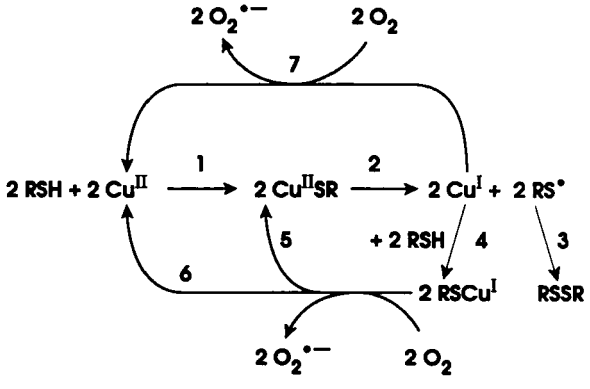

FIGURE 5 Schematic diagram of reactions for Copper(II)-induced oxidation of thiols and reoxidation of Cu(I) by oxygen.

Although Cu(I) is not stable in aqueous solutions,[20] a number of Cu(I) cationic or anionic complexes are stable in aqueous solution and some have been used for treatment of a variety of diseases.[7,21] A stable complex is formed between Cu(I) and GSH whereas the complex between Cu(I) and cysteine is much less stable.[22] Complexes of GSH and Cu(I) have been suggested to be intermediates in cellular metabolism and storage of Cu(I).[23] GSH inhibited free radical formation from Cu(I) possibly by stabilization of Cu(I) through chelation.[24] This is the mechanism that is proposed here to explain the difference in rates of oxidation of thiols by Cu(II). Mi and Zuberbühler reported that the rate-limiting observable step for the Cu(II)-catalyzed oxidation of ascorbic acid in aqueous acetonitrile was the rate of reoxidation of Cu(I) by oxygen.[25]

EXPERIMENTAL

Materials. Copper sulfate (5 H_2O) was purchased from Allied Corp. (Morristown, NJ). Mercaptoethanol was obtained from Serva Fine Biochemicals Inc. (Westbury, NY). Acetonitrile, optima grade, and 3MM chromatography paper (Whatman) were obtained from Fisher Scientific (Fair Lawn, NJ). The other compounds were obtained from Sigma Chemical Co. (St. Louis, MO). Pre-coated TLC plastic sheets with a cellulose layer (EM Reagents) were purchased from Alltech Associates (Deerfield, IL). The aqueous reagents were prepared in water purified with a Barnstead NANOpure II system to reduce contamination by metal ions. Because Cu(I) is unstable in aqueous media,[20] it was prepared under nitrogen at a concentration of 10 mM in acetonitrile just before the experiments were started.

Assay of thiols and Cu(I). Oxidation of thiols by Cu(II) was assayed by the DTNB method of Beutler et al.[26] The thiol (200 μM) was incubated in 50 mM TRIS buffer, pH 7.5, at 30°C either without Cu(II) or with Cu(II) at concentrations varying from 40 to 160 μM. At intervals, samples were removed, mixed with the DTNB reagent and after 5 min read at 412 nm.

Cu(I) was determined by the method of Zak[27] by measuring the absorbance of the Cu(I)-2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid (bathocuproinedisulfonic acid) complex at 485 nm. The only changes in the method were commercial bathocuproinedisulfonic acid was used and 0.1 M Tris buffer (final concentration), pH 7.5, was used in place of 45% sodium acetate.

Oxygen uptake. Oxygen uptake experiments were monitored using a Clark oxygen electrode (Yellow Springs Instrument Co.). The incubations were carried out at 30°C in 50 mM Tris buffer, pH 7.5 in a total volume of 3 ml. Usually the thiol was the last component added to the incubation chambers. When Cu(I) was used, it was added last.

Chromatography. Chromatography of the products of Cu(II)-induced oxidation of L-cysteine was on 0.1 mm cellulose pre-coated TLC plastic sheets in 95% ethanol:acetic acid:water (65:1:34)[28] and in butanol:acetone:acetic acid:water (35:35:10:20).[9] Chromatography of the products of Cu(II)-induced oxidation of GSH was on Whatman grade 3MM chromatography paper in 95% ethanol:water (7:3).[29] The products were detected by spraying with 0.25% ninhydrin in acetone.[30] Standards of L-cysteine, L-cystine, L-cysteine sulfinic acid, L-cysteine sulfonic acid, GSH, and GSSG were chromatographed with the products formed from copper-induced oxidation of L-cysteine and GSH.

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