Kinetic Studies on the Role of Dioxygen in the Copper-Catalyzed Autoxidation of Cysteine

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Kinetic studies on a role of dioxygen in the copper-catalyzed autoxidation of cysteine in glycylglycine–phosphate buffers have been carried out. The rate of autoxidation was obtained by measuring the consumption of cysteine. It has been revealed that cysteine is oxidized by a "sequential mechanism". The reaction pathway can be shown by Eqs. 1, 2, 3, and 4; cysteine is oxidized not only by copper(II) species, but also by copper(I)–O_2 adducts:

$$\text{Cu(II)–L} + \text{CyS}^- \rightleftarrows \text{L–Cu(II)–CyS}^- \quad (1)$$

$$\text{L–Cu(II)–CyS}^- \rightarrow \text{Cu(I)–L} + \text{CyS}\cdot \quad (2)$$

$$\text{Cu(I)–L} + \text{O}_2 \rightleftarrows \text{L–Cu(I)–O}_2 \quad (3)$$

$$\text{L–Cu(I)–O}_2 + \text{CyS}^- + 2\text{H}^+ \rightarrow \text{Cu(II)–L} + \text{CyS}\cdot + \text{H}_2\text{O}_2 \quad (4)$$

Here, L represents ligands including cysteine. The oxidation step (4) catalyzed by the Cu(I)–O_2 species was proposed to be rate-determining.

Copper complexes, together with iron complexes, are the most widely studied redox catalysts.[1,2] In biological systems, copper-containing enzymes catalyze oxygen transport, electron transfer, oxidation, and oxygenation.[2] Those reactions, except for oxygen transport, involve an electron transfer from donor to acceptor. In the oxidase reaction, dioxygen functions as the electron acceptor, receiving either two or four electrons. The Cu(II) in the enzymes associates with the electron donor forming an intermediate, in which electrons are transferred from the donor to the Cu(II) to yield Cu(I) species. Subsequently, the electron is transferred to dioxygen from the Cu(I) which is concurrently reoxidized to Cu(II). Thus, the copper is considered to be turned over between the two oxidation states, and the dioxygen is reduced to either H_2O_2 or H_2O. We have been studying, from the viewpoint of bioinorganic chemistry, the autoxidation of aminothiols catalyzed by copper complexes, in which the thiols function as the electron donor.

Copper ions effectively catalyze the autoxidation of sulfanyl compounds, such as cysteine and other related substrates,[3] and various amounts of H_2O_2 are produced.[4] Since H_2O_2 upon producing was consumed to oxidize the sulfanyl compounds, the stoichiometry between the formation of H_2O_2 and the consumption of sulfanyl compounds had remained uncertain. In a previous paper we reported that principally one molar H_2O_2 was formed upon the oxidation of two molars cysteine.[5] Thus, the primary process can be written as follows;

$$2\text{CySH} + \text{O}_2 \rightarrow \text{CyS–SCy} + \text{H}_2\text{O}_2,$$

where CySH and CyS–SCy denote cysteine and cystine, respectively.

Since both Cu(I) and Cu(II) ions possess strong affinities for cysteine,[6] the reaction mechanism appears to be complicate.[7] As stated above, the turnover of copper between the oxidized and reduced states is considered to mediate the electron transfer from cysteine to dioxygen. Thus, the role of dioxygen has been assumed to be related to the reactivation of the catalytically inactive Cu(I) species. In contrast, it has been reported that ascorbic acid is oxidized by a direct attack of dioxygen on the Cu(II)-substrate complex.[8] Assuming that ascorbate, or electron donors like aminothiols, is oxidized by this mechanism, an oxido-reductive turnover of the copper ion does not occur; the copper must be in a single oxidation state during the course of reaction. Thus, the role of dioxygen in the autoxidation by redox-active metal ions and complexes has hitherto been ambiguous. In order to verify the role of dioxygen in autoxidation, we attempted to reexamine the copper-catalyzed autoxidation of cysteine. In this study it was revealed that cysteine was oxidized by a "sequential mechanism".[9] Cysteine was oxidized not only by the Cu(II) species, but also by the Cu(I)–O_2 adducts.

Experimental

Materials. A stock solution of Cu(II) was prepared

from copper chips of 99.999% purity (Kishida Chem. & Co., Tokyo). The chips, which were washed successively with alcohol and ether, were dissolved in a small amount of concd HNO_3. Thus-prepared Cu(II) solution was diluted to the desired concentration with purified water, which was deionized and distilled three times with all glass apparatus; the first distillation was from alkaline permanganate. The concentration of the Cu(II) solution was checked by titration with a standardized 0.01 M EDTA (1 M=1 mol dm^{-3}) using murexide as an indicator.[10] Solutions of cysteine (Sigma Chem. Co., Mo.) and other related sulfanyl compounds (Sigma Chem. Co., Mo.) were freshly prepared just prior to use, and determined by spectrophotometry using 6,6′-dinitro-3,3′-dithiodibenzoic acid (Wako Pure Chem. Ind., Osaka, abbreviated as DTNB).[11] Superoxide dismutase, abbreviated as SOD, was a commercially available product (Truett Lab., Tx., Lot 579180); and its specific activity was 3000+U/mg, as assayed by the method of McCord and Fridovich.[12]

Kinetic Procedure by Conventional Spectrophotometry. The oxidation was conducted in a 100 ml water-jacketted beaker under aerobic (O_2/N_2 purged) and anaerobic (N_2 purged) conditions over the pH range from 6.2 to 7.8 at 20 °C. A Cu(II) solution of either 1.38×10^{-6} or 1.80×10^{-6} M in 0.015 M glycylglycine–0.01 M phosphate buffer ($I=0.1$ M with $NaNO_3$) was thermostatted under appropriate circumstances for 20 min prior to kinetic runs. The reaction was started by adding the cysteine solution last. During the reaction, an O_2/N_2 gas mixture was introduced at a rate of 100 ml min^{-1}, and vigorous agitation by a magnetic stirrer was continued. Aliquots were withdrawn at periodic intervals from the reaction mixtures and were used to assay either cysteine or hydrogen peroxode by spectrophotometry. Determinations of cysteine and H_2O_2 were carried out by using DTNB[11] and $TiCl_4$,[13] respectively, on a Hitachi 101 spectrophotometer. The dioxygen dissolved in the medium was determined both before and after kinetic runs with a Beckman Fieldlab oxygen analyzer, which had been calibrated against air-saturated water.

An absorbance-time plot gave a straight line for over a 50% reaction. The rate in the steady state (v) was obtained from the slope, and kinetic constants, including V and K, were determined by Lineweaver–Burke analyses.

Observation by Stopped-Flow Spectrophotometry. The association of Cu(II) with the substrate and reduction of the copper coupled with cysteine oxidation were observed under both aerobic and anaerobic conditions by using a stopped-flow technique. Solutions of 5.01×10^{-5} M Cu(II) and 4.00×10^{-3} M cysteine at pH 7.4 in 0.015 M glycylglycine–0.01 M phosphate buffer, which had been thermostatted at 20 °C under either aerobic (O_2 purged) or anaerobic (N_2 purged) conditions for 20 min, were mixed under O_2 or N_2 at 8 kg cm^{-2}, and subsequent changes in the absorbance at 335 and 385 nm were recorded on a Union RA-401 stopped-flow spectrophotometer. The dead time of the stopped-flow apparatus was 1.2 ms using a 5 mm quartz cell.

Results

The roles of dioxygen in the autoxidation have not been well understood, though it is known that the copper ion and complexes effectively catalyze the autoxidation of cysteine, and that the rate depends on the concentrations of both dioxygen and cysteine, as well as that of copper. A double-reciprocal plot of the rate against the concentration of the dissolved dioxygen, $[O_2]$, gave a straight line, which indicated that cysteine was autoxidized with the saturation kinetics. The double-reciprocal plots at different pHs from 6.5 to 7.4 are shown in Fig. 1, where [CySH] is fixed at 2.00×10^{-3} M. The ordinate intercept represents $1/V$, where V denotes the autoxidation rate at infinite concentration of dioxygen. The intercept on the abscissa represents $-1/K$, where K corresponds to the concentration of dioxygen at $v=V/2$, indicating the apparent dissociation constant of the Cu(I)–dioxygen adducts. In the enzyme reaction K is called the Michaelis constant. The values of V and K at pH 7.10 and at $[CySH]=2.00\times10^{-3}$ M were given as 6.7×10^{-6} M s^{-1} and 3.80×10^{-3} M, respectively. Thus, the kinetic examination clearly indicated the association of dioxygen with the catalyst. The overall reaction obeys the Michaelis–Menten-type mechanism. Those linear plots were observed below pH 7.5. On the contrary; the rates of the autoxidation of glutathione and homocysteine were found to be linearly proportional to $[O_2]$, as observed in ascorbate autoxidation. Those differences in the kinetic behaviors might have resulted from the differences in the coordination mode between the substrate and the copper, and in the ability of association between the copper and dioxygen. Cysteine is a bidentate ligand with N and S donors forming thermodynamically stable, but kineti-

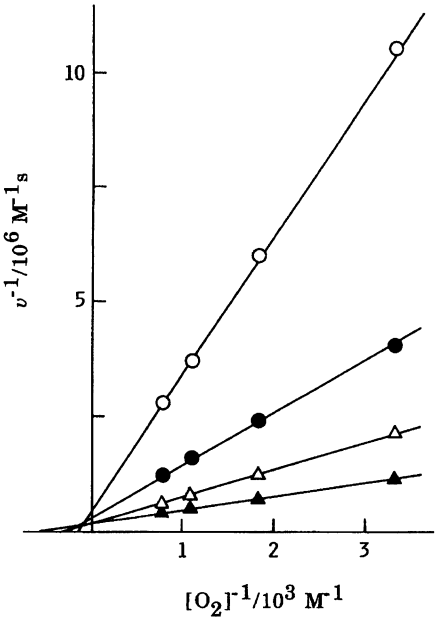


Fig. 1. Double reciprocal plot of the autoxidation rate against the concentration of dissolved dioxygen; pH 6.55 (○), pH 6.85 (●), pH 7.10 (△), pH 7.40 (▲). $[CySH]_0=2.00\times10^{-3}$ M, $[Cu(II)]_0=1.36\times10^{-6}$ M, $T=20$ °C, $I=0.1$ M ($NaNO_3$).

cally labile, complexes with both Cu(II) and Cu(I),[6,14] while glutathione and probably homocysteine are monodentate with an S donor forming thermodynamically rather unstable complexes.[15] A detailed discussion will be presented elsewhere.

Dioxygen was reduced to H_2O_2, which could be detected by a reaction with $TiCl_4$. During the initial stage of the reaction, the rate of cysteine consumption was equal to that of the peroxide formation, as shown in Fig. 2; the consumption of two molar cysteine was coupled with the formation of one molar H_2O_2. Thus, cysteine was apparently autoxidized by a two-equivalents electron transfer, and the Cu(II) species was reduced. However, since the peroxide produced intermediately was used for the oxidation of cysteine, the stoichiometry mentioned above was not satisfied as the oxidation proceeded. Under the anaerobic condition, hydrogen peroxide oxidized cysteine with the stoichiometric relation; one molar H_2O_2 oxidized two molars cysteine, as shown in Fig. 3. If the reoxidation of the Cu(I) species involves two one-electron transfer steps, a superoxide ion would be produced. Superoxide has been proposed as a candidate for reactive species,[16] and the enzyme SOD disproportionates superoxide ion to dioxygen and hydrogen peroxide.[11] The addition of varying concentrations of SOD did not modify the rate of autoxidation, as shown in Table 1. Neither acceleration nor inhibition was observed. In addition, the superoxide ion could not be detected by colorimetry with epinephrine, which has been shown to undergo one-electron oxidation by O_2^-, yielding a pigment, called adrenochrome,

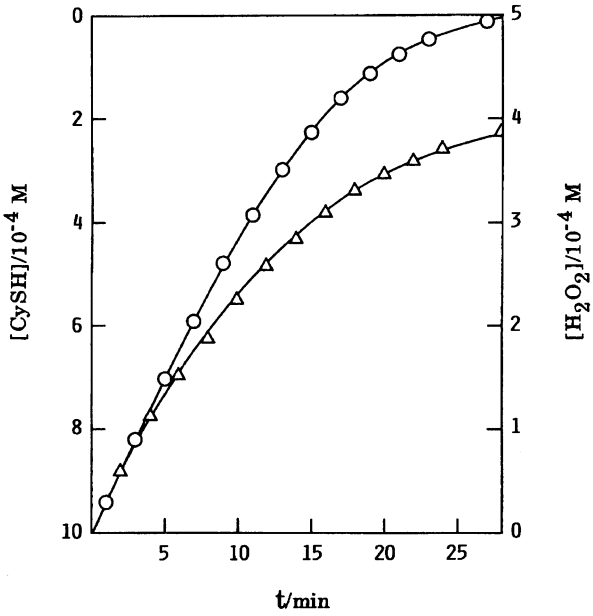


Fig. 2. Time course for the consumption of cysteine and the production of hydrogen peroxide under an aerobic condition; CySH (○), H_2O_2 (△) $[CySH]_0=1.00\times10^{-3}$ M, $[Cu(II)]_0=1.36\times10^{-6}$ M, pH=7.1, $T=20$ °C, $I=0.1$ M ($NaNO_3$).

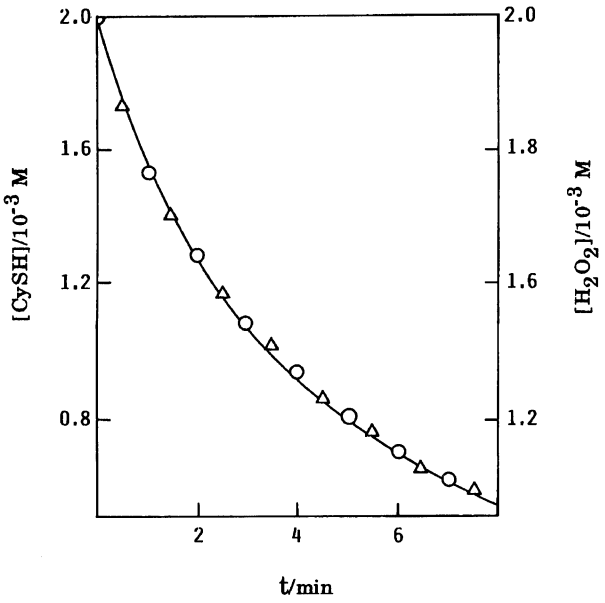


Fig. 3. Time course for the consumption of cysteine and hydrogen peroxide under nitrogen; CySH (○), H_2O_2 (△) $[CySH]_0=2.00\times10^{-3}$ M, $[H_2O_2]=2.00\times10^{-3}$ M, $[Cu(II)]_0=1.40\times10^{-6}$ M, pH=7.4, $T=20$ °C, $I=0.1$ M ($NaNO_3$).

Table 1. Effect of Superoxide Dismutase on the Copper-Catalyzed Autoxidation of Cysteine at pH 7.4

$[SOD]/10^{-7}$ M	$v/10^{-4}$ M min^{-1}	v/v_0
0	7.65	1.00
0.6	7.89	1.03
1.2	8.15	1.06
2.5	7.90	1.03
4.9	7.95	1.04

$[Cu(II)]=1.80\times10^{-6}$ M, $[CySH]_0=4.88\times10^{-3}$ M.

with $\lambda_{max}=485$ nm.[17] Those findings suggest that the superoxide ion is not generated and, even if produced transiently, it does not function as an oxidant for the autoxidation of cysteine.

We previously reported that the pH-rate profile for the copper-catalyzed autoxidation displayed a bell-shaped curve with a maximum at pH 7.4.[5] The pH profile for the kinetic parameters, including V and K, were examined again. The pH profiles of V and K are shown in Fig. 4. Both the pH–V and pH–K plots show bell-shaped curves. The V reached a maximum at pH 7.1—7.2, decreasing dramatically thereafter, while the maximum for K was observed at pH 6.6. This indicated that the ability of binding between the copper and dioxygen became minimized at around pH 6.6, but that the autoxidation rate increased above this pH. The parameters could not be determined in the pH range above 7.4, because the double-reciprocal plot did not give a straight line, as stated later.

The rate of autoxidation depended on the concentration of cysteine. However, since cysteine has strong

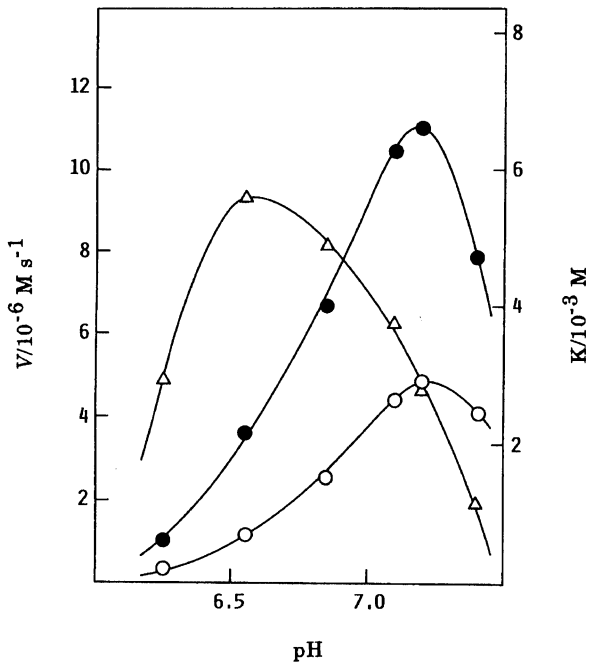


Fig. 4. pH Dependence of the kinetic parameters for the autoxidation of cysteine; V_{max}: $[CySH]_0 = 1.00\times10^{-3}$ M (○), $[CySH]_0 = 4.00\times10^{-3}$ M (●), $K_{0.5}$: (△).

affinities for both Cu(I) and Cu(II),[6,14] the rate-[CySH] profile appeared to be complicated. Firstly, the double-reciprocal plots did not give straight lines. Secondly, when the supply of dioxygen was insufficient above pH 7.5, the rate which initially increased decreased with increasing [CySH], as shown in Fig. 5. Increasing the concentrations of CySH over 2×10^{-3} M apparently retarded its oxidation. On the contrary, the autoxidation rate of glutathione was linearly proportional to the substrate concentrations, though the reaction was slow. Probably, cysteine functions not only as a substrate, but also as an inhibitor.

Discussion

Cu(II) complexes associate successively with cysteine and other related sulfanyl compounds to form transients, as shown in Eqs. 1a and 1b:

$$\text{Cu(II)–L} + \text{CyS}^- \rightleftarrows \text{L–Cu(II)–CyS}^- \quad (1a)$$

$$\text{L–Cu(II)–CyS}^- + \text{CyS}^- \rightleftarrows {}^-\text{CyS–Cu(II)–CyS}^- + \text{L} \quad (1b)$$

The ternary complex, L–Cu(II)–SCy$^-$, has been characterized by stopped-flow spectroscopic methods, where L stands for peptides[18] or polyamino-polycarboxylates.[19] Cu(II) in a neutral glycylglycine solution is known to coordinate with the peptide, forming Cu(II)(H_{-1}GlyGly), in which the peptide bond is deprotonated (Chart 1). The reaction (1a) was very fast, so that $k_{(1a)+}$, which was greater than 10^7 $M^{-1}s^{-1}$, could not be accurately determined by the conventional stopped-flow technique. The rate of the backward reaction in Eq. 1a was negligibly small, as compared with that of the forward reaction. In the second reaction, the rate constant ($k_{(1b)+}$) and the binding constant ($\log K_{(1b)}$) were 3.1×10^3 $M^{-1}s^{-1}$ and 3.15 at pH 9.5, respectively.[20] Then, in the presence of a large excess of CySH, the L–Cu(II)–CyS$^-$ complex upon forming spontaneously reacted with another CyS$^-$ to yield a binary complex, Cu(II)$(CyS^-)_2$, which was the main species

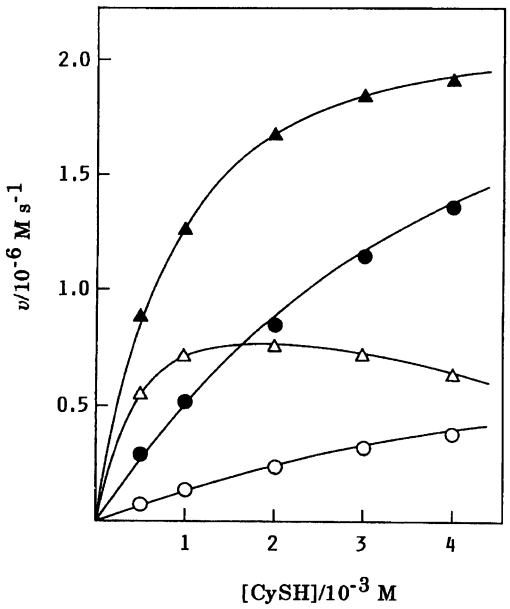
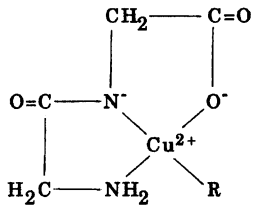
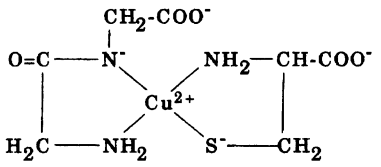


Fig. 5. Dependence of autoxidation rate on the concentrations of cysteine; pH 6.85: $[O_2] = 3.00\times10^{-4}$ M (○), $[O_2] = 1.26\times10^{-3}$ M (●), pH 7.75: $[O_2] = 3.00\times10^{-4}$ M (△), $[O_2] = 1.26\times10^{-3}$ M (▲).



Cu(II)(H_{-1}GlyGly)

R = H_2O, OH^- or HPO_4^{2-}



(CyS$^-$)Cu(II)(H_{-1}GlyGly)

Chart 1.

for Cu(II), and would function as a reaction intermediate.

Cu(II) was found to be reduced by cysteine under both aerobic and anaerobic conditions. Then, the first step involving electron transfer in the intermediates can be shown to be

$$\text{L–Cu(II)–CyS}^- \rightarrow \text{Cu(I)–L} + \text{CyS}\cdot, \quad (2)$$

where L denotes ligands including cysteine, and CyS^- and CyS· represent the thiolate ion and the sulfanyl radical, respectively. The coordination modes of Cu(II)–L, Cu(I)–L, or both, are expected to control the oxidation rate. The autoxidation of CySH catalyzed by copper complexes of polyamino-polycarboxylate was shown to depend on the stability constants of the Cu(II) complexes.[21] The sulfanyl radical, which could not be detected, would be rapidly combined to yield cystine.[22]

A subsequent reaction may be the oxygenation of Cu(I) species,

$$\text{Cu(I)–L} + \text{O}_2 \rightleftarrows \text{L–Cu(I)–O}_2. \quad (3)$$

The dioxygen binding of Cu(I) has been considered to be quasireversible. The equilibrium and rate constants for the L–Cu(II)–O_2 formation were estimated to be 27 M^{-1} and 9×10^5 $M^{-1}\,s^{-1}$, respectively, and those for the L–Cu(I)–O_2–Cu(II)–L formation from L–Cu(I)–O_2 to be 8.5×10^5 M^{-1} and 2.5×10^5 $M^{-1}\,s^{-1}$, respectively, at −50 °C in 50%/DMF, where L represents tris[(2-pyridyl)methyl]amine.[23] Both of the equilibrium constants strongly decrease with increasing temperature.[23] This indicates that the L–Cu(I)–O_2 adduct upon producing reacts promptly with other reductants, Cu(I)–L, or probably CyS^-. Thus, both the kinetic and thermodynamic data could be accounted for by assuming the formation of oxygen-adducts.[24] Then, concerning the role of dioxygen in the autoxidation, two kinds of mechanism expressed by the following equations:

$$\text{L–Cu(I)–O}_2 + \text{CyS}^- + 2\text{H}^+ \rightarrow \text{Cu(II)–L} + \text{CyS}\cdot + \text{H}_2\text{O}_2 \quad (4a)$$

and

$$\text{L–Cu(I)–O}_2 + \text{Cu(I)–L} + 2\text{H}^+ \rightarrow 2\text{Cu(II)–L} + \text{H}_2\text{O}_2 \quad (4b)$$

Both mechanisms involve a two-electron transfer. Reaction (4b) would involve L–Cu(I)–O_2–Cu(I)–L as an intermediate. However, since dioxygen reactions of Cu(I) have been shown to be first-order in Cu(I) and in O_2,[25] the reaction could be treated kinetically as

$$\text{L–Cu(I)–O}_2 \rightarrow \text{Cu(II)–L} + \text{O}_2^-. \quad (4c)$$

If the reaction can be elucidated by the pathway, namely a "sequential mechanism", shown by Eqs. 1, 2, 3, and 4a, where the dioxygen-adduct directly attacks on the substrate, the rate expression is given by

$$v = k_2[\text{Cu(II)–CyS}^-] + k_{4a}[\text{Cu(I)–O}_2][\text{CyS}^-]. \quad (5)$$

For simplicity, Cu(I)–L and Cu(II)–L are hereafter abbreviated as Cu(I) and Cu(II), respectively.

Using the steady state approximation, the concentrations of the intermediates are represented by

$$[\text{Cu(II)–CyS}^-] = \{k_1/(k_{-1}+k_2)\}[\text{Cu(II)}][\text{CyS}^-] \quad (6)$$

and

$$[\text{Cu(I)–O}_2] = \{k_3/(k_{-3}+k_{4a}[\text{CyS}^-])\}[\text{Cu(I)}][\text{O}_2]. \quad (7)$$

Applying this approximation to the concentration of Cu(II), we obtain

$$k_{4a}[\text{Cu(I)–O}_2] + k_{-1}[\text{Cu(II)–CyS}^-] - k_1[\text{Cu(II)}][\text{CyS}^-] = 0. \quad (8)$$

Then, Eq. 7 may be rewritten as

$$[\text{Cu(I)–O}_2] = \{k_1/(k_{-1}+k_2)\}(k_2/k_{4a})[\text{Cu(II)}]. \quad (9)$$

From Eqs. 6 and 9, we obtain

$$[\text{Cu(II)–CyS}^-]/[\text{Cu(I)–O}_2] = (k_{4a}/k_2)[\text{CyS}^-]. \quad (10)$$

It is indicated that molar ratio of the active species in the steady state is related to the ratio of the rate constants and $[CyS^-]$.

The rate expression (5) can be rearranged to

$$V_{max}/v = 1 + (K_{1-2}/[\text{CyS}^-]) + (k_2/k_{4a}[\text{CyS}^-])\{1 + (K_{3-4a}/[\text{O}_2])\}, \quad (11)$$

where V_{max}, K_{1-2} and K_{3-4a} represent $(k_2+k_{4a})[Cu]_0$, $(k_{-1}+k_2)/k_1$ and $(k_{-3}+k_{4a}[CyS^-])/k_3$, respectively. The term $[Cu]_0$ denotes the total concentrations of copper shown in

$$[\text{Cu}]_0 = [\text{Cu(I)}] + [\text{Cu(I)–O}_2] + [\text{Cu(II)}] + [\text{Cu(II)–CyS}^-]. \quad (12)$$

If the autoxidation can be elucidated by the pathway, namely a "ping-pong mechanism", shown by Eqs. 1, 2, 3, and 4b, where dioxygen reoxidizes the Cu(I), but does not oxidize the substrate, the rate expression would be given by

$$v = k_2[\text{Cu(II)–CyS}^-]. \quad (13)$$

Using the steady state approximation, the concentrations of the intermediates are given by

$$[\text{Cu(II)–CyS}^-] = \{k_1/(k_{-1}+k_2)\}[\text{Cu(II)}][\text{CyS}^-] \quad (6)$$

and

$$[\text{Cu(I)O}_2] = \{k_3/(k_{-3}+k_{4b})\}[\text{Cu(I)}][\text{O}_2] = \{k_1/(k_{-1}+k_2)\}(k_2/k_{4b})[\text{Cu(II)}][\text{CyS}^-]. \quad (14)$$

The ratio of the intermediates is given in

$$[\text{Cu(II)–CyS}]/[\text{Cu(I)–O}_2] = (k_{4b}/k_2). \quad (15)$$

The rate expression (13) can be rearranged to

$$V_{max}/v=1+(K_{1-2}/[CyS^-])+(k_2/k_{4b})\{1+(K_{3-4A}/[O_2])\}, \quad (16)$$

where V_{max}, K_{1-2}, and K_{3-4b} represent $k_2[Cu]_0$, $(k_{-1}+k_2)/k_1$ and $(k_{-3}+k_{4b})/k_3$, respectively.

Experimentally, those two mechanisms are easily distinguished. By holding one of the substrates at a constant level and by varying the second, a family of lines in a reciprocal plot is given. Dioxygen was arranged as the variable substrate and cysteine as the changing fixed substrate. The lines intersect at a point on the abscissa, as shown in Fig. 6, which supports that the autoxidation can be explained by Eq. 13. If the pathway is elucidated by Eq. 16, a family of lines should be parallel.

The formation and subsequent decay of the transients during the course of oxidation is presented in Fig. 7. An intermediate produced first was the ternary complex, $(CyS^-)Cu(II)(H_{-1}GlyGly)$, with λ_{max} at 335 nm. The formation of the ternary complex was extremely fast, so that the reaction had been completed within the dead-time of the instrument, 1.2 ms, and thereby the kinetic traces were not recorded in Fig. 7. The increase for the initial 100 ms was due to the formation of $Cu(II)(CyS^-)_2$, with λ_{max} at 335 and 385 nm, from the ternary complex; the subsequent decrease was due to a reduction of the binary complex. It was indicated that the ligand-exchange to produce $Cu(II)$–$(CyS^-)_2$ was faster than its reduction. Under the aerobic condition at 8 kg cm^{-2} O_2, the reaction was equilibrated after 20 s from the start to reach a steady state, where

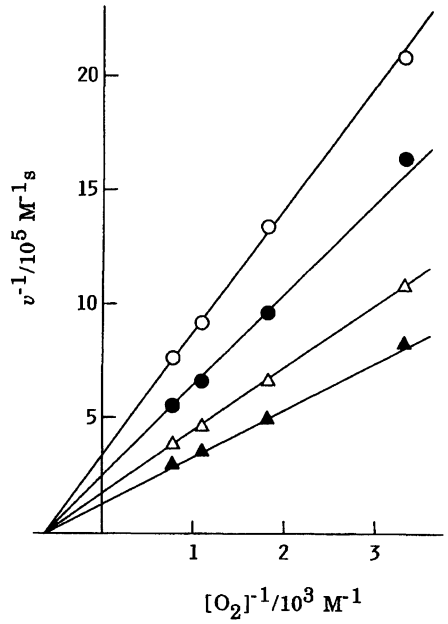


Fig. 6. Double reciprocal plot of the autoxidation rate against the concentration of dissolved dioxygen at pH 7.4; Cysteine is arranged as the changing fixed substrate. $[CySH]_0$: 5.00×10^{-4} M (○), 1.00×10^{-3} M (●), 2.00×10^{-3} M (△), 4.00×10^{-3} M (▲).

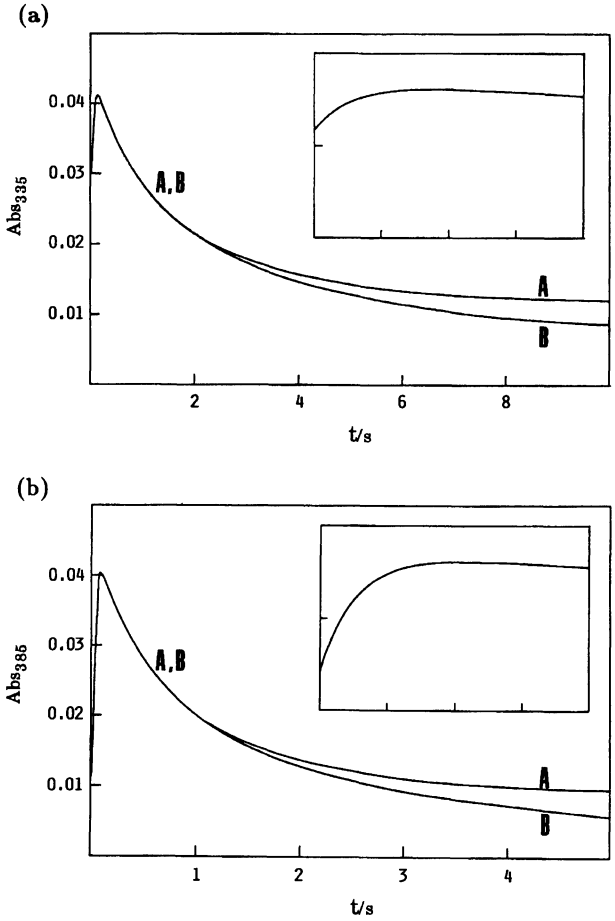


Fig. 7. Stopped-fow kinetic traces for the copper-catalyzed oxidation of cysteine under aerobic and anaerobic conditions; (a) λ=335 nm, (b) λ=385 nm, A; under 8 kg cm^{-1} O_2, B; under 8 kg cm^{-1} N_2, $[Cu(II)]_0=2.5\times10^{-5}$ M, $[CySH]_0=2.00\times10^{-3}$ M. Inserted are the traces for initial 200 ms.

the ratio of the concentrations of the Cu(I)-to-Cu(II) species was approximately 4/1. The rate of Cu(II)–L reduction (k_2) was faster, probably one order of magnitude, than that of Cu(I)–L reoxidation; k_{4a} and the ratio of [Cu(II)]/[Cu(I)] increased roughly with increasing $[CyS^-]$. In contrast, cysteine had been oxidized almost completely within 20 s under nitrogen. The main species for Cu(II) in the steady state was suggested to be $Cu(II)$–$(CyS^-)_2$ by spectroscopic measurements.

When $k_{-3}\gg k_{4a}[CyS]$, K_{3-4a} is reduced to (k_{-3}/k_3), that is the dissociation constant of L–Cu(I)–O_2. A relatively large value for K in cysteine autoxidation was suggestive of a weak association of L–Cu(I) and dioxygen.

The addition of a strong chelating agent for Cu(II) ion contributes to either a stimulation or inhibition of the oxidation, by which the reaction mechanism is modified. Ethylendiaminetetraacetate(EDTA) and *N*-(2-hydoxyethyl)-ethylenediamine-*N*,*N*′,*N*′-tetraacetate (HEDTA) stimulated autoxidation, and the family of lines in a reciprocal plot was parallel,[5] suggestive of the possibility

of a "ping-pong mechanism". The reaction mechanism would be determined by the reactivity of Cu(II)–O_2; this is an alternative of oxidizing the Cu(I) species or the sulfanyl compounds.

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