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Manometric Study of the Copper-Catalyzed Oxidation of Cysteine

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The copper–catalyzed oxidation of cysteine was followed from the consumption of oxygen using the conventional Warburg method and from the formation of hydrogen peroxide using the spectrophotometric method. The rate of oxygen uptake varied with pH and showed a maximum at pH 7.2. Hydrogen peroxide was produced progressively during the oxidation, which indicated the possibility of the successive four equivalent reduction of oxygen *via* hydrogen peroxide to water. The amounts of oxygen uptake and of hydrogen peroxide formed decreased with the increase of pH. From the peroxide formation and the oxygen consumption, the mode of the utilization of molecular oxygen was discussed. In the low pH region, the two equivalent redox–reaction, in which oxygen is reduced to hydrogen peroxide, may be predominant. As pH increases, hydrogen peroxide formed is utilized for the reoxidation of copper(I) ion, and thereupon the successivefour equivalent redox–reaction becomes predominant.

Recently, an enzyme catalyzing the oxidation of 2-mercaptoethylamine to hypotaurine was found to contain copper, iron and zinc ions.[2] Copper and iron ions in this enzyme might mediate the electron transfer between the substrate and oxygen molecules. The metal ion is turned over, during the course of the reaction, between the high– and low–valence states, and thereupon electron is transfered from the substrate to the high valence metal ion and from the low valence metal to oxygen molecule. The sulfhydryl group is autoxidized to disulfide, sulfinic and sulfonic acids. In the disulfide formation, the turn–over of the metal catalyst is probably coupled either with the two equivalent reduction of oxygen to hydrogen peroxide or with the four equivalnt reduction of oxygen to water. The present work was undertaken to investigate the copper–catalyzed oxidation of cysteine in order to elucidate some of the features of the biological oxidation.

In some copper–containing enzymes, *i.e.*, galactose oxidase[3] and diamine oxidase,[4] the turn–over of copper is coupled with the two equivalent reduction of oxygen and hydrogen peroxide is accumulated as the final product from oxygen. In other enzymes, *i.e.*, laccase[5] and ascorbic acid oxidase,[6] the turn–over is coupled with the four equivalent reduction of oxygen and water is the final product. In the latter enzymes, since hydrogen peroxide is not utilized efficiently for the oxidation, it is considered that oxygen is reduced directly to water but not *via* hydrogen peroxide, *i.e.*, the simultaneous four equivalent reduction.[7] In the chemical oxidation, oxygen may be reduced *via* hydrogen peroxide to water. The present paper dealt with the utilization of the molecular oxygen in the copper–catalyzed oxidation. The determination of the rate was done by measuring oxygen consumption.

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2) D. Cavallini, S. Duprè, R. Scandurra, M.T. Graziani and F. Cotta-Ramusino, *European J. Biochem.*, **4**, 209 (1968).
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Experimental

Material——The Cu(II) solution was prepared from copper sheet, 99.999% purity. The copper sheet accurately weighed was dissolved in a small amount of conc. HNO_3 and then diluted with then twice distilled water. This stock solution, standardized with the complexometric titration method if necessary,[8] was diluted to a desired concentration with 0.1N KNO_3. The cysteine solution was prepared just before use from commercially available L-cysteine HCl monohydrate. The buffer solutions used were as follows; 0.02M phosphate, pH 5.9 to 7.1, 0.02M glycylglycine, pH 6.8 to 8.4 and 0.02M glycine, pH 8.4 to 9.5. The ionic strength of all the solutions were adjusted to 0.1 with KNO_3.

Kinetic Procedure——The consumption of oxygen was followed with the conventional Warburg manometric technique at 20°. The main compartment of the reaction vessel contained 2.0 ml of 1.00×10^{-2}M cysteine, 5.0 ml of the buffer solution and 2.0 ml of 0.1N KNO_3, and the side arm contained 1.0 ml of 1.40×10^{-5}M copper solution. Routinely, the total volume of the reaction mixtures was 10 ml. Being connected with the manometer, the reaction vessel was thermostatted at 20° and the solution was saturated with pure oxygen. After 20 min of temperature equilibration, the catalyst was tipped from the side arm and the reaction was started. The oxygen uptake was read every 1 min thereafter. During the measurement, the vessel was shaken mechanically at the rate of 135 oscillations/min. After the uptake of oxygen reached to a constant level, the reaction was stopped and H_2O_2 produced was determined spectrophotometrically with $TiCl_4$.[9]

In another experiment, the formation of H_2O_2 was followed spectrophotometrically. The composition of the reaction mixtures was same as the manometric measurement. During the measurement, the oxygen gas presaturated with 0.1NKNO_3 was bubbled continuously into medium at 100 ml/min.

Result and Discussion

It is postulated that the first step in the metal–catalyzed oxidation is the formation of the metal–substrate complex, which is decomposed subsequently to the low valence metal ion and probably the free radical of the substrate.[10] The molecular oxygen may play a role in the reoxidation of the low valence metal ion, which is catalytically inactive, to the high valence metal ion. The reoxidation of the low valence metal ion, copper (I) in the present case, is expressed as follows:

$$2Cu^{+} + O_2 + 2H^{+} \rightleftarrows 2Cu^{2+} + H_2O_2 \quad (1)$$

$$2Cu^{+} + H_2O_2 + 2H^{+} \rightleftarrows 2Cu^{2+} + 2H_2O \quad (2)$$

Provided that the reactions (1) and (2) proceed very rapidly, this assumption may be valid, the rate of oxygen uptake can be used as the rate of cysteine oxidation.

The consumption of gaseous oxygen increased linearly in the initial stage. The rate of the reaction was determined graphically from the initial linear part of the reaction curve. The reaction curve, plotted the oxygen consumption against the reaction time, was shown in Fig. 1. The rate of oxygen uptake and the total amount of oxygen uptake, 0.25 to 0.5 equivalent with respect to the substrate, were appeared to vary with pH. The pH dependence of the rate and the amount of oxygen uptake were shown in Fig. 2. Both curves displayed maxima near pH 7.

Molecular oxygen is utilized for the reoxidation of the catalytically inactive copper (I) ion. If hydrogen peroxide produced in the reaction (1) has not an ability to reactivate the catalyst, the peroxide is the final product of oxygen and the over–all reaction for the cysteine oxidation may be shown by the reaction (3);

$$2CySH + O_2 \rightleftarrows CyS\text{-}SCy + H_2O_2 \quad (3)$$

8) G. Schwarzenbach, "Die komplexometrische Titration," Ferdinand Enke Verlag, Stuttgart, 1955, p.68.
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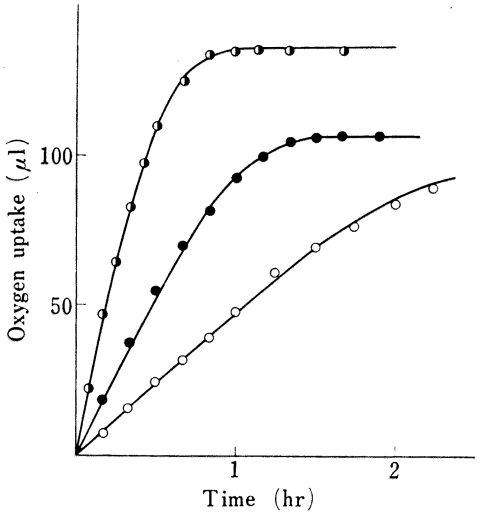

Fig. 1. Typical Reaction Curves of the Oxidation of Cysteine

—○—○—: pH 6.2 —◑—◑—: pH 7.4
—●—●—: pH 8.4
copper ion: 1.40×10^{-6}M cysteine: 2.00×10^{-3}M

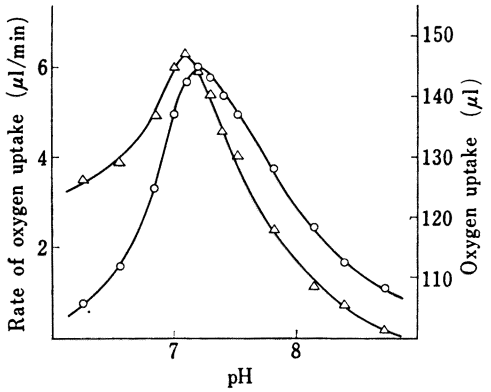

Fig. 2. Effect of pH on Oxygen Uptake

—○—○—: rate of oxygen uptake
—△—△—: amount of oxygen uptake
copper: 1.40×10^{-6}M cysteine: 2.00×10^{-3}M

where CySH and CyS-SCy represent cysteine and cystine, respectively. In this case, the rate of oxygen uptake corresponds exactly to the oxidation rate. The rate of oxygen uptake is equal to that of peroxide formation. The amount of oxygen consumption will be half equivalent with respect to cysteine. If the reaction (2), as well as (1), contributes to the reactivation of the catalyst, both the oxygen consumption and the peroxide formation will reduce in accordance with the increasing contribution of the step (2). The reactivation of the catalyst mentioned above is coupled with the successive four equivalent reduction of oxygen *via* hydrogen peroxide to water. Besides this mechanism, the contribution of the simultaneous four equivalent reduction, not *via* hydrogen peroxide, may be possible.

$$4Cu^{+} + O_2 + 4H^{+} \rightleftarrows 4Cu^{2+} + 2H_2O \qquad (4)$$

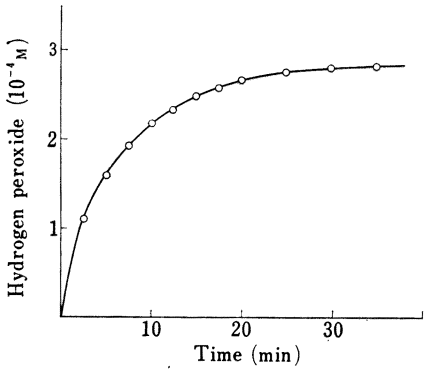

Fig. 3. Hydrogen Peroxide Formation in the Oxidation of Cysteine

copper: 1.40×10^{-6}M cysteine: 2.00×10^{-3}M pH 7.4

According to this redox-system, hydrogen peroxide is never formed in any step of the reaction. On the contrary, in the mechanism of the successive four equivalent redox-reaction, hydrogen peroxide should be detected except in the extreme case where the rate of the step (2) is far rapid as compared with that of (1). The formation curve of hydrogen peroxide shown in Fig. 3 indicates the impossibility of the simultaneous four equivalent redox-reaction.

The total amounts of oxygen uptake and of hydrogen peroxide formed, measured at four different pH values and in the definite concentration of the substrate, were presented in Table I. The maximum value for the oxygen uptake was appeared at pH 7.2. The ratio of

the peroxide formation to the oxygen uptake decreased with the increase of pH. Since hydrogen peroxide produced in the step (1) may be partly utilized for the reoxidation of the catalytically inactive copper (I) ion, the over-all reaction concerning the reactivation of the catalyst may be rewritten as follows;

$$2(1+n)Cu^{+} + O_2 + 2(1+n)H^{+} \rightleftarrows 2(1+n)Cu^{2+} + 2nH_2O + (1-n)H_2O_2 \qquad (5)$$

where n, a positive real number less than unity, means the extent of the contribution of the step (2) in the reactivation. From the concentration ratio of hydrogen peroxide to oxygen uptake, the n value can be estimated:

$$n = 1 - [H_2O_2]/[O_2] \qquad (6)$$

It is described statistically that the reactivation of copper (I) ion is the 2 $(1+n)$ equivalent oxidation. The result shown in Table I indicates that the peroxide is utilized undoubtedly for the reoxidation of copper (I) ion and that the reactivation is inclined to the successive four equivalent redox reaction beyond pH 7.

Table I. Oxygen Consumption and Hydrogen Peroxide Formation at Various pH's

pH	H_2O_2 formation 10^{-4}M	O_2 consumption μl	$[O_2]/[CySH]_0$[a]	n
6.7	3.16	266	0.28	0.43
6.9	3.28	284	0.30	0.44
7.4	2.20	268	0.28	0.60
8.2	0.31	230	0.24	0.94

a) Mole of oxygen consumed for the oxidation of 1 molar cysteine.
total concentration of copper, $[Cu]_0$: 1.40×10^{-6}M
initial concentration of cysteine, $[CySH]_0$: 2.00×10^{-3}M

The peroxide formation and the oxygen uptake were appeared to vary, depending on the substrate concentration. Some examples were presented in Table II. Though the real rate for the oxidation, $d[CySH]/dt$, should increase with the substrate concentration in any pH region, the rate of oxygen uptake did not always display the concentration dependence as shown in Table II: The rate increases with concentration of the substrate at pH 6.9, while it did not increase and showed somewhat a constant level at pH 7.4. The peroxide formation did not depend regularily upon the substrate concentration. Those irregular relations may come from the different participation of the step (2) in the reactivation of the catalyst, because the n value indicates that the successive four equivalent redox reaction proceeds effectively as

Table II. Oxygen Consumption and Hydrogen Peroxide Formation

pH	Cysteine 10^{-3}M	Rate μl/min	H_2O_2 formation 10^{-4}M	O_2 consumption μl	n
6.9	0.50	3.93	1.83	102	0.13
6.9	1.00	6.52	2.65	176	0.28
6.9	2.00	8.45	3.22	290	0.47
6.9	4.00	10.73	2.63	492	0.74
7.4	0.50	9.78	1.86	106	0.15
7.4	1.00	9.16	2.32	164	0.32
7.4	2.00	9.50	2.20	266	0.62
7.4	4.00	9.39	0.62	452	0.93

total concentration of copper: 1.40×10^{-6}M

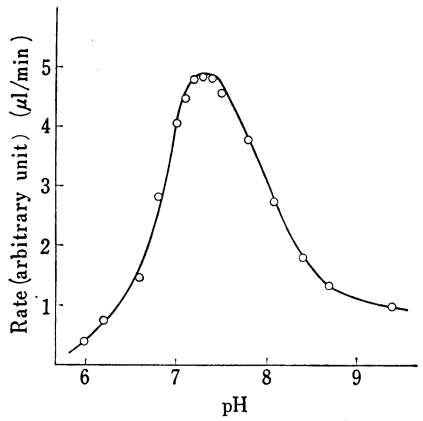

Fig. 4. pH Dependence of the Corrected Rate of Oxygen Uptake

experimental details as under Fig. 2

the concentration of the substrate increases. The oxidation with hydrogen peroxide would depend on the concentration of the substrate.

The mode of the reaction was thus different according to the pH variation. Since the total amounts of oxygen uptake vary depending on the pH variation, the apparent rate has to be corrected relative to the amount of oxygen consumption under the corresponding condition. The pH variation of the corrected rate was presented in Fig. 4. The maximum was appeared at pH 7.3—4. The maximum rate in the iron–catalyzed oxidation is appeared at pH 8.[11] The reason why the maximum rate in the copper–catalyzed oxidation is appeared in the lower pH region would be explained from the thermodynamically strong interaction of the metal to the substrate.

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