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Mechanism of Copper-Catalyzed Oxidation of Glutathione

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The mechanism of copper-catalyzed glutathione oxidation was investigated using oxygen consumption, thiol depletion, spectroscopy and hydroxyl radical detection. The mechanism of oxidation has kinetics which appear biphasic. During the first reaction phase a stoichiometric amount of oxygen is consumed (1 mole oxygen per 4 moles thiol) with minimal •OH production. In the second reaction phase, additional (excess) oxygen is consumed at an increased rate and with significant hydrogen peroxide and •OH production. The kinetic and spectroscopic data suggest that copper forms a catalytic complex with glutathione (1 mole copper per 2 moles glutathione). Our proposed reaction mechanism assumes two parallel processes (superoxide-dependent and peroxide-dependent) for the first reaction phase and superoxide-independent for the second phase. Our current results indicate that glutathione, usually considered as an antioxidant, can act as prooxidant at physiological conditions and therefore can participate in cellular radical damage.

Keywords: Glutathione, oxidation, copper, hydroxyl radical, superoxide radical, hydrogen peroxide

INTRODUCTION

Glutathione (γ-glutamylcysteinylglycine, GSH) is one of the major components of the cellular antioxidant defense. It plays multiple roles, acting as a substrate for glutathione peroxidase and glutathione S-transferase and as a direct scavenger of free radicals.

The thiol group of glutathione can react with molecular oxygen[1,2] resulting in glutathione disulfide. This reaction is catalyzed by transition metals (copper and iron) and can lead to the generation of reactive oxygen species (ROS) including superoxide $O_2^{\bullet -}$, hydrogen peroxide H_2O_2 and hydroxyl radical •OH. However, data about the mechanism of glutathione oxidation in the presence of copper and the involvement of ROS are not clear.

Generation of superoxide in the copper-mediated oxidation of GSH has been considered to result from oxygen reduction by low-valent metal,[3] diglutathione anion radical[4] or both

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metal and glutathione.[1,2] Generation of H_2O_2 has been explained by the dismutation of superoxide,[1,2] but also by its reduction by thiol.[3] Depletion of GSH by superoxide, in a chain reaction which completely excludes the intermediate generation of hydrogen peroxide has also been proposed.[4] Generation of •OH has generally been presumed to result from a metal-catalyzed Haber–Weiss or Fenton reaction.[1–3] Cleavage of DNA[5] and oxidation of lipids[6] by copper–thiol systems assume the production of hydroxyl radicals, but this has not been directly detected and quantitated. An inhibition of •OH production by GSH during the cuprous-driven Fenton reaction was also reported.[7]

The stoichiometry of the reaction is also not certain. The main reaction product is generally considered to be oxidized glutathione (GSSG). However, the generation of glutathione sulfonic acid was shown in the oxidation of GSH by superoxide, generated in a xanthine-oxidase system.[4] Since superoxide is an intermediate of oxygen reduction,[1–3] one could also presume the production of glutathione sulfonic acid during metal catalyzed GSH oxidations.

Most previously proposed reaction mechanisms[1–4] have considered glutathione as a simple reductant for $Cu^{2+} \leftrightarrow Cu^{+}$ redox cycling. However, various reports have also suggested the formation of complexes between reduced copper and reduced glutathione[8,9] and oxidized copper and oxidized glutathione.[10] The latter complex was found to have superoxide dismutation activity.[11] Since the complexing agents significantly influence the involvement of copper in the generation of free radicals,[12] the effect of copper chelation by glutathione on the mechanism of its catalytic action should be considered.

In the current work we investigate the mechanism of copper-mediated GSH oxidation and the involvement of reactive oxygen intermediates in this process. We performed the reaction at physiological temperatures in simple phosphate buffer: pH 7.4 at 37°C. We used the methods of fluorescence and absorption spectroscopy and oxygen consumption for the detection of reactive oxygen species. Superoxide dismutase (SOD) and catalase were used to determine the involvement of superoxide and hydrogen peroxide in the reaction. Our results suggest a new mechanism of copper-catalyzed oxidation of glutathione which helps to explain various controversies in the literature.

MATERIALS AND METHODS

All reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) or Sigma Chemical Co. (St. Louis, MO, USA) and were used without additional purification, except for $CuSO_4 \cdot 5H_2O$, which was recrystallized prior to use. Buffer solutions were prepared using Milli-Q water (18 MΩcm) using 99.999% $Na_2HPO_4 \cdot 7H_2O$ and 99.999% H_3PO_4.

The reactions were performed at 37°C in 40 mM sodium phosphate buffer, pH 7.40. Preliminary experiments showed that varying the buffer concentration from 10 to 100 mM did not affect the kinetics of the process (data not shown).

Oxygen consumption was measured using a Clark oxygen electrode with amplifier system from YSI, Inc. (Yellow Springs, Ohio). Since this apparatus contains plastics which could carry over trace metal impurities, key observations were repeated using an all glass and ceramic measuring system from Oxygen Sensors Inc. (Norristown, PA). An involvement of superoxide and hydrogen peroxide was determined from the effects of SOD and catalase. Modification of oxygen consumption by superoxide dismutase and catalase required active enzyme, since heat inactivation eliminated all enzyme effects.

The production of •OH was estimated by including coumarin-3-carboxylic acid (3-CCA) in the reaction buffer.[13,14] Hydroxyl radicals produce only one fluorescent product,

7-hydroxycoumarin-3-carboxylic acid (7-OHC-CA). Fluorescence was measured using an SPF-500 spectrofluorometer (SLM Instrument Co, Urbana, IL) with excitation at 400 nm and emission at 450 nm. We used a 1 mM concentration of 3-CCA probe in order to minimize the competitive scavenging of •OH by GSH and to enhance sensitivity.

Absorption spectra were detected using a DW-2000 spectrophotometer (SLM Instrument Co, Urbana, IL, USA). Absorbance at 400–450 nm prior to and after the experiments did not exceed 0.05 for all the samples, thus avoiding any inner filter effect for the fluorescence measurements. The concentration of free thiol was determined by addition of an aliquot of solution to 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) buffered by phosphate to pH 7.4, then measuring absorbance at 412 nm. The thiol concentration was determined from calibration curves obtained using reference samples with the same concentration of copper.

RESULTS

The kinetics of oxygen consumption and •OH production demonstrated two phases in the copper-catalyzed oxidation of GSH (Figure 1). During the first reaction phase the solution of 0.1 mM GSH plus 10 μM of Cu^{2+} consumed an amount of oxygen close to that predicted by reaction (1):

$$4GSH + O_2 \rightarrow 2GSSG + 2H_2O. \qquad (1)$$

The involvement of superoxide and hydrogen peroxide in the reaction was examined by adding superoxide dismutase and catalase. Superoxide dismutase caused a 20% decrease of the oxygen consumption rate but did not affect the total amount of oxygen consumed. Catalase caused a 30% decrease of the oxygen consumption rate and decreased the total amount of oxygen consumed (Figure 1). The effect of both enzymes in the mixture was additive. Heat denatured enzymes did not affect the kinetics of oxygen

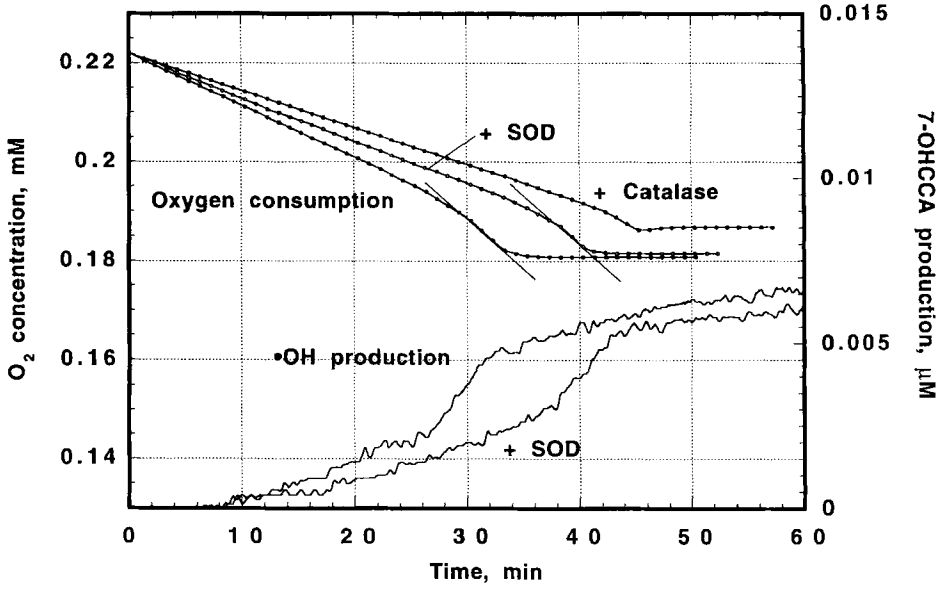

FIGURE 1 Kinetics of oxygen consumption and •OH production by 0.1 mM GSH and 10 μM Cu^{2+} at 37°C in 40 mM phosphate buffer (pH = 7.40) and the effects of catalase (10 U/ml) and superoxide dismutase (10 U/ml). Catalase completely prevents •OH generation (data not shown).

consumption (data not shown). These results indicate the generation of both superoxide and hydrogen peroxide during the copper-catalyzed *oxidation* of glutathione. Neither enzyme eliminated the biphasic nature of oxygen consumed and did not change the total amount of oxygen used as predicted by Eq. (1).

Copper-catalyzed oxidation of GSH in the first phase was accompanied by the production of a small amount of 7-OHCCA (0.008% of consumed oxygen), reflecting •OH addition to 3-CCA (Figure 1). The production of •OH was halved by superoxide dismutase but this enzyme did not change the total amount of 7-OHCCA produced (Figure 1). No 7-OHCCA was produced in the presence of catalase (data not shown).

At the end of the first reaction phase the rate of oxygen consumption suddenly increases more than 2-fold (Figure 1). The accelerated consumption of oxygen is accompanied by the simultaneous increase of •OH production (Figure 1). These effects indicate the alteration of the reaction mechanism and appearance of the second reaction phase of copper-catalyzed glutathione oxidation. During this second reaction phase the solution consumes excess oxygen, as compared to the stoichiometry of reaction (1). The excess oxygen consumption was equal to $50 \pm 5\%$ for 0.1 mM GSH (Figure 1). It was similar to the stoichiometry of glutathione oxidation $GSH : O_2 = 3.6 : 1.4$ (54% excess of oxygen) in a superoxide-generating system.[4]

The concentration of free thiol, as detected by DTNB reduction, decreased during the first reaction phase (Figure 2). Because the overall oxidation was very slow in this model system, it was possible to measure the apparent free thiol concentration immediately after copper addition using standard mixing techniques. Addition of copper to GSH before mixing with DTNB decreased the 412 nm absorption in a copper-dependent manner suggesting a 1:1 complex which eliminated reactivity with DTNB (Figure 2, inset). This was not caused by an interaction of copper with the reduced DTNB chromophore since adding copper after reaction of GSH with

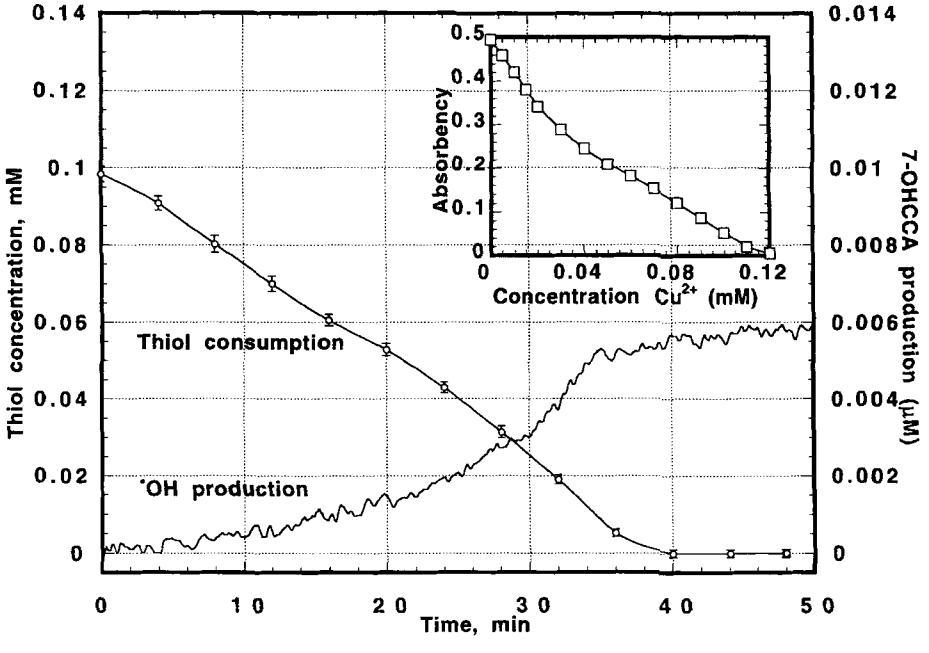

FIGURE 2 Consumption of free thiol during autoxidation of 0.1 mM GSH and 10 μM Cu^{2+} detected by DTNB method. Inset: Effect of copper concentration on the thiol detection from 0.1 mM GSH.

DTNB caused only a very slow oxidation of the chromophore (data not shown). Two additional broad absorption maxima at 635 nm and 810 nm appeared in the spectrum of Cu^{2+} (770 nm) after GSH addition, also demonstrating the chelation of copper by GSH (data not shown).

Increasing the Cu^{2+} concentration caused an acceleration of oxygen consumption in both reaction phases (Figure 3). The total amount of consumed oxygen did not depend on copper concentration, but the proportion of oxygen consumed during the first phase decreased as the copper concentration increased. No initial phase was observed when the copper concentration increased to half the GSH concentration. Under conditions where GSH was in large excess over copper, the excess oxygen increased with GSH concentration (Figure 4). However, it is difficult to follow the precise stoichiometry of the reactions under such circumstances.

The •OH yield in the second phase depended on the Cu^{2+} concentration and significantly increased when the Cu^{2+} concentration exceeded half the GSH concentration (Figure 5). These data also indicate the involvement of copper–glutathione complex 1 : 2 in the reaction.

DISCUSSION

Our current results demonstrate several features of copper-catalyzed GSH oxidation which have not been documented previously. These include the biphasic kinetics of the process, with accelerated consumption of oxygen and production of hydroxyl radical in the second phase. We have also demonstrated that the production of •OH is independent of superoxide, commonly assumed to mediate the redox cycling of the metal. The expected stoichiometry of 4 moles GSH consuming 1 mole of oxygen occurs during the first reaction phase and the rate, but not the extent, of this reaction are affected by both superoxide and hydrogen peroxide. This suggested the presence of two independent and parallel pathways for

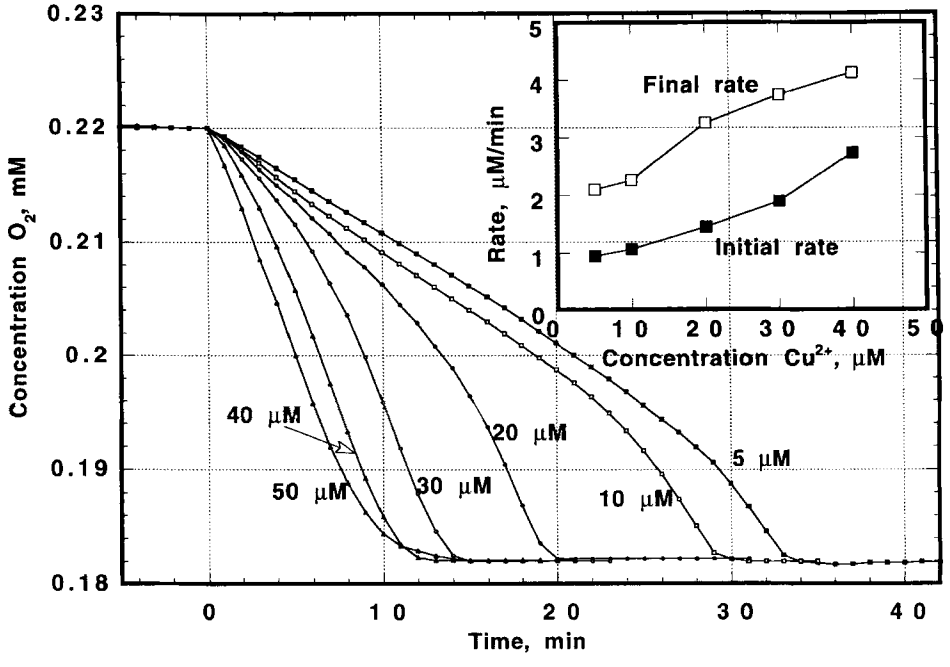

FIGURE 3 Effect of Cu^{2+} on the oxygen consumption by 0.1 mM GSH.

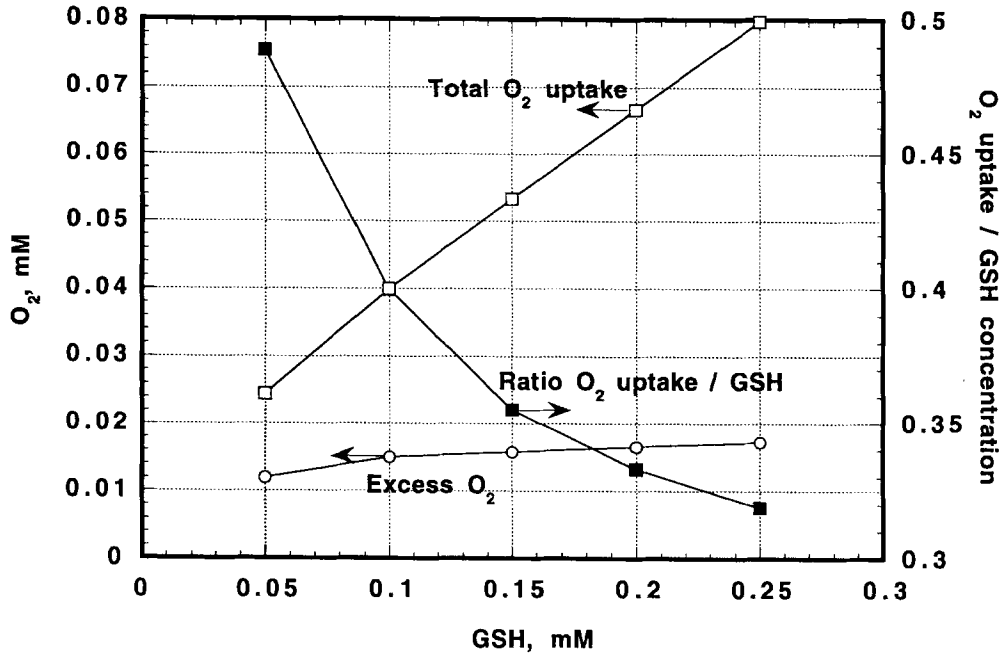

FIGURE 4 Stoichiometry of GSH autoxidation in presence of 10 μM Cu^{2+}.

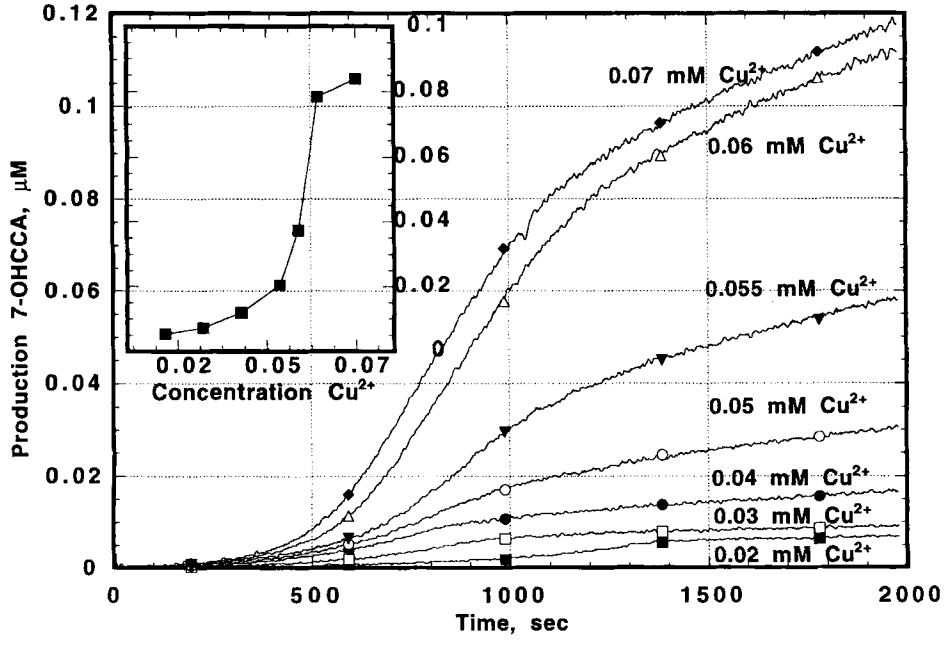

FIGURE 5 Effect of Cu^{2+} concentration on the kinetics of •OH generation by 0.1 mM GSH.

modification of a common intermediate. GSH appears to form a very strong complex with copper at a 1 : 1 ratio, and also a weaker complex at a 2 : 1 ratio. The effect of copper concentration on the thiol detection by DTNB (Figure 2, inset) shows a strong binding of the first GSH molecule to Cu^{2+}. Data on production of •OH (Figure 5) support the binding of a second GSH molecule with formation of complex which is similar to copper–cysteine $Cu^{2+}(CysS^{-})_2$.[15] Both complexes are involved in hydroxyl radical production, which requires the presence of hydrogen peroxide but not superoxide.

A reaction scheme consistent with these observations is shown in Figure 6. For a large excess of GSH over copper, we assume the formation of a complex between Cu^{2+} and two glutathione molecules, with the cysteine thiol moiety and the glutamine α-aminogroup moiety forming the coordination bonds (Figure 6).

$$Cu^{2+} + 2GSH \leftrightarrow Cu^{2+}(GS^{-})_2 + 2H^{+}. \quad (2)$$

Spectroscopic data show both hypsochromic (635 nm) and bathochromic (810 nm) shifts of the absorption of hydrated Cu^{2+} (770 nm).

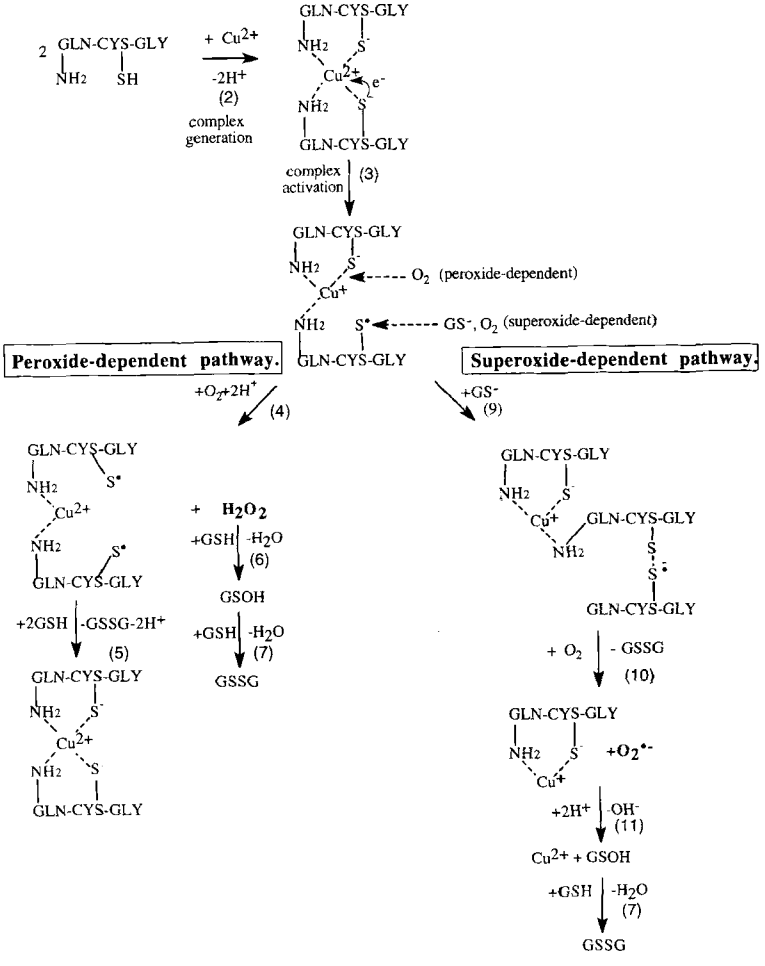

FIGURE 6 Mechanism of copper-catalyzed GSH autoxidation.

A similar hypsochromic shift occurs for copper ion complexed with glycine or glutamine. Binding of cupric ion to the glutamic α-aminogroup has been described for its complex with oxidized glutathione.[10] The bathochromic band indicates the chelation of copper by the thiol group and we have previously demonstrated a qualitatively similar chelation with the thiol(s) of dithiothreitol.[14] Both additional maxima are very broad and have decreased extinction coefficients in comparison with Cu^{2+} absorption, preventing the determination of complex stability from spectroscopic data.

The complex is activated by electron transfer from S^- to Cu^{2+}:

$$Cu^{2+}(GS^-)_2 = Cu^+(GS^-)(GS^\bullet). \qquad (3)$$

The thiyl sulfur in $GS^\bullet$ is no longer bound to the metal atom due to loss of its negative charge, and the electron transfer is irreversible. Because $GS^\bullet$ also coordinates with copper through the alpha aminogroup of its glutamine moiety, reaction (3) does not cause a complete complex dissociation. This represents a significant feature of the current reaction mechanism, which allows the binding of reduced or oxidized metal ion to both thiol and thiyl radical through the whole reaction. Such binding explains the biphasic mechanism of the overall oxidation process (see following).

Oxygen may now attack the complex at two sites, the reduced metal or the thiyl sulfur. The first pathway is initiated by reduction of oxygen by cupric ion. Because Cu^+ in the molecule of activated complex is bound to another strong reductant S^-, the simultaneous oxidation of both atoms has to be considered as the most probable process. This results in the two-electron reduction of oxygen with production of hydrogen peroxide but without intermediate superoxide generation:

$$Cu^+(GS^-)(GS^\bullet) + O_2 + 2H^+ = Cu^{2+}(GS^\bullet)_2 + H_2O_2. \qquad (4)$$

Due to the absence of copper–sulfur bonds, the two sulfur radicals produced in reaction (4) are free to dimerize, and the resulting weak complex of oxidized copper (via one or two amines) is displaced by two additional glutathiones, recovering the catalyst:

$$Cu^{2+}(GS^\bullet)_2 + 2GSH = Cu^{2+}(GS^-)_2 + GSSG + 2H^+. \qquad (5)$$

It is not possible to rule out the release of free $GS^\bullet$ in reaction (5) and its further involvement in side reactions (see below).

Hydrogen peroxide has previously been found to be a product of thiol oxidation.[2,3] It may be reduced by thiols without the production of radical intermediates or oxygen depletion:[16]

$$H_2O_2 + GSH = GSOH + H_2O \qquad (6)$$

$$GSOH + GSH = GSSG + H_2O. \qquad (7)$$

Reactions (4–7) represent a peroxide-mediated superoxide-independent pathway of GSH reduction. Intermediate generation of hydrogen peroxide explains the deceleration of the first phase reaction rate by catalase (Figure 1). The 30% reduction of the reaction rate by catalase suggests the consumption of 60% of oxygen through this pathway.

The independent effect of SOD infers the existence of another simultaneous pathway with the intermediate generation of superoxide anion. Although sulfur radicals are not easily oxidized, they react with GS^-,[17] producing the strong reductant diglutathione anion radical $GSSG^{\bullet-}$ ($E_0 = -1.5$ V[18]):

$$GS^\bullet + GS^- \leftrightarrow GSSG^{\bullet-}. \qquad (8)$$

An analogous reaction occurs with the activated complex, since thiyl sulfur is not bound to copper:

$$Cu^+(GS^-)(GS^\bullet) + GS^- = Cu^+(GS^-)(GSSG^{\bullet-}). \qquad (9)$$

Oxygen reacts with $GSSG^{\bullet-}$ producing superoxide anion:[4,18]

$$Cu^{+}(GS^{-})(GSSG^{\bullet-}) + O_2 = Cu^{+}(GS^{-}) + GSSG + O_2^{\bullet-} \quad (10)$$

which may then react directly with the other product, cuprous glutathionate:

$$Cu^{+}(GS^{-}) + O_2^{\bullet-} + 2H^{+} = GSOH + Cu^{2+} + OH^{-} \quad (11)$$

with further reduction of sulfenic acid (GSOH) in reaction (7). It is possible that reaction (11) is accentuated by the immediate proximity of reactants.

As an alternative mechanism, consider the possible chain oxidation of GSH by superoxide described in:[4]

$$GSH + O_2^{\bullet-} = GSO^{\bullet} + OH^{-} \quad (12)$$

$$GSO^{\bullet} + GSH = GS^{\bullet} + GSOH \quad (13)$$

$$GS^{\bullet} + GS^{-} \leftrightarrow GSSG^{\bullet-} \quad (8)$$

$$(GSSG^{\bullet-}) + O_2 = GSSG + O_2^{\bullet-}. \quad (10a)$$

The participation of superoxide in these reactions would prevent the copper dependence of the reaction rate and would not allow the stoichiometry of oxygen consumption to be unaffected by SOD. This does not appear to apply in the present circumstances. Note that this process is peroxide- and copper-independent, even though all of the reactants are available from our reaction scheme (Figure 6).

Reactions (9–11) represent a superoxide-mediated peroxide-independent pathway of GSH oxidation. The 20% decrease of the reaction rate by superoxide dismutase suggests that about 40% of consumed oxygen is utilized by this pathway. Additive effects of SOD and catalase mixture suggests the independence of superoxide- and peroxide-mediated pathways and excludes superoxide dismutation as the actual source of peroxide generation in copper-catalyzed GSH oxidation.

Existence of two simultaneous pathways of oxygen reduction is a possible explanation of disagreements in the literature data. Winterbourn and Metodiewa[4] used a superoxide-generating system with chelation of trace metals, excluding the peroxide-mediated reactions and enforcing the superoxide-dependent chain process. Other authors[1–3] investigated the reaction in the presence of transition metals and detected the generation of both superoxide and hydrogen peroxide.

Reactions (1–11) do not predict an intermediate generation of hydroxyl radicals, although the simultaneously generated H_2O_2 and reduced copper could react in a Fenton-type reaction. Indeed, the low amount of 7-OHCCA produced vs oxygen consumed (Figures 1 and 2) is consistent with data by Mason *et al.*[19] suggesting no hydroxyl radical production in similar chemical model systems. The production of 7-OHCCA in our studies is also much lower than was found for the oxidation of ferrous–polyphosphate complex.[20,21] In radiochemical systems where the production of $^{\bullet}OH$ has been accurately quantitated,[22] the yield of 7-OHCCA in the reaction of $^{\bullet}OH$ with CCA is about 4%. These results suggest the conversion of only 0.2% of consumed oxygen into $^{\bullet}OH$ during the first phase of copper catalyzed oxidation of GSH.

In the second reaction phase GSH consumed an excess of oxygen as compared with the stoichiometric ratio $GSH:O_2$ 4:1. The rate of both oxygen consumption and hydroxyl radical production increased significantly after complete oxidation of free GSH. This was most clearly demonstrated by adding copper at 50% or more of the initial GSH concentration (Figures 3 and 5). We suggest that the main process during the second reaction phase is the autoxidation of the copper-glutathione complex. The absence of free thiol excludes the utilization of thiyl radicals generating superoxide in reactions (8–9). This explains the superoxide-independent character of hydroxyl radical production. Excess oxygen consumption suggests the generation of

products with a higher oxidation state of sulfur[4] and hydroxyl radicals are most likely produced from the simultaneous presence of reduced metal and hydrogen peroxide.

Examples of such oxidation products are the GSOH produced in reactions (5) and (11) as well as the reaction of thiyl radical with oxygen:[4,17,18]

$$GS^{\bullet} + O_2 \leftrightarrow GSOO^{\bullet}. \quad (14)$$

Glutathione peroxyl radical reacts with oxygen after isomerization into sulfonyl radical:

$$GSOO^{\bullet} \rightarrow GSO_2^{\bullet}. \quad (15)$$

$$GSO_2^{\bullet} + O_2 = GSO_2OO^{\bullet}. \quad (16)$$

with final production of glutathione sulfonic acid. Although we have no direct evidence for such highly peroxidized products, they may be inferred from the excess oxygen consumption during the second reaction phase.

As the free glutathione concentration decreases, a build up of hydrogen peroxide may occur since reaction (5) is slow. Thus, near the end of the stoichiometric glutathione oxidation the reactants may be dominated by a 1 : 1 complex of glutathione and copper, and peroxide. The peroxide may directly oxidize cuprous ion producing hydroxyl radical. Our proposed superoxide-independent mechanisms of peroxide and hydroxyl radical generation agrees with the data of Albro *et al.*[2] who detected only a slight inhibitory effect of superoxide dismutase on the accumulation of hydrogen peroxide in metal-catalyzed oxidation of glutathione.

The proposed mechanism of GSH oxidation confirms the possible dual biological role of this compound as an antioxidant and pro-oxidant.[12] The utilization of superoxide and hydrogen peroxide at high GSH concentration occurs with minimal generation of hydroxyl radicals, as it appears during the first reaction phase. The complete oxidation of glutathione in cellular systems is excluded because of their millimolar glutathione concentration, which is maintained by the enzyme system for GSSG reduction (glutathione reductase and NADPH, generated by pentose cycle).[23] This would prevent the appearance of the second reaction phase of glutathione oxidation except under conditions of severe oxidative stress.

GSH could also act as pro-oxidant at low concentration in the presence of copper. This may occur due to release of GSH from the cells under oxidative stress conditions[24] or viral infection.[25] With ceruloplasmin as a source of copper for thiol oxidation,[26] the glutathione autoxidation at these conditions can be extended to the second phase with production of ROS. This implicates a possible involvement of glutathione oxidation as a component of the oxidative stress damage.

CONCLUSION

The autoxidation of glutathione is catalyzed by copper, which forms a complex with two molecules of glutathione. The reaction has a complex kinetics, consisting of two phases. During the first phase the stoichiometric amount of oxygen is consumed without significant $^{\bullet}OH$ production. We propose two parallel mechanisms (superoxide-dependent and peroxide-dependent) for this reaction. In the second reaction phase the oxidation of a copper–glutathione complex occurs with consumption of excess oxygen at increased rate and with significant $^{\bullet}OH$ production. These results indicate that glutathione can act not only as an antioxidant, but also as prooxidant under certain conditions.

Acknowledgments

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