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Mechanism of Copper-Catalyzed Autoxidation of Cysteine

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The kinetics of copper-catalyzed autoxidation of cysteine and its derivatives were investigated using oxygen consumption, spectroscopy and hydroxyl radical detection by fluorescence of a coumarin probe. The process has complex two-phase kinetics. During the first phase a stoichiometric amount of oxygen (0.25 moles per mole of thiol) is consumed without production of hydroxyl radicals. In the second reaction phase excess oxygen is consumed in a hydrogen peroxide-mediated process with significant $^{\bullet}OH$ production. The reaction rate in the second phase is decreased for cysteine derivatives with a free aminogroup and increased for compounds with a modified aminogroup. The kinetic data suggest the catalytic action of copper in the form of a cysteine complex. The reaction mechanism consists of two simultaneous reactions (superoxide-dependent and peroxide-dependent) in the first phase, and peroxide-dependent in the second phase. The second reaction phase begins after oxidation of free thiol. This consists of a Fenton-type reaction between cuprous–cysteinyl complex and following oxidation of cysteinyl radical to sulfonate with the consumption of excessive oxygen and significant production of hydroxyl radicals.

Keywords: Cysteine, copper, hydroxyl radical, hydrogen peroxide

INTRODUCTION

Cysteine reacts with molecular oxygen in neutral and alkaline solutions forming cystine. Various aspects of the mechanism of this reaction (autoxidation) were discussed in numerous papers.[1–5] Cysteine autoxidation is catalyzed by transition metals (copper and iron) and leads to generation of several reactive intermediates: superoxide anion $O_2^{\bullet -}$,[1] hydrogen peroxide H_2O_2,[2] hydroxyl radical $^{\bullet}OH$[3] and cysteinyl radical $CysS^{\bullet}$.[4] The role of transition metal ion is generally considered in two main aspects. It acts as an electron carrier from thiol to oxygen and the catalyst of $^{\bullet}OH$ generation in Haber–Weiss reaction between superoxide and peroxide. However, cysteine is a chelator of iron[3] and copper.[4] This chelation suggests that the reaction catalyst is not the free metal ion, but its complex with cysteine. The location of catalyst and reactant in the same molecule suggests a possible involvement of metal ion not only in the initiation of reaction, but also in other steps of the process.

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Considering the chelation of copper we characterized the biphasic mechanisms of copper-catalyzed autoxidation of dithiothreitol[6] and glutathione.[7] The autoxidation of glutathione consists of two phases. The first one includes a classical stoichiometric oxidation of glutathione to glutathione disulfide by two simultaneous mechanisms (superoxide-dependent and peroxide-dependent). In the second reaction phase we detected the consumption of excessive oxygen and significant •OH production in the peroxide-dependent process. We consider that the mechanism of copper-catalyzed cysteine autoxidation also has to be reexamined concerning the interaction between catalyst and reagent.

In this work we investigated the copper-catalyzed autoxidation of cysteine and its derivatives. This reaction may have a practical application due to recent findings of high cysteine concentration in tumor cells.[8] It also can be related to neurotoxicity of cysteine, which may be mediated by products of its oxidation.[9]

The oxidation of cysteine derivatives also has to be considered in their application as radioprotectors.[2] To investigate the reaction kinetics and mechanism, we used the methods of fluorescence and oxygen consumption. Hydroxyl radical was detected by coumarin fluorescent probe. Superoxide dismutase (SOD) and catalase were used to determine the involvement of superoxide and hydrogen peroxide in the reaction. To characterize the role of cysteine functional groups in the process, we tested the compounds with blocked aminogroup (N-acetylcysteine), carboxygroup (cysteine ethyl ester) and absent carboxygroup (cysteamine). Considering the experimental results, we proposed the mechanism of copper-catalyzed autoxidation of cysteine derivatives.

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 $CuSO_4 \cdot 5H_2O$, which was recrystallized before use. Buffer solutions were prepared using Milli-Q water (18 MΩ cm) using 99.999% $Na_2HPO_4 \cdot 7H_2O$ and 99.999% H_3PO_4.

The reactions were performed at 37°C in sodium phosphate buffer, pH = 7.40. We used high 40 mM buffer concentration to prevent pH instability, because the oxidation of thiol is strongly pH-dependent.[2] Our preliminary experiments show, that in the presence of thiols this buffer does not precipitate copper at used concentration less then 100 µM. Since the concentration of buffer did not affect the reaction kinetics, our results can be applied to the physiological 5–10 mM phosphate concentration.

The rate of the reaction was measured by the consumption of oxygen. This method is preferable in comparison with spectroscopic detection by DTNB, since copper affects the results of thiol measurements.[6] 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).

Production of •OH was estimated by the fluorescent probe coumarin-3-carboxylic acid (CCA). This method is based on the detection of the fluorescence (excitation at 400 nm and emission at 450 nm) of 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA), which is one of the products of the reaction between CCA and •OH.[9] The method can be used for the detection of copper-mediated hydroxyl free radicals.[10] The advantages of the method are selectivity, high sensitivity and measurement of real-time kinetics of •OH production. Fluorescence measurements were accomplished using an SPF-500 spectrofluorometer (SLM Instrument Co, Urbana, IL, USA). The absorption spectra were detected using a DW-2000 spectrophotometer (SLM Instrument Co, Urbana, IL, USA). The absorbance at

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400–450 nm before and after the experiment did not exceed 0.05 for all the samples, which allowed us to avoid an inner filter effect.

The role of other reactive oxygen species (superoxide anion and hydrogen peroxide) in the reaction was determined using specific enzymes. SOD and catalase effects on the reaction kinetics were determined at high enzyme concentrations (up to 100 U/mL) due to competition between the enzyme and transition metals in the reaction with superoxide and peroxide at neutral pH.[11] This enzyme concentration, however, was low as compared with copper, allowing to prevent effect of metal binding.

RESULTS

The kinetics of copper-catalyzed autoxidation of cysteine and glutathione[7] are similar. In both cases it consists of two phases with different characteristics of oxygen consumption and •OH production. During the first phase oxygen is consumed with constant rate. The total oxygen consumption during this phase is in agreement with the stoichiometry of reaction

$$4CysSH + O_2 = 2(CysS)_2 + 2H_2O \quad (1)$$

The production of •OH radicals during the first reaction phase is low (Figure 1).

In the second reaction phase excess of oxygen over the quantity required by reaction (1) is consumed with decreased rate. It is accompanied by significant •OH production (Figure 1). The amount of excess oxygen consumption for 0.1 mM cysteine is about 60% as required by reaction (1).

To find the conditions under which the second reaction phase begins, we performed the reaction at comparable concentrations of reagents (0.1–0.2 mM cysteine and 0.05 mM Cu^{2+}). As it is shown on Figure 2, the consumption of oxygen during the first phase depends on copper concentration. The quantity of consumed oxygen is equal to $\frac{1}{4}$ of the excess of cysteine over copper. Subsequently, the second phase begins after oxidation of the free cysteine by Equation (1) at equal concentrations of unoxidized cysteine and copper.

The reaction rates in both phases were determined from the linear part of the oxygen

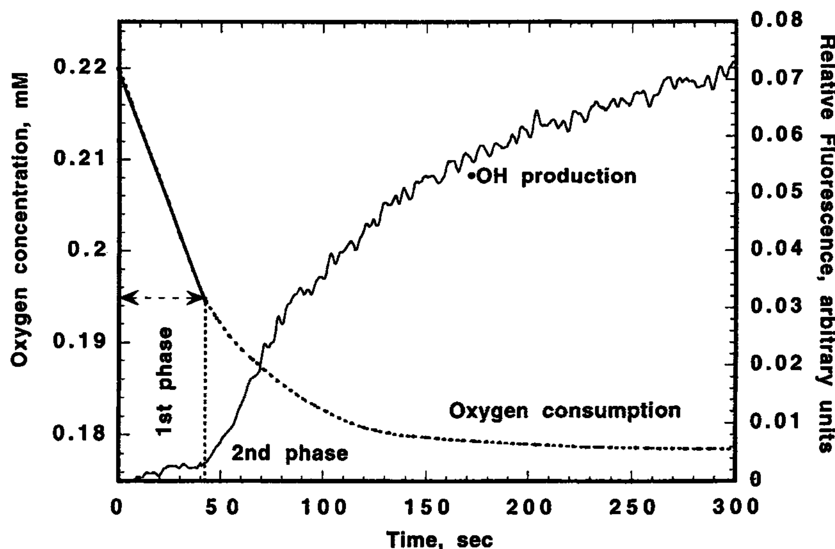

FIGURE 1 Kinetics of oxygen consumption and •OH production by 0.1 mM cysteine at 37°C in 40 mM phosphate buffer (pH = 7.40) catalyzed by 10 µM Cu^{2+}.

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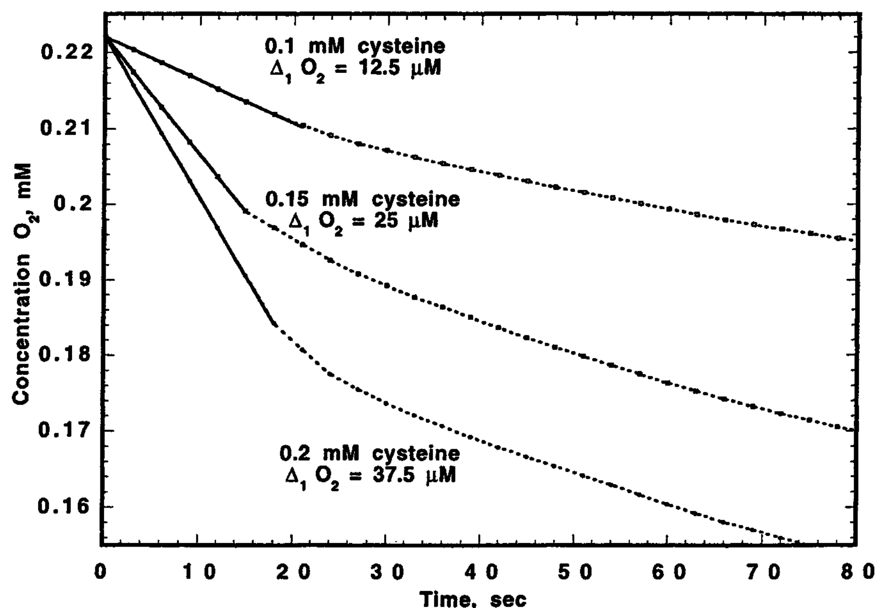

FIGURE 2 Effect of cysteine concentration on the oxygen consumption during the first reaction phase in the presence of 50 μM Cu^{2+}. The oxygen consumed during the first reaction phase oxidizes the excess of cysteine over copper with stoichiometry $RSH:O_2=4$.

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consumption curve. They depend on the concentration of cysteine and copper. The initial reaction rate in the first phase (V_1) in the presence of 5 μM Cu^{2+} is described by the equation

$$V_1 = 1.6 \cdot 10^{-6}[\text{Cys}]/(8.8 \cdot 10^{-4} + [\text{Cys}]) \quad (2)$$

The reaction rate in the second phase V_2 at 50 μM Cu^{2+} is equal:

$$V_2 = 7.9 \cdot 10^{-7}[\text{Cys}]/(9 \cdot 10^{-4} + [\text{Cys}]) \quad (3)$$

At a fixed cysteine concentration (0.1 mM), the effect of copper on the initial reaction rate is described by

$$V_1 = 1.51 \cdot 10^{-6}[\text{Cu}]/(5.12 \cdot 10^{-6} + [\text{Cu}]) \quad (4)$$

for the first phase, and

$$V_2 = 3.5 \cdot 10^{-7}[\text{Cu}]/(5.2 \cdot 10^{-6} + [\text{Cu}]) \quad (5)$$

for the second phase. Figure 3 shows an example of the concentration dependence of the reaction rate in inverted coordinates, which was used for the calculation of the coefficients in Equations (2)–(5). Equations (2)–(5) are typical for reactions with generation of intermediate compounds. The equation coefficients were used for the calculation of kinetic parameters, which are specified in the discussion and presented in Table I.

TABLE I Kinetic parameters of autoxidation of cysteine derivatives

Compound	K_1, M	K_2, M	k_1, s^{-1}	k_2, s^{-1}
Cysteine	$5.1 \cdot 10^{-6}$	$8.8 \cdot 10^{-4}$	0.32	0.075
Cysteine ethyl ester	$6.7 \cdot 10^{-5}$	$2.8 \cdot 10^{-4}$	0.37	0.12
Cysteamine	$1.7 \cdot 10^{-6}$	$2.5 \cdot 10^{-4}$	0.13	0.072
N-Acetylcysteine	$1.8 \cdot 10^{-5}$	$2.6 \cdot 10^{-4}$	0.013	0.14

The biphasic mechanism of cysteine autoxidation is similar to the reaction of oxygen with other thiols and infers the chelation of catalyst by the molecule of reagent.[6,7] To consider the possible involvement of carboxyl and aminogroup in the chelation of copper, we investigated the oxidation of the cysteine derivatives with modified aminogroup (N-acetylcysteine), modified carboxygroup (cysteine ethyl ester) and absent

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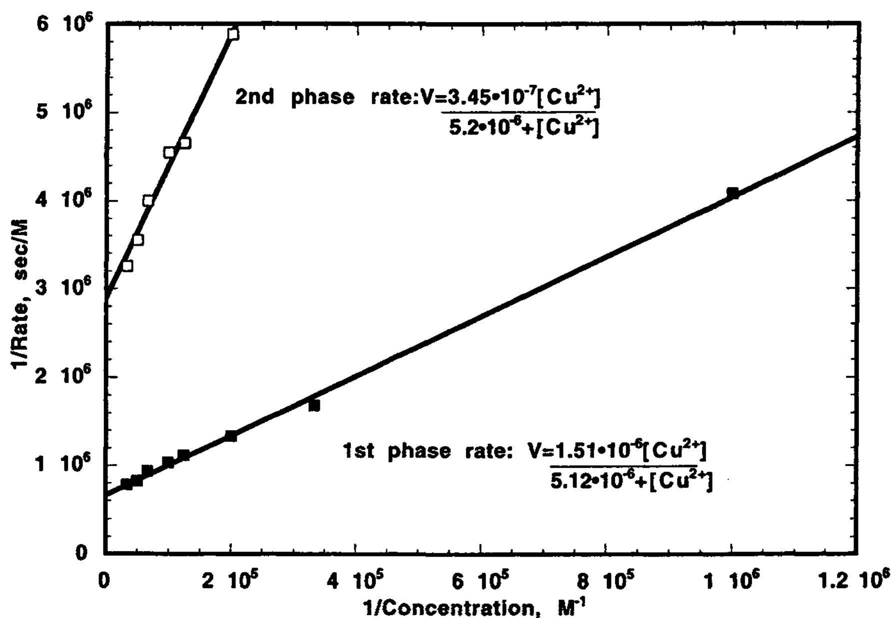

FIGURE 3 Dependence of the rate of oxygen consumption by 0.1 mM cysteine on the concentration of Cu^{2+}.

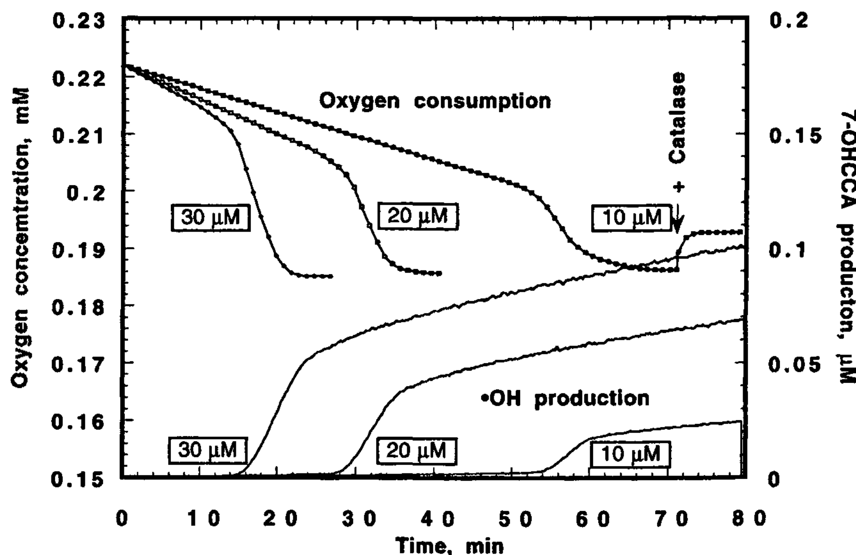

FIGURE 4 Oxygen consumption and •OH production by 0.1 mM N-acetylcysteine at different Cu^{2+} concentrations. Notice the beginning of the second reaction phase for Cu^{2+} : N-acetylcysteine 1 : 2. The effect addition of 100 U/mL catalase after the reaction is shown for 10 μM Cu^{2+}.

carboxygroup (cysteamine). All these compounds had biphasic kinetics of the oxidation which were similar to these described above. Figure 4 represents an example of the kinetics of oxygen consumption and •OH production by the cysteine derivative N-acetylcysteine. The excess oxygen consumption was not changed after modification of the cysteine molecule (cf. Figures 1 and 4). The reaction rates for the cysteine derivatives were described by equations similar to (2)–(5). The kinetic parameters of oxidation of the cysteine derivatives are presented in Table I.

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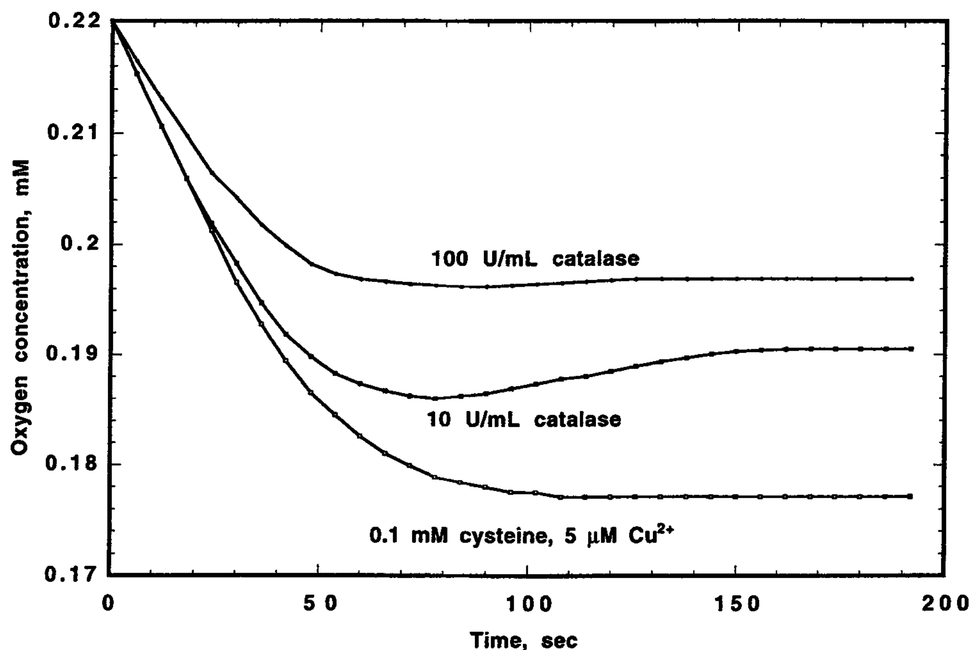

FIGURE 5 Effect of catalase on the oxygen consumption by 0.1 mM cysteine, catalyzed by 5 μM Cu^{2+}.

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Compounds with the free aminogroup (cysteine, cysteamine and cysteine ethyl ester) have a slower rate of oxygen consumption during the second phase in comparison with the first phase. The blockage of cysteine aminogroup in N-acetylcysteine significantly decreases the rate of oxygen consumption during the first phase, but does not affect the second reaction phase. It causes an acceleration of the oxygen consumption rate during the second reaction period (Figure 4). This type of kinetics was described earlier for autoxidation of glutathione.[7] Another major difference between cysteine and its derivatives is the concentration of the reagents required for the beginning of the second phase. The second reaction phase for cysteine derivatives begins when the concentration of unoxidized thiol is two-fold higher than copper in contrast with equimolar concentrations for cysteine. Figure 4 shows as an example this effect for N-acetylcysteine.

Effects of catalase and SOD during the first reaction phase were similar for cysteine and glutathione. Addition of SOD caused a 20% decrease of the reaction rate. Catalase decelerated the reaction about 30%. Because the oxidation of cysteine is significantly faster in comparison with glutathione, these effects can be clearly detected only at high enzyme concentration (Figure 5). We also characterized the effect of catalase on the reaction rate in the second phase. Low concentration of catalase (less than 30 U/mL) decreased the amount of excess oxygen consumption and caused a return of the oxygen into the system after the reaction (Figure 5). High catalase concentration (100 U/mL) completely prevented the excess oxygen consumption. Addition of catalase after the reaction completion caused a production of the part of excess oxygen (Figure 4). These results show, that hydrogen peroxide is one of the reaction products and plays an important role in the existence of the second reaction phase.

DISCUSSION

The mechanism of copper-catalyzed autoxidation of cysteine derivatives has common features with the process described for glutathione.[7]

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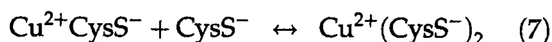
In the current work we performed a more detailed kinetic investigation and specified the role of hydrogen peroxide in the reaction mechanism.

We consider, that the reaction catalyst is a copper–thiol complex 1 : 2. The formation of this complex and its participation in copper-catalyzed cysteine oxidation was described by Cavallini *et al.*[5] The generation of the complex and intramolecular electron transfer are the rate-limiting steps of the process.

Two reactions are involved in the complex generation:

$$Cu^{2+} + CysS^- \leftrightarrow Cu^{2+}CysS^- \quad (6)$$



$$Cu^{2+}CysS^- + CysS^- \leftrightarrow Cu^{2+}(CysS^-)_2 \quad (7)$$

with equilibrium constants of complex dissociation:

$$K_1 = \frac{[Cu^{2+}][CysS^-]}{[Cu^{2+}CysS^-]} \quad (8)$$

$$K_2 = \frac{[Cu^{2+}CysS^-][CysS^-]}{[Cu^{2+}(CysS^-)_2]} \quad (9)$$

We presume that the rate-limiting step is the electron transfer from sulfur to copper, as it occurs during glutathione oxidation.[7] Because sulfur loses its negative charge and ability to bind copper, this reaction is irreversible:

$$Cu^{2+}(CysS^-)_2 \rightarrow Cu^+CysS^\bullet CysS^- \quad (10)$$

The initial reaction rate in the first phase is determined by reaction (10):

$$V_1 = k_1[Cu^{2+}(CysS^-)_2] \quad (11)$$

Total concentration of copper in the solution can be determined as

$$[Cu^{2+}] = [Cu^{2+}]_f + [Cu^{2+}CysS^-] + [Cu^{2+}(CysS^-)_2] \quad (12)$$

The concentration of free copper $[Cu^{2+}]_f$ can be found from (8):

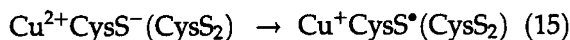
$$[Cu^{2+}]_f = \frac{K_1[Cu^{2+}CysS^-]}{[CysS^-]} \quad (13)$$

After the substitution of (13) in Equation (12), the value $[Cu^{2+}CysS^-]$ can be excluded from (9). It gives the reaction rate as

$$V_1 = \frac{k_1[Cu^{2+}][CysS^-]}{K_2(1 + K_1/[CysS^-]) + [CysS^-]} \quad (14)$$

At high cysteine concentration $K_1/[CysS^-] \ll 1$ can be neglected. Comparison of (14) and (2) gives the values $K_2 = 8.8 \cdot 10^{-4}$ M and $k_1 = 3.2 \cdot 10^{-1}\ s^{-1}$. Analogous calculations with exclusion of cysteine concentration in Equation (9) give a value $K_1 = 5.1 \cdot 10^{-6}$ M from Equation (4). Comparison of these results with literature data shows, that constants of cupric–cysteine complex dissociation are lower than published values for cupric–ammonia[12] $K_1 = 4.9 \cdot 10^{-5}$ and $K_2 = 2.1 \cdot 10^{-4}$. The stronger chelation of copper by cysteine molecule suggests a possible involvement of sulfur atom and carboxyl group in the complex formation.

In the second reaction phase, the values of constants K_1 and K_2 remain the same [cf. Equations (2) with (3) and (4) with (5)]. The rate-limiting constant in the second phase is about 4-fold lower [cf. Equations (4) and (5)]. The initiation of the second phase at equal concentration of cysteine and copper (Figure 2) suggests that the limiting step is the transfer of the electron in the complex with equal concentrations of copper and thiol. The same values of K_2 for both phases [Equations (2) and (3)] show that the stability of this complex is similar to copper–cysteine 1 : 2. Most probably, the copper atom is bound to cysteine and another ligand with similar binding constant, apparently cystine. In this presumption, the rate-limiting step in the second phase is the reaction



$$Cu^{2+}CysS^-(CysS_2) \rightarrow Cu^+CysS^\bullet(CysS_2) \quad (15)$$

with the constant $k_2 = 7.5 \cdot 10^{-2}\ s^{-1}$.

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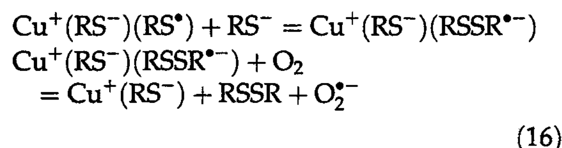
Analogous calculations were used for the determination of the equilibrium and rate constants for the derivatives of cysteine. The values of the constants are included in Table I. The results show the significance of both amino- and carboxy-group for the binding of the first thiol molecule to copper. Blocking or exclusion of one of these groups makes the complex less stable (cf. higher values of K_1 for cysteine ethyl ester and cysteamine in comparison with cysteine). The chemical structure of the cysteine derivative is not significant for the binding of the second thiol molecule (cf. K_2 values). It suggests the binding of second ligand through the thiol group.

The most important is the correlation between the rate-limiting constant of the first phase k_1 and the chemical structure of the compound. The blockage of free aminogroup in N-acetylcysteine significantly (25-fold) decreases the rate of its oxidation in comparison with cysteine. This effect shows that the close location of free aminogroup and thiol group is crucial for the thiol autoxidation. The aminogroup in the vicinity of the thiol group considerably increases the rate of thiol oxidation by site-specific binding of copper ion. In the absence of this aminogroup thiol molecules are stable to autoxidation, as it is seen for glutathione,[7] N-acetylcysteine and dithiothreitol.[6]

The constant rate of the second phase k_2 does not correlate with the structure of cysteine derivatives, suggesting the independent action of the thiol group in electron transfer to copper. It provides an unusual effect of the acceleration of oxygen consumption in the second phase for the compounds with blocked cysteine aminogroup, N-acetylcysteine and glutathione. On the other hand, k_2 correlates with the constant of complex dissociation K_1, showing the lower rate of the second phase for the compounds with more stable complexes (cysteine and cysteamine).

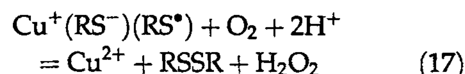
Effects of catalase and SOD allow us to clarify the mechanism of excessive oxygen consumption and $^{\bullet}OH$ production in the second reaction phase. The enzyme effects in the first reaction phase are similar to glutathione oxidation, suggesting the same mechanisms of oxygen consumption. According to our earlier studies,[7] the reaction is initiated by intramolecular electron transfer, described by the reaction (10). About 40% of oxygen is converted into superoxide anion in thiol-mediated reaction:



$$\begin{aligned} &Cu^+(RS^-)(RS^\bullet) + RS^- = Cu^+(RS^-)(RSSR^{\bullet-}) \\ &Cu^+(RS^-)(RSSR^{\bullet-}) + O_2 \\ &\quad = Cu^+(RS^-) + RSSR + O_2^{\bullet-} \end{aligned} \tag{16}$$

Superoxide is reduced by cuprous–thiol complex by two-electron mechanism without production of hydrogen peroxide and hydroxyl radical.[7]

The remaining 60% of oxygen is converted into hydrogen peroxide by two-electron reduction. This process is described by equation

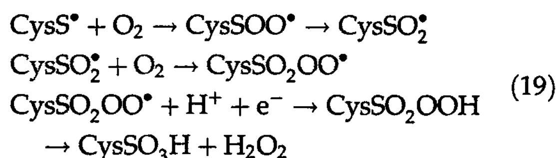


$$\begin{aligned} &Cu^+(RS^-)(RS^\bullet) + O_2 + 2H^+ \\ &\quad = Cu^{2+} + RSSR + H_2O_2 \end{aligned} \tag{17}$$

Hydrogen peroxide can be reduced by two molecules of free thiol without hydroxyl radical production:

$$\begin{aligned} H_2O_2 + RS^- &= RSOH + OH^- \\ RSOH + RS^- &= RSSR + OH^- \end{aligned} \tag{18}$$

Reactions (16)–(18) describe the mechanism of thiol oxidation in the first reaction phase. The mechanism of the second phase was explained as the reaction of thiol radicals with oxygen in the absence of free thiol in solution.[7] This is accompanied by consumption of excessive oxygen and production of hydrogen peroxide by the sequence of the reactions, described previously:[7,13,14]



$$\begin{aligned} &CysS^\bullet + O_2 \rightarrow CysSOO^\bullet \rightarrow CysSO_2^\bullet \\ &CysSO_2^\bullet + O_2 \rightarrow CysSO_2OO^\bullet \\ &CysSO_2OO^\bullet + H^+ + e^- \rightarrow CysSO_2OOH \\ &\quad \rightarrow CysSO_3H + H_2O_2 \end{aligned} \tag{19}$$

The results of current investigation support the assumption that the absence of free thiol is the condition for initiation of the second reaction phase and production of hydroxyl radicals. During the cysteine autoxidation, the second phase

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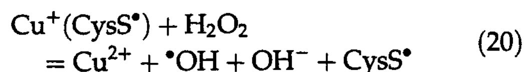
begins at equal concentrations of free cysteine and copper (Figure 2). The second phase for cysteine derivatives begins at two-fold excess of unoxidized thiol (Figure 4). This difference between cysteine and its derivatives can be explained from the ligand structure. As shown above, the cysteine molecule has three binding sites (thiol, amine and carboxyl). Binding of cysteine to four-coordinating copper ion necessarily involves the chelation of thiol group for an equimolar complex, but this group can be unbound for the second cysteine molecule. Subsequently, free thiol is absent in the solution only for the equimolar copper–cysteine complex. In the case of cysteine derivatives one of the groups (amine or carboxyl) is blocked, and the molecules have two binding sites. Binding of two ligand molecules to copper ion essentially involves the interaction through both thiol group. According to this, free thiol is absent in the solution for the complex of copper with two molecules of cysteine derivatives.

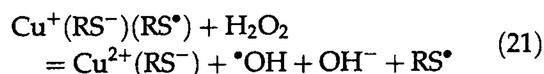
According to the mechanism of the first phase, free thiol is involved in reactions (16) and (18). An absence of free thiol in solution blocks the superoxide generation by reaction (16) and hydrogen peroxide utilization by reaction (18). However, the generation of hydrogen peroxide in reaction (17) does not require free thiol. Subsequently, an absence of free thiol in the second reaction phase should cause the accumulation of hydrogen peroxide in solution. Our current results support this presumption. Addition of catalase after the reaction completion causes production of oxygen (Figure 4), showing the generation of hydrogen peroxide after the second reaction phase. Addition of catalase in the middle of the first phase caused the decreasing of the reaction rate without oxygen production, suggesting the action of H_2O_2 only as an intermediate compound in the first phase. A high concentration of catalase completely blocks the second phase of the reaction (Figure 5). These data show the H_2O_2 generation in the second reaction phase. The prevention of second reaction phase by H_2O_2 elimination suggests hydrogen peroxide as a precursor of hydroxyl radical.

Effects of catalase (Figures 4 and 5) and cysteine concentration (Figure 2) suggest that the second phase is initiated by the reaction between hydrogen peroxide and cuprous–cysteinyl complex:



$$Cu^{+}(CysS^{\bullet}) + H_2O_2 = Cu^{2+} + {}^{\bullet}OH + OH^{-} + CysS^{\bullet} \quad (20)$$

For the cysteine derivatives the second phase begins at 2-fold excess of thiol over copper (Figure 4). In this case the product of this reaction is equimolar complex:



$$Cu^{+}(RS^{-})(RS^{\bullet}) + H_2O_2 = Cu^{2+}(RS^{-}) + {}^{\bullet}OH + OH^{-} + RS^{\bullet} \quad (21)$$

which can reenter the reaction (20) after the intramolecular electron transfer by reaction (15).

The reaction (20), (21) can be considered as the Fenton-type reaction between thiol-chelated cuprous and hydrogen peroxide. This process is the mechanism of $^{\bullet}OH$ production in the second reaction phase (Figures 1 and 4). It also causes a significant production of cysteinyl radical after the oxidation of free thiol. Cysteinyl radical consumes an additional amount of oxygen, producing cysteine sulfonic acid by the sequence of the reactions (19) and reproducing the same amount of hydrogen peroxide.

Slow decomposition of the hydrogen peroxide after the reaction causes the residual production of $^{\bullet}OH$ after the completion of oxygen consumption in the second phase, as it is seen in Figure 4. High concentrations of catalase totally prevent reaction (17) and excludes the second reaction phase, excessive oxygen consumption and $^{\bullet}OH$ production.

The proposed reaction mechanism allows the calculation of the amount of excess oxygen consumption and peroxide production. Oxidation of 0.1 mM of thiol in the first phase requires 0.025 mM O_2. The production of hydrogen peroxide by reaction (17) is equal to 60% of consumed oxygen, or 0.015 mM H_2O_2. Reaction (20) generates the same amount of cysteinyl radical. For complete conversion into cysteine sulfonic acid by reactions (19), cysteinyl radical requires 1.5 molecule of oxygen, or 0.0225 mM O_2. It suggests 1.9-fold

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excess of oxygen consumption during the whole process. Copper-catalyzed autoxidation of 0.1 mM thiol gives 1.6 oxygen excess (see Figures 1, 2, 4, 5) due to partial involvement of hydrogen peroxide into reaction (18). The amount of hydrogen peroxide after the reaction completion should remain constant. Addition of catalase after the reaction causes the production of 6.5 μM oxygen (Figure 4), indicating the residual 0.013 mM of H_2O_2.

The amount of excessive oxygen consumption is determined by production of hydrogen peroxide and does not depend on the type of thiol and copper concentration. Because of this, all the examined cysteine derivatives consume the same amount of oxygen, despite the different rates of oxidation during the first and second reaction phases. An increase of copper concentration does not affect total oxygen consumption, although it changes both the rate and amount of oxygen consumption during the first phase.

The reaction mechanism also explains the independence of the second phase rate constant k_2 from the thiol structure and its correlation with K_1. The structure of the organic part of the radical has a low effect on the reactivity of thiol radical with oxygen. However, higher stability of complex (lower K_1) favors the annihilation of hydroxyl and thiol radical immediately after their generation in reaction (20). It decreases the rate of thiol radical-driven second phase for cysteine and cysteamine in comparison with cysteine derivatives.

Reactions (20), (21) are the explanation of the existence and particularities of the second reaction phase. It is the main adjustment of the mechanism of copper-catalyzed autoxidation of

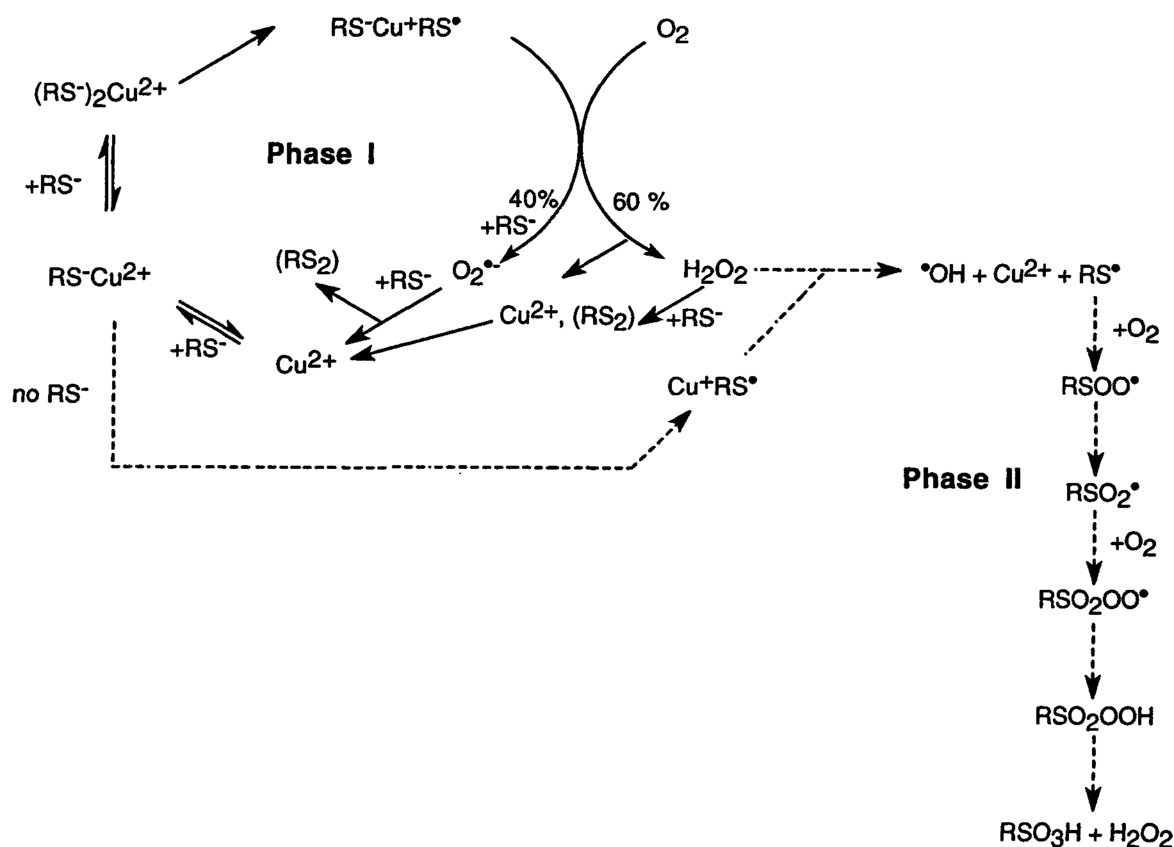

FIGURE 6 Mechanism of copper-catalyzed cysteine autoxidation; 1st phase – solid lines, 2nd phase – broken lines.

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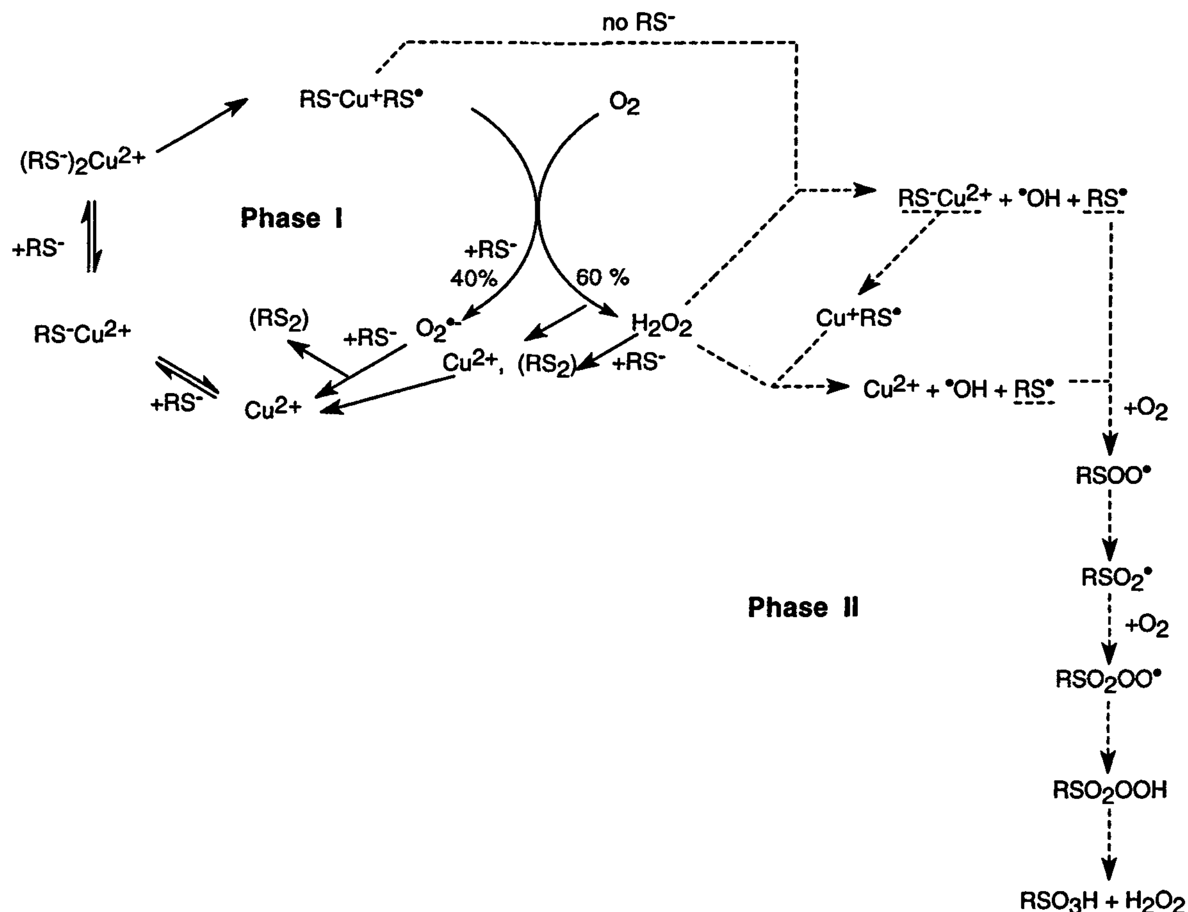

FIGURE 7 Mechanism of copper-catalyzed autoxidation of cysteine derivatives; 1st phase – solid lines, 2nd phase – broken lines.

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cysteine derivatives, proposed in our earlier work.[7] The scheme of the reaction mechanism for cysteine autoxidation is shown in Figure 6. The autoxidation of cysteine derivatives has different conditions of the second phase initiation, which is shown in Figure 7.

CONCLUSION

The kinetics of copper-catalyzed autoxidation of cysteine derivatives is a two-phase process with involvement of several reactions. Such a complexity emerges due to the interaction between catalyst and reagent during the reaction. The possibility of such interaction has to be considered at the investigation of various biological thiol redox processes, including radiation-related radical processes and the action of radioprotectors.

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