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Copper(II)-Poly(L-histidine) as an Enzyme Model for L-Ascorbate Oxidase

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The autoxidation catalyzed by copper(II)-poly(L-histidine) was studied by a rapid-mixing spectrophotometric method. The reaction obeys Michaelis-Menten kinetics. The double reciprocal plots of the velocity against the concentrations, *i.e.* the Lineweaver-Burk plots, of ascorbate and of oxygen gave a straight line. A kinetic analysis of the Lineweaver-Burk plot of the reaction reveals that the autoxidation of ascorbate by Cu(II)-PLH proceeds a so-called Ping-Pong mechanism. Copper(II) ion associates with PLH. The rate of ascorbate autoxidation appeared to depend on the concentration of copper(II) ion in the PLH phase, which is designated as the average binding number. The copper(II) ion concentrated in the PLH phase is highly reactive and is likely to be perturbed by the addition of inert electrolytes. The labile copper(II) ion, which is concentrated in the polymer phase, may be the catalytically active species.

The copper-catalyzed autoxidation of ascorbate has been widely studied as a model of the enzyme reaction.[1,2] The catalytic activity of copper(II) ion is substantially dependent on the nature of the ligand and the coordination environment around the metal ion. Chloride ion was found to induce an increase in the catalytic activity of the copper(II) ion,[3] while ethylenediaminetetraacetic acid[4] or 1,10-phenanthroline[5] inhibited the oxidation. Of much interest is that the copper(II) complex of poly(L-histidine) (Cu(II)-PLH) shows a higher catalytic activity than copper(II) ions in their aqua or amino acidato complexes.[6] On the other hand, other cationic poly(amino acid)s, such as poly(L-lysine) and poly(L-arginine), exhibited little effect on the acceleration of the reaction.[4] The specific effect of PLH on the reaction, which is abolished in the presence of moderate concentration of inert electrolyte, is interpreted by the electrostatic effect of the polyelectrolyte on the concentration of the substrate involved in the close vicinity of the bound copper(II) ion.[6]

We consider that PLH participates not only in the concentration of the substrate by the electrostatic effect in the vicinity of the catalytically active site, but also in the modification of catalytic activity of copper(II) ion by the Cu(II)-PLH interaction. The purpose of this study is to examine how the reaction mechanism of autoxidation and the catalytic activity of copper(II) ions are altered in the presence of PLH.

Experimental

Materials. PLH was a product of Sigma Chemical Co. (lot 98c-5026, degree of polymerization 58, approximate molecular weight based on viscosity measurement 8000). The polymer dissolved in 0.01 mol dm^{-3} acetic acid was dialyzed against doubly distilled water and then lyophylized. L-Ascorbic acid, copper(II) perchlorate, and acetic acid were reagent grade chemicals, which were commercially available, and used without further purification. A stock solution of copper(II) perchlorate dissolved in doubly distilled water was standardized by the conventional complexometric titration with EDTA. The copper(II) solution was diluted by acetate buffer to an appropriate concentration. The ascorbic acid solution was freshly prepared just before kinetic runs.

Kinetic Procedure. The oxidation of ascorbate was conducted at 25 °C in a quartz flow-cell by using a rapid-mixing technique with a Union MX-7 mixing apparatus. Routinely, the reaction was carried out in 0.02 mol dm^{-3} acetate buffer and under 100% oxygen. The solutions of copper(II) ion and ascorbate were saturated with pure oxygen or nitrogen-oxygen mixture by bubbling prior to the kinetic run. The concentration of oxygen dissolved in the medium was determined electrometrically with a Beckman Fieldlab oxygen analyzer, which had been calibrated against air-saturated water. The reaction was started by mixing rapidly both the reactants. After the start of the reaction, the decrease of ascorbate concentration was recorded spectrophotometrically at 260 nm with a Union 401 spectrophotometer. In the kinetic experiment, initial rates were determined at pH 3.6, 4.0, and 4.4 by a graphical method. The pH value of the solution was measured on a TOA DENPA HM-5A pH meter.

Equilibrium Dialysis. Equilibrium dialysis was performed according to the method of Yamaoka *et al.*[7] A 4 ml plastic dialysis cell which was seperated to two chambers by a Visking cellulose membrane was used. In one chamber(A) was contained 2 ml of copper(II) solution containing various amounts of PLH, and in another (B) 2 ml of copper(II) solution. The initial concentrations of copper(II) in both chambers are set to be equal. The dialysis cell was shaken at room temperature for 18 h to reach an equilibrium and then the concentration of copper(II) in the chamber(B) was determined by atomic absorption spectrophotometry with a HITACHI 170-70 Zeeman spectrometer. Since copper(II) adsorbed on the cellulose membrane is very small, total amount of copper(II) in the cell is considered to be constant. If PLH has an ability to bind with copper(II) ion, the copper(II) ion in the chamber (B) diffuses across the chamber (A). Then, the concentration of copper(II) increases in the chamber (A), in which both free and PLH-bound copper(II) ions are contained. Under the equilibrium condition, the concentration of free copper(II) ion, [Cu^{2+}], is equal in both chambers. The difference of the copper(II) concentration between the initial and equilibrated states corresponds to the quantity of the PLH-bound copper(II). The equation describing those relations is shown in Eq. 1.

$$[\mathrm{Cu}^{2+}]_{\mathrm{bound}} = \{[\mathrm{Cu}^{2+}]_{\mathrm{o}} - [\mathrm{Cu}^{2+}]\} \times \{(V_1 + V_2)/V_1\}, \qquad (1)$$

where [Cu^{2+}]$_{\mathrm{bound}}$ and [Cu^{2+}]$_{\mathrm{o}}$ represent the concentration of

PLH-bound copper(II) ion in the chamber(A) and the initial concentration of copper(II) ion, and V_1 and V_2 are the volumes of the solutions in the chambers(A) and (B), respectively. The concentration of bound copper(II) ion in the polymer phase constructing by PLH, which is usually termed as the average binding number, $\bar{\nu}$, is given by Eq. 2.

$$\bar{\nu} = [\mathrm{Cu}^{2+}]_{\mathrm{bound}}/[\mathrm{PLH}]_{\mathrm{total}} \tag{2}$$

Results

1) Effect of PLH. The copper-catalyzed autoxidation of ascorbate was enhanced by the addition of PLH. In Table 1 is summarized the rate of oxidation at various concentrations of ascorbate. Obviously, the rate was observed to increase at least fivefold by the addition of 5 mg dm^{-3} PLH over the range of 0.5—10×10^{-5} mol dm^{-3} ascorbate. The reaction obeys the Michaelis-Menten mechanism. In the absence of PLH, the rate increased linearly with an increase of the ascorbate concentration.

The catalytic activity of copper(II) ion depends closely on the concentration of PLH added. Though PLH itself does not possess the catalytic activity, it enhances the autoxidation of ascorbate probably by complexing with copper(II) ion. When the rate is plotted against the amounts of PLH added, the rate profile displays a bell-shaped curve with the maximum varied depending on the pH of the medium. Some examples are shown in Fig. 1, where the total concentration of copper(II) ion is fixed at constant.

2) Effect of pH. The rate of the autoxidation varied depending on pH as shown in Fig. 2. The rate of oxidation catalyzed by free copper(II) ion increased with pH throughout the range measured, whereas the Cu(II)-PLH-catalyzed oxidation showed a different behavior. The pH-rate profile displayed a bell-shaped curve with a maximum at pH 4.3.

3) Effect of Varying Concentrations of Ascorbate. In the copper-catalyzed autoxidation, the double reciprocal plot of the rate against the ascorbate concentration, *i.e.* Lineweaver-Burk plot, gives a straight line. The

TABLE 1. RATE OF ASCORBATE OXIDATION IN THE PRESENCE AND ABSENCE OF PLH

[Ascorbate]	V_{Cu}	$V_{\mathrm{Cu\text{-}PLH}}$	$V_{\mathrm{Cu\text{-}PLH}}$
10^{-5}mol dm^{-3}	10^{-6}mol dm^{-3} min^{-1}	10^{-6}mol dm^{-3} min^{-1}	V_{Cu}
0.5	0.3	4.5	15
1.0	0.5	7.3	15
2.5	1.4	13	9
5.0	2.4	18	8
10	5.0	24	5

$[\mathrm{Cu}^{2+}]_0=6.0\times10^{-6}$mol dm^{-3}, [PLH]=5 mg dm^{-3}, pH 4.0 (0.02 mol dm^{-3} acetate buffer).

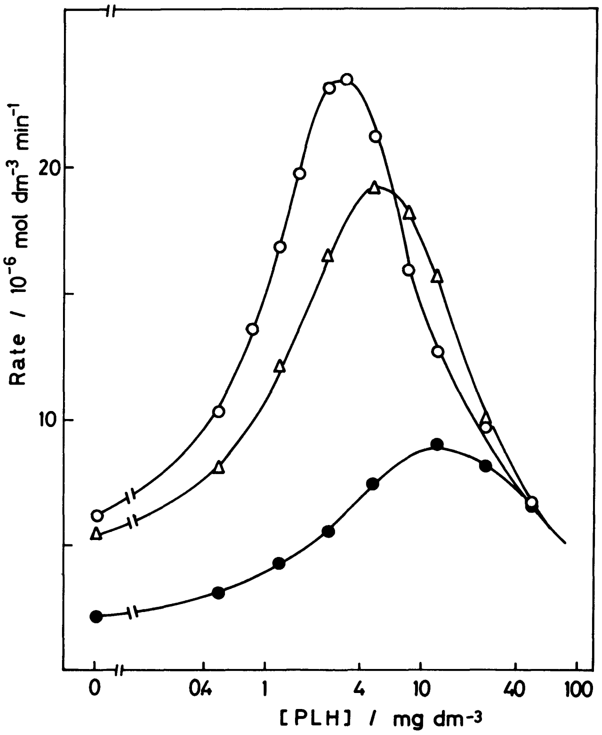

Fig. 1. Plot of the oxidation rate against PLH concentration.
$[\mathrm{Cu}^{2+}]_0=6.0\times10^{-6}$ mol dm^{-3}, [Ascorbate]$=5.0\times10^{-5}$ mol dm^{-3}, pH=3.6(●), 4.0(△), and 4.4(○).

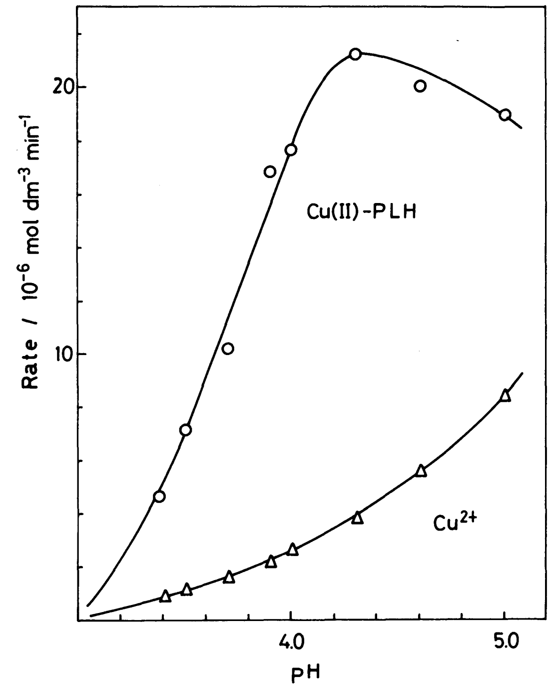

Fig. 2. pH profile of oxidation rate.
$[\mathrm{Cu}^{2+}]_0=6.0\times10^{-6}$ mol dm^{-3}, [PLH]=5 mg dm^{-3}, [Ascorbate]$=5.0\times10^{-5}$ mol dm^{-3}.

families of straight lines of those double reciprocal plots obtained by changing the concentrations of oxygen intersect at a point on the abscissa as shown in Fig. 3A. The intercepts at the ordinate and at the abscissa correspond respectively to the reciprocals of the apparent maximal velocity, V'_{max}, and of the half saturation concentration of ascorbate, $K_{0.5}$, for the reaction. The value of V'_{max} and $K_{0.5}$ at 5×10^{-5} mol dm^{-3} ascorbic acid are shown in Table 2. On the other hand, in the Cu(II)-PLH-catalyzed oxidation, the double reciprocal plot of the rate against the ascorbate concentration at a fixed oxygen concentration gives straight line as shown in Fig. 3B. A series of the double reciprocal plots for the various concentrations of oxygen appears to be parallel. V'_{max} and $K_{0.5}$ at 5×10^{-5} mol dm^{-3} ascorbic acid are given in Table 2.

4) Effect of Varying Concentrations of Oxygen. Both the rates of the copper(II)- and Cu(II)-PLH-

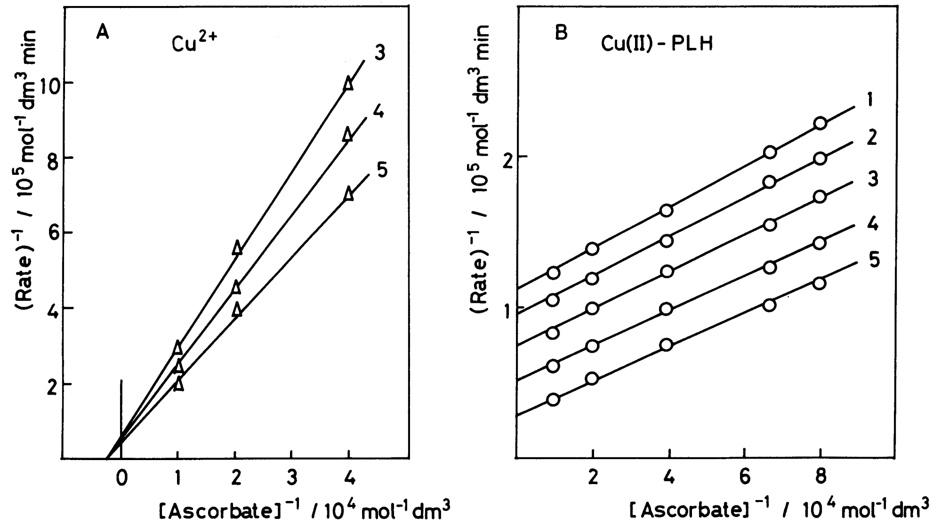

Fig. 3. Double reciprocal plot of the rate against the concentration of ascorbate.
$[Cu^{2+}]_0=6.0\times10^{-6}$ mol dm^{-3}, [PLH]=5 mg dm^{-3}, $[O_2]$=(1) 0.27, (2) 0.43, (3) 0.55, (4) 0.87, and (5) 1.34×10^{-3} mol dm^{-3}, pH 4.0.

Table 2. Kinetic parameter for Cu(II)-PLH- and copper(II)-catalyzed oxidation

Substrate	$K_{0.5}/10^{-4}$ mol dm^{-3}		$V'_{\mathrm{max}}/10^{-5}$ mol dm^{-3} min^{-1}	
	Cu(II)-PLH	Cu^{2+}	Cu(II)-PLH	Cu^{2+}
Ascorbate	0.3	3.3	3.0	2.0
Oxygen	12.0	5.3	3.2	0.4

[Ascorbate]=5×10^{-5} mol dm^{-3}, [Oxygen]=1.3×10^{-3} mol dm^{-3}, $[Cu^{2+}]=6\times10^{-6}$ mol dm^{-3}, [PLH]=5 mg dm^{-3}, pH 4.0 (0.02 mol dm^{-3} acetate buffer).

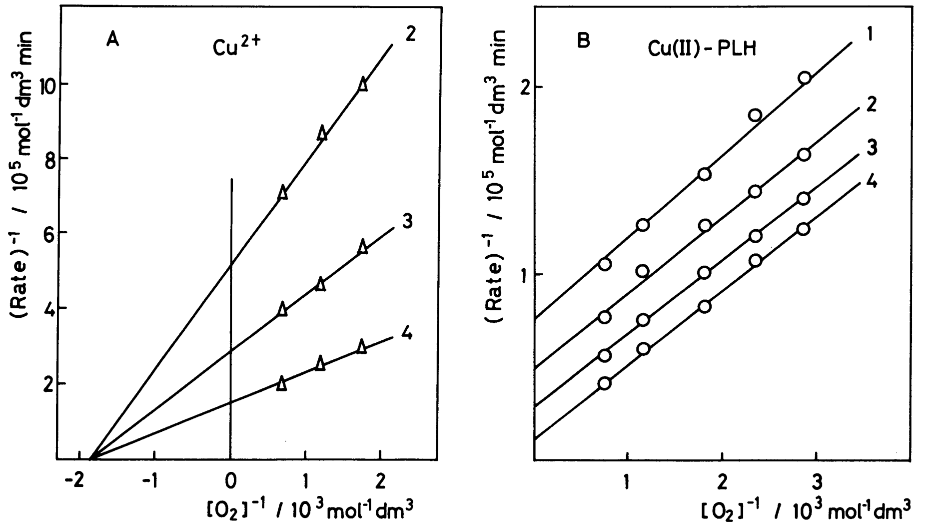

Fig. 4. Double reciprocal plot of the rate against the concentration of oxygen.
$[Cu^{2+}]_0=6.0\times10^{-6}$ mol dm^{-3}, [PLH]=5 mg dm^{-3}, [Ascorbate]=(1) 1.5, (2) 2.5, (3) 5.0, and (4) 10×10^{-4} mol dm^{-3}, pH 4.0.

catalyzed oxidation depended on the concentration of oxygen. In the copper(II)-catalyzed autoxidation, the families of the double reciprocal plots obtained by changing the concentrations of ascorbate intersect at a point on the abscissa as shown in Fig. 4A. The value of V'_{max} and $K_{0.5}$ at 1.3×10^{-3} mol dm^{-3} oxygen are shown in Table 2. In the Cu(II)–PLH-catalyzed reaction, a series of the double reciprocal plots for the various concentrations of ascorbate appears to be parallel as shown in Fig. 4B. V'_{max} and $K_{0.5}$ at 1.3×10^{-3} mol dm^{-3} oxygen are shown in Table 2. The presence of parallel Lineweaver-Burk plots for various concentrations of another reactant indicates a possibility that the reaction proceeds by a Ping-Pong mechanism[8] as discussed later.

5) Effect of Anion. Inert electrolyte such as acetate ion modified the catalytic activity. The effect of anion at various concentrations on the rate is shown in Fig. 5. The effect of anion is shown to vary depending on the concentration of PLH-bound copper(II) and pH. At pH 4.4, the addition of increasing amounts of acetate ion brings a rapid reduction in the rate at 1.1 mmol Cu/g PLH, while a slow reduction at 0.97 mmol Cu/g PLH. These results indicate that the highly active Cu(II)–PLH complex which contains more than 1.0 mmol Cu/g PLH is apt to undergo strucural perturbation by inert electrolyte. At pH 4.0, such distinct effect by anion was not shown.

Discussion

1) Reaction Mechanism. The presence of PLH enhances remarkably the catalytic activity of copper(II) ion and alters the mechanism of the reaction. In the copper(II)-catalyzed autoxidation, a series of the Lineweaver-Burk plots for the various concentrations of ascorbate or oxygen intersect on the abscissa as shown in Figs. 3A and 4A. In the autoxidation catalyzed by Cu(II)–PLH, on the contrary, a series of the Lineweaver-Burk plots was parallel. Parallel Lineweaver-Burk plots observed for various concentrations of another reactant, which are obtained in the reaction by several oxidase such as ascorbate oxidase,[9] are explained by the Ping-Pong mechanism. In the Ping-Pong mechanism, the oxidation of the substrate by the catalyst (Eq. 3) and the reoxidation of the catalyst (Eq. 4) proceed alternatively.

$$\mathrm{S} + \mathrm{Cu(II)^*} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{Cu(II)^*\text{-}S} \xrightarrow{k_2} \mathrm{Cu(I)^*} + \mathrm{P}, \qquad (3)$$

$$\mathrm{Cu(I)^*} + \mathrm{O_2} \xrightarrow{k_3} \mathrm{Cu(II)^*} + \mathrm{O_2^-}, \qquad (4)$$

where Cu(II)* and Cu(I)* stand for the oxidized and reduced forms of the catalyst, respectively. S and P stand for the substrate and the product, respectively. The values of k are rate constants. O_2^- produced is probably either dismuted to O_2 and H_2O_2 or reduced to H_2O_2. The mechanism for Cu(II)–PLH-catalyzed oxidation resembles kinetically that of the enzyme reaction.

The steady state rate equation for this mechanism is

$$V_{max}/v = 1 + K_{0.5}/[\mathrm{S}] + k_2/k_3 \times 1/[\mathrm{O_2}], \qquad (5)$$

where $K_{0.5}$ represents the Michaelis constant, *i.e.* the half saturation concentration of ascorbate, of Eq. 3. From the plot of $1/v$, where v represents the apparent maximal velocity of ascorbate oxidation at various oxygen concentrations, against $1/[O_2]$, it is possible to obtain the true maximal velocity (V_{max}) at infinite concentrations of ascorbate and oxygen; V_{max} was obtained as 1.7×10^{-4} mol dm^{-3}min^{-1}.

2) Cu(II)–PLH Binding and the Catalytic Activity. Cu(II)–PLH appears to be an excellent reaction model

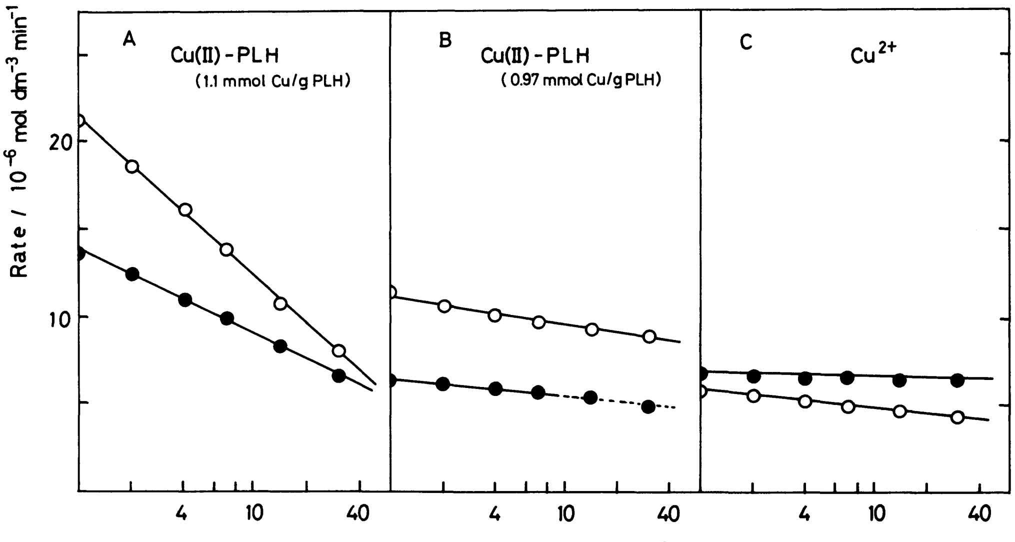

Fig. 5. Effects of various concentrations of anion on the oxidation rate.
$[Cu^{2+}]_0=6.0\times10^{-6}$ mol dm^{-3}, [Ascorbate]$=7.0\times10^{-5}$mol dm^{-3}, anion=AcO$^-$(○) and ClO_4^-(●), [PLH]= (A) 2 mg dm^{-3}, (B) 8 mg dm^{-3}, and (C) 0 mg dm^{-3}, pH 4.4.

Table 3. Relation of oxidation rate and concentration of PLH-bound Copper(II)

[PLH]	pH 4.0				pH 4.4			
	V	$\bar{\nu}$	bound-Cu	free-Cu	V	$\bar{\nu}$	bound-Cu	free-Cu
mg dm^{-3}	10^{-6}mol dm^{-3}min^{-1}	mmol g^{-1}	%	%	10^{-6}mol dm^{-3}min^{-1}	mmol g^{-1}	%	%
0	5.2	0	0	100	6.4	0	0	100
0.5	8.1	0.96	5	95	10.3	1.13	5	95
1.2	12.2	0.89	10	90	16.8	1.11	12	88
2.4	16.5	0.82	18	82	23.1	1.09	24	76
4.8	19.2	0.74	32	68	21.2	1.04	45	55
8.0	18.2	0.64	47	53	15.8	0.97	70	30
12	15.6	0.53	58	42	12.5	0.84	92	8
24	10.0	0.33	72	28	8.6	0.46	100	0
48	6.6	0.21	92	0	6.6	0.23	100	0

for ascorbate oxidase. The binding of copper(II) ion to PLH, which is confirmed by the equilibrium dialysis experiment, seems necessary for the enhancement of the reaction. PLH is shown to bind copper(II) ion at low pH, starting at pH 3, with a maximum binding constant at pH 5.[10] The catalytically inactive poly(amino acid)s do not associate with copper(II) ion. Therefore, the catalytic activity is expected to be related to the amount of the PLH-bound copper(II) ion, which locates in the polymer phase and is evaluated by the average binding number ($\bar{\nu}$) given by Eq. 2.

The quantity of PLH-bound copper(II), $[\mathrm{Cu}^{2+}]_{\mathrm{bound}}$, was obtainable from the equilibrium dialysis experiment under the same experimental conditions. The values of $\bar{\nu}$ under the same conditions as the kinetic run are shown in Table 3 along with the rate observed. In the Cu(II)-PLH system, both PLH-bound and free copper(II) ion always exist in the medium and both possess the catalytic activity. Then, the observed rate of the oxidation, V_{obsd}, may be expressed as the sum of the rates due to PLH-bound copper(II) ion, $V_{\mathrm{bound\,Cu}}$ and due to free copper(II) ion, V_{Cu}.

$$V_{\mathrm{obsd}} = V_{\mathrm{bound\,Cu}} + V_{\mathrm{Cu}}. \qquad (6)$$

Since the value of V_{Cu} is linearly proportional to the concentration of free copper(II) ion, which had been examined by a separate experiment. V_{Cu} can be estimated in each kinetic run. Accordingly, the rate of oxidation by Cu(II)-PLH, $V_{\mathrm{bound\,Cu}}$, is obtainable by Eq. 6. Under the experimental conditions, the total concentration of copper(II) ion is kept at constant. But, since the amounts of PLH are variable, the concentration of copper(II) ion in the polymer phase, which corresponds to $\bar{\nu}$, is variable. Therefore, the rate per the PLH-bound copper(II) ion, which is designated as k_{sp}, should be expressed by $V_{\mathrm{bound\,Cu}}/[\mathrm{Cu}^{2+}]_{\mathrm{bound}}$. We tried to obtain a correlation between k_{sp} and $\bar{\nu}$. If PLH acts merely as chelating agent for copper(II) ion, k_{sp} is expected to be a constant irrespective of the variation of $\bar{\nu}$. However, as shown in Fig. 6, the plots of k_{sp} against $\bar{\nu}$ at pH 4.0 and 4.4 indicate that the specific rate, k_{sp}, increases with $\bar{\nu}$, but not proportional to $\bar{\nu}$. As shown above, the binding number of copper(II) ion per PLH corresponds to the concentration of copper(II) ion located in the polymer matrix of PLH. Ascorbate is also suggested to be concentrated in the polymer phase.[6] Then, the encounter of the copper(II) ion in PLH phase with ascorbate anion may increase with an increase of $\bar{\nu}$. Under the conditions at $\bar{\nu}<1.0$ mmol Cu(II)/g PLH, k_{sp} is larger at pH 4.0 than at pH 4.4. This may be explained by the pH dependence of the ligand exchange rate between copper(II) ion and PLH. A ^{1}H NMR study of the Cu(II)-PLH interaction indicates that the ligand exchange of copper(II) ion in the PLH matrix occurs rapidly under weakly acidic conditions, reducing with increase of pH over pH 3.5.[11] It is suggested that the Cu(II)-PLH binding is rather labile at pH 4.0 and rather tight at pH 4.4. Hence, labile copper(II) ion in PLH phase is assumed

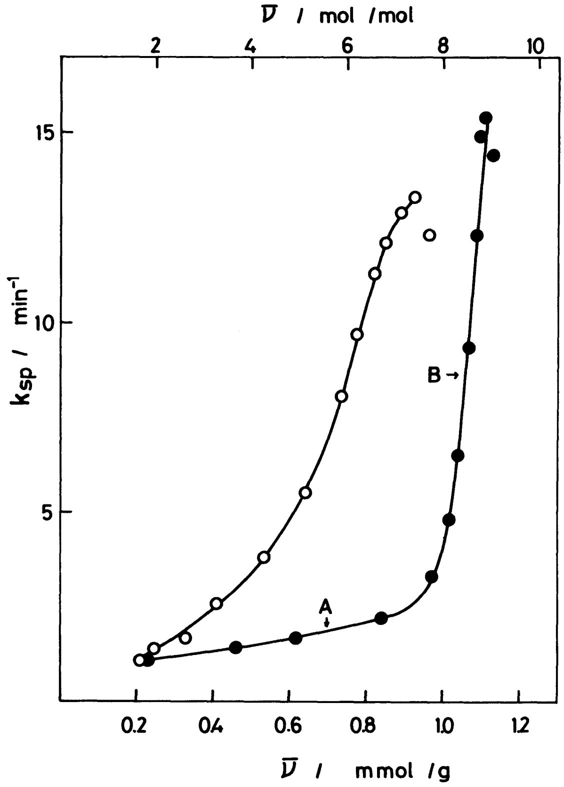

Fig. 6. Relation of Cu(II)-PLH binding and catalytic activity.
pH=4.0(○) and 4.4(●).

to be catalytically active. Of much interest is that the plot at pH 4.4 appears to be composed of two phases, which can be distinguished clearly and is designated A and B, respectively. k_{sp} begins to increase drastically from $\bar{\nu}$=1.0 mmol Cu/g PLH. Provided that the apparent molecular weight of the PLH used is 8000, 1.0 mmol Cu/g PLH corresponds to 8 mol Cu/mol PLH. The PLH used can bind maximally with 9 molar copper(II) at pH 4.4. The mode of interaction of copper(II) ion with PLH also changes dramatically from $\bar{\nu}$=1.0 mmol Cu/g PLH, which is deduced from the effect of electrolytes on the rate of oxidation. As shown in Fig. 5, at $\bar{\nu}$=1.1 mmol Cu/g PLH, where the bound copper(II) ion is in the phase (B), the rate decreased rapidly by the addition of acetate anion. In the phase (A) where $\bar{\nu}$ is 0.97 mmol Cu/g PLH, on the contrary, the rate decreased a little by the addition of the electrolyte. The copper(II) ion in the phase (B) interacts very weakly with PLH, being easily perturbed by electrolyte as compared with the copper(II) ion in the phase (A). These findings support that the bound copper(II) ion which is labile in the matrix of PLH, is a highly catalytic active species.

In conclusion, we suggest that specific effect of PLH on the ascorbate oxidation is not only the concentration of the copper(II) ion and/or ascorbate in the matrix of PLH, but also the formation of a labile and catalytically active copper(II) ion.

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