Uricase Model Reactions of Polylysine–Cu(II) Complexes#

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Oxidation of uric acid was catalyzed by Cu(II) complexes of polylysines. The presence of the polylysine as the second ligand (in addition to the first ligand; the substrate) showed an enhancement of the intrinsic catalytic activity of Cu(II) ions. Accounting for the apparent substrate inhibition phenomenon, due to the absorbance interference by the intermediate, approximate K_m value for the model systems were determined to be around 30—40 μM, which are better than that of the free Cu(II) system, 60—80 μM, and comparable or even better than the K_m values for some enzymatic reactions. From the CD spectrum the conformation of the polymer ligands is known to influence the catalytic activity. A preference of partly ordered structure is implied.

Various compounds comprising polymers or molecular associations have been successfully applied as enzyme models in order to understand the mechanisms of the enzyme actions.[1—5] In a number of enzymes, metal ions are known to play important roles in catalytic activity, and therefore many model studies have been performed on polymer–metal complexes.[6—10] Among these, polypeptide–metal complexes have attracted interest, since they are considered to have intermediary properties of both the metal proteins and the synthetic model complexes; we could examine the contribution of higher order structure of the polymer in a relatively simplified manner.

In the most of the cases, however, free metal ions can exert some intrinsic catalytic activity for the reaction. In some cases, the addition of the polymeric ligand such as polypeptide would reduce the apparent catalytic activity of the system, by excluding the substrate (the first ligand) from the metal coordination sphere. To obtain an excess (positive) catalytic activity by the presence of the second ligand (polymer), we must introduce a system having an appropriate strength of ligation and a proper cooperation of the catalytic residues on polymers.

In the present study, we take polylysine complexes with Cu(II), on which some of the reactions have already been tested for the catalytic possibility so far, such as the oxidation of ascorbic acid or some hydrolytic reactions.[11,12] Here we studied the oxidation of uric acid as an oxidase model. The mechanism of uricase is still ambiguous, in spite of a long history of extensive studies on both enzyme and model systems.[13—21] Practical application of the enzyme or model system to the clinical assay[22,23] also attracts our attention. In the system studied here, we could find excess (positive) catalytic activity due to the presence of the polypeptide ligand. We could also observe the contribution of the secondary structure of the peptide chain to the catalytic activity.

Experimental

Materials: Uric acid and copper(II) chloride were purchased from Wako Chemicals (Osaka, Japan). Their concentrations in aqueous solution were determined spectrophotometrically and conductometrically. Poly-L-lysine (PLL) was obtained from the Peptide Institute (Minoo, Japan) as a hydrobromide or hydrochloride. Poly-DL-lysine (PDLL) hydrobromide (type VII-B) was obtained from Sigma (St. Louis, MO, USA). Their concentrations in aqueous solutions were determined by a back-titration using a conductometric method. Poly(ethyleneimine) (PEI),[24] poly(2-vinylimidazole) (PVIm),[25] poly(acrylic acid) (PAA),[26] and copoly(diethyldiallyl ammonium bromide/SO_2) (DACS)[27] were as reported in the cited references. Other chemicals were commercially available reagents. Uricase from *Candida utilis* was obtained from Toyobo Co. (Lot. 7474; Osaka, Japan) and that from hog liver was purchased form Boehringer-Mannheim (Lot. 1387173; Mannheim, Germany).

Methods: Ultraviolet and visible spectra were mea-

#Abbreviations used: PLL, poly-L-lysine; PDLL, poly-DL-lysine; PEI, poly(ethyleneimine); PVIm, poly(2-vinylimidazole); PAA, poly(acrylic acid); DACS, copoly(diethyldiallyl ammonium bromide/SO_2).

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sured on a spectrophotometer of Union SM 401 (Union Giken, Hirakata, Japan) and pH values of the solutions were determined by Hitachi-Horiba (Kyoto, Japan) F-7ss pH meter. Oxygen concentration in the reactant solution was equilibrated by bubbling the decarboxylized air, N_2, or O_2 gas for at least 15 min at a constant temperature. The final oxygen concentration was determined by an oxygen meter of Union Ox-55001, to which was attached a combination oxygen electrode of YSI4001 (Yellowsprings Instrument, Yellowsprings, OH, USA). Conductance of the solution was measured by a high frequency conductometer (Wayne-Kerr B331 MKII). The circular dichroic spectrum was measured on a Jovin-Yvon-Union Dichrograph Mark III-J (Union Giken) or J-720 (JASCO, Tokyo, Japan).

Results and Discussion

(1) Effect of Polymer on Cu(II)-Catalyzed Oxidation of Uric Acid: In the course of the oxidation of uric acid by free Cu(II) ion, the UV absorbance changed as shown in Fig. 1-a at the indicated wavelength. The absorbance change in the uricase-catalyzed reaction is shown in Fig. 1-b. In the enzymatic oxidation, the maximum absorbance observed at around 293 nm, due to the uric acid (U), is disappearing and the absorbance at lower (260 nm) and higher (324 nm) wavelength showed a transient increase. This has been interpreted as the formation of an intermediate (or the initial product of enzymatic reaction), which is decomposed nonenzymatically to allantoin or its enol form (A). The postulated structure of the intermediate was 1-carbohydoxy-2,4,6,8-tetraaza-3,7-dioxo-4-ene-bicyclo (3,3,0) octane,[15] but recent chemical studies favored an intermediate having a structure of 5-hydroxyisouric acid.[20,21] In a medium free from borate, this initial intermediate (I_0) is converted via 2-oxo-4,5-dihydroxy-4-carbohydroxy-5-ureidoimidazolidine (I_1) and its dehydrated form (2-oxo-4-hydroxy-4-carbohydroxy-5-ureidoimidazolidine: I_1').[15,21] (Eq. 1).

$$U \rightarrow I_0 \rightarrow I_1 \rightarrow I_1' \rightarrow A. \quad (1)$$

The non-enzymatic oxidation showed a very slow absorbance decrease at 293 nm after a certain extent of the reaction and that at 324 nm showed a pseudo-stationary level after that. The non-enzymatic reaction showed different changes at 260 and 324 nm, while the absorbance increases at these wavelengths are parallel in the enzymatic reaction. These results will be related to the inhibitory action of Cu(II) or other heavy metal ions on the degradation process of the intermediate(s), by forming rather stable metal–ligand complexes.[13] A possible participation of I_1, which shows a moderate absorbance peak at around 275 nm,[15] could be different in the non-enzymatic catalysis. The amount of the accumulated intermediate, when estimated at 324 nm, was approximately proportional to the amount of Cu(II) in the non-enzymatic reaction.

As the first step to study the polymeric model of uricase, we studied the effect of additions of polymers on the Cu(II)-catalyzed reactions at the apparent stationary state, as shown in Fig. 2. PEI started an exponential decrease of the intermediate and a complete blocking of the 293 nm decrease, while PLL stimulated the intermediate accumulation and accelerated the substrate consumption. PVIm, though not shown here, exhibited a similar result as PLL. The effect of PEI is considered, therefore, to be similar to that of EDTA or other chelating reagents to restore the heavy metal inhibition of the intermediate decomposition. The chelating ability of PEI towards Cu(II) is so strong[28] that the catalytic potential of the metal ion for the intermediate formation is almost diminished. In contrast to this,

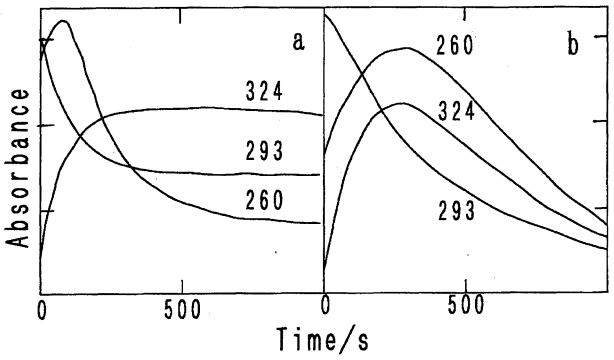

Fig. 1. UV absorbance change during the oxidation of uric acid. a) Cu(II)-catalyzed reaction; [Cu(II)]=6.7 μM, [uric acid]=69 μM. 0.05 M phosphate buffer (pH 10.4), 25 °C, and air saturated. Ordinate unit; 0.025 OD for 293 nm, 0.005 OD for 260 and 324 nm. b) Uricase-catalyzed reaction; [uricase (hog)]=11 nM, [uric acid]=69 μM. 0.05 M phosphate buffer (pH 8.3), 25 °C, and air saturated. Ordinate unit; 0.25 OD for 293 nm and 0.05 OD for 260 and 324 nm.

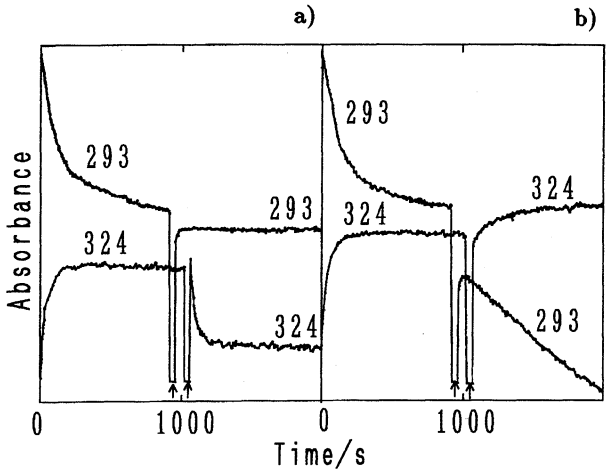

Fig. 2. Effect of polymers on the Cu(II)-catalyzed oxidation of uric acid. [Cu(II)]=6.7 μM, [uric acid]=69 μM. 0.05 M phosphate buffer (pH 10.4), 25 °C, and air saturated. Arrows indicate the addition of the polymer. a) PEI; [PEI]=35 μM monomer unit. b) PLL; [PLL]=67 μM monomer unit. Ordinate; 0.2 OD/full scale for 293 nm and 0.02 OD for 324 nm.

PLL and PVIm accelerated the intermediate formation step.

(2) Oxidation of Uric Acid by Polymer–Cu(II) Complexes: When the mixture of polymer and Cu(II) ion was added to the initial reaction mixture, the rate of the reaction changed depending on the properties of polymer ligands. In Table 1 the relative reaction rates of the polymer–Cu(II) system at initial stage are listed with reference to the free Cu(II) under similar conditions. As expected from the result of Fig. 2-a, PEI–Cu(II) has no catalytic activity. The results of the quaternary cationic polymer (DACS) and the anionic polymer (PAA) can be explained as a partial separation of the two oppositely charged reactants (uric acid (−1 or −2) and Cu(II) (+2)) in the presence of a polyion of either charge: an effect of polyelectrolyte on the reaction between oppositely charged ions.[29] Though PVIm–Cu(II) was expected to show a positive catalysis, its effect on the initial rate was very small. On the contrary, two polylysine–Cu(II) systems showed higher catalytic activity than the control, in the initial phase of the reaction. As for the turnover of the catalysis, however, the poly-DL-lysine–Cu(II) and Cu(II) systems were inferior to the PVIm–Cu(II) system, as known from the absorbance decrease at 293 nm after three hours.

In Fig. 3 the substrate concentration dependences of the initial velocity of the uric acid oxidation catalyzed by polylysine–Cu(II) systems are compared with those of enzymatic and free Cu(II) systems. The rates are described in terms of OD/s, since the extinction coefficients of the intermediates were not exactly evaluated. The initial rates obtained at 293 nm and at 324 nm for two uricases (e and f) showed different dependences. The former showed an apparent substrate inhibition. Though the apparent substrate inhibition of this enzyme, detected at 293 nm, had attracted some discussion,[13,16,30] Priest et al. showed that such a phenomenon was not due to the intrinsic properties of the enzyme but to the artifact by the spectrophotometric properties of the intermediate(s) and the substrate.[16] Non-enzymatic oxidation by the polylysine–Cu(II) complexes (a,b) also showed apparent substrate inhibitions when observed at 293 nm, like that shown by the free Cu(II) system (c).

The rates observed from the increase at 324 nm gave the ordinary saturation profiles for these model systems, though free Cu(II) systems (c,d) showed some sigmoidal dependences. The characteristics of the free Cu(II) system did not alter by changing the concentration of the other substrate, oxygen, for a rage from 2 to 100%. This insensitivity of initial reaction velocity on the oxygen concentration indicates that the turnover of the catalytic reaction in this model system is not so high and the affinity of oxygen molecule to Cu(II)–uric acid complex is high. The variation of the ratio of initial velocity (described in OD/s) at 293 nm to that 324 nm for different catalytic systems are 1.4—1.6 for Cu(II) systems, 2.0 for pig uricase and 2.5 for the yeast enzyme. This difference reflects the sensitive balance of the rate processes (intermediate formation and decompositions).

We can now estimate the approximate values of K_m for the model systems. PLL–Cu(II) and PDLL–Cu(II) gave around 30—40 μM. These values are better than that of the free Cu(II) system, 60—80 μM, and not too bad compared with the K_m values for the enzymatic reaction, 10 and 10—20 μM for the hog and yeast enzyme, respectively. The model polylysine system gave better K_m values than some of the reported values for other microbial uricases, e.g., 200 and 100 μM for that from *Arthrobacter pasceus*,[31] and *Bacilllus fastidiosus*,[32] respectively.

Figure 4 shows the pH-dependence of the PLL– and PDLL–Cu(II) catalyses. It also contains the pH profiles of the free Cu(II) (—). Generally the rate is accelerated with increasing pH, which will be related to the general base catalysis of the non-borate buffer systems for this reaction.[15] Both PLL–Cu(II) (○) and PDLL–Cu(II) (□) exhibited higher catalytic efficiency on the initial velocity than the free Cu(II), more than twice of the latter in the certain range of pH (9—9.5 for PLL and 10—10.5 for PDLL). PLL–Cu(II) showed a higher rate than PDLL–Cu(II) at relatively lower pH, while the latter showed better catalytic activity than PLL–Cu(II) at pH>9.5.

Table 1. Influence of the Presence of Various Polymers on the Oxidation Reaction of Uric Acid Catalyzed by Free Cu^{2+} Ion [a]

Added polymer	[Polymer]/[Metal][b]	Relative activity	Turnover[c]
None	0	1.0	2.0
PEI	5	0.0	—
DACS	10	0.8	—
PAA	25	0.7	—
PVIm	10	0.3	4.0
PLL	20	1.2	—
PDLL	20	1.7	2.1

a) 0.05 M Phosphate buffer (pH 10.2), 26 °C. $[Cu^{2+}]=6.7$ μM, [uric acid]=67 μM. —, not measured. b) In monomer unit for polymer. c) Calculated from [disappeared substrate in 3 h]/[Cu(II)].

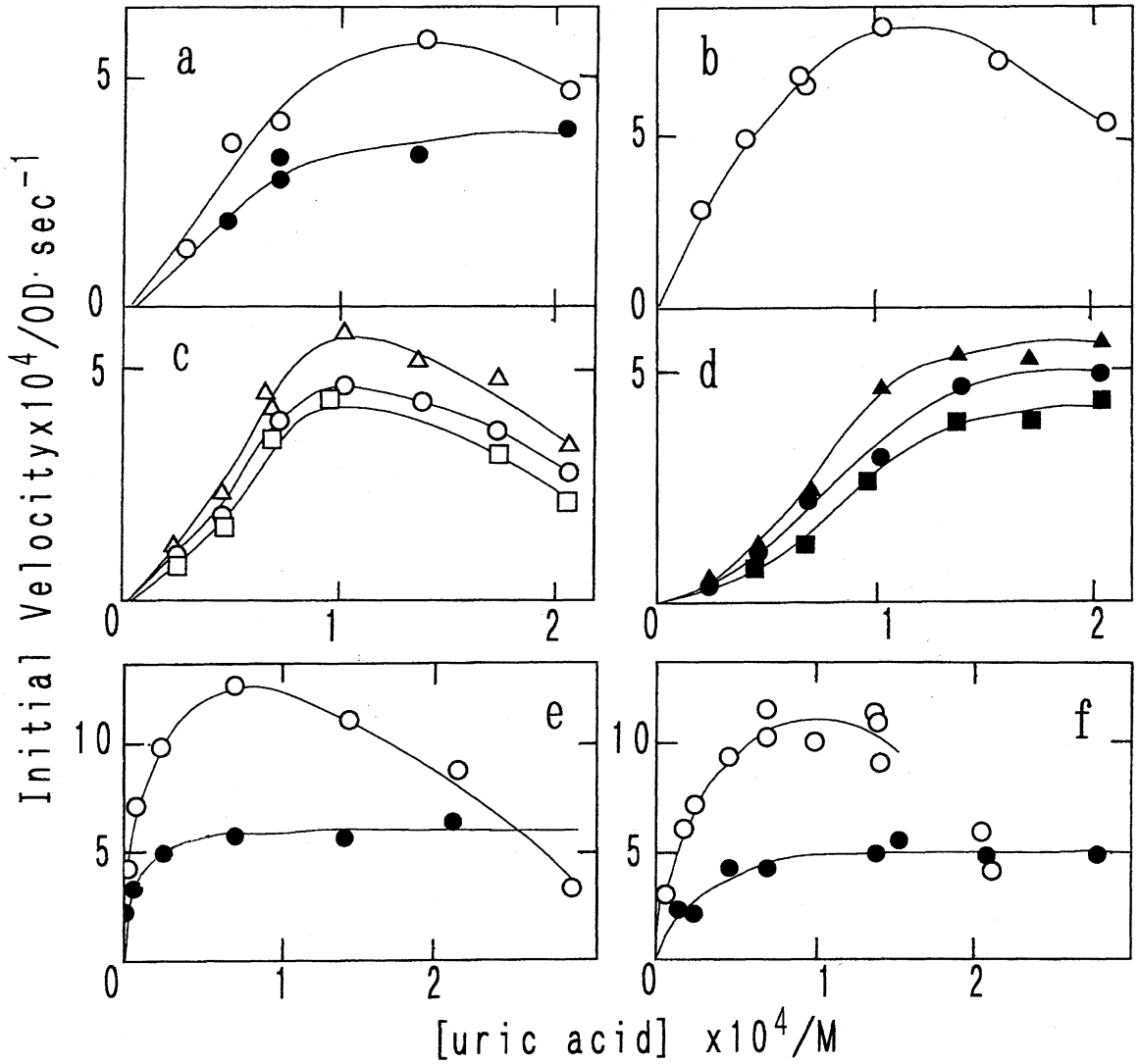

Fig. 3. Substrate concentration dependences of the velocity of the uric acid oxidation reaction by various systems. For each, open symbols are measured at 293 nm and closed ones at 324 nm. At 25 °C, and 0.05 M phosphate. a) PLL–Cu(II); [Cu(II)]=6.7 μM, [PLL]=67 μM monomer unit, pH 10.3, air saturated. b) PDLL–Cu(II); [Cu(II)]=6.7 μM, [PDLL]=130 μM monomer unit, pH 10.3, air saturated. c) Free Cu(II); [Cu(II)]=6.7 μM, pH 10.4. △, O_2 saturated; ○, air saturated (21%); □, 2% O_2. d) Free Cu(II); Same as in c). e) Hog liver uricase; [enzyme]=11 nM, pH 8.3. f) Candida uricase; [enzyme]=28 nM, pH 8.4.

These excess catalytic activities of the model polymeric system over free Cu(II) will be accounted for by the following factors.

1) A kind of general polyelectrolyte catalysis will be exhibited by a cationic polymer (Cu(II)-coordinated polylysine) to accumulate the anionic substrate in the vicinity of a polymer.

2) Some assistance will be offered from the excess ε-amino groups of polylysine during the catalytic process by virtue of the interaction with the substituents at C-2 and C-8 positions of uric acid, which may be similar to that which was considered in the enzymatic catalysis.

3) The degradation of the intermediate is facilitated by producing an amine adduct of allantoin[33] with free ε-NH_2.

(3) CD Spectrum of PLL and PLL–Cu(II): PLL is known to change its secondary structure from coil to helix at around pH 10 (at 25 °C), though there are some disputes about the ordered structure of PLL–Cu(II) complex in the neutral to alkaline range.[34—38]

Figure 5 shows the effect of Cu(II) ion addition on the CD spectrum of PLL in phosphate buffer. An addition of Cu(II) ion increased the apparent ellipticity. Figure 6 contains the pH dependence of the apparent ellipticity at 222 nm of PLL solution in the presence and absence of Cu(II) ion at 25 °C. The polymer metal ratio was varied from 20 to 5 and the midpoint of the ordered-structure formation (apparent pK_a) shifted about 0.4 unit towards the lower pH side. The ordered structure maintains the characteristics of the α-helical one.

When we compare this pH profile with the pH depen-

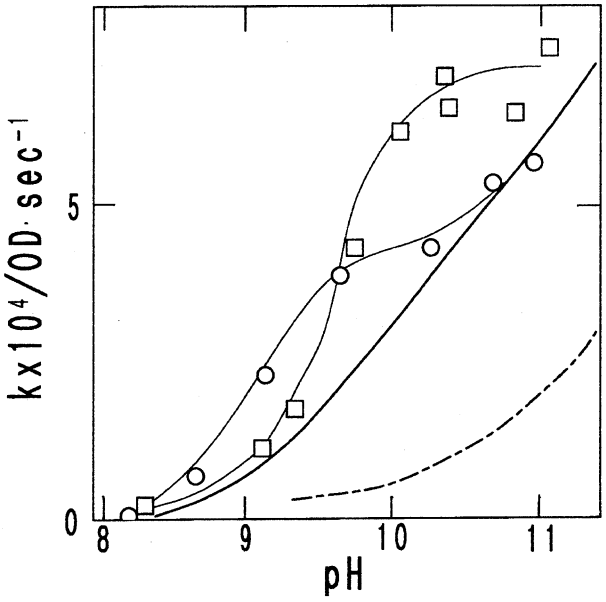

Fig. 4. pH dependence of uric acid oxidations at 25 °C. 0.05 M phosphate buffer, measured at 293 nm. —, free Cu(II)-catalyzed; [Cu(II)]=6.7 μM. ○, PLL-Cu(II); [Cu(II)]=6.7 μM, [PLL]=130 μM (monomer). □, PDLL-CU(II); [Cu(II)]=6.7 μM, [PDLL]=130 μM (monomer). -·-, spontaneous; [EDTA]=17 μM. For each; [uric acid]=69 μM, air saturated.

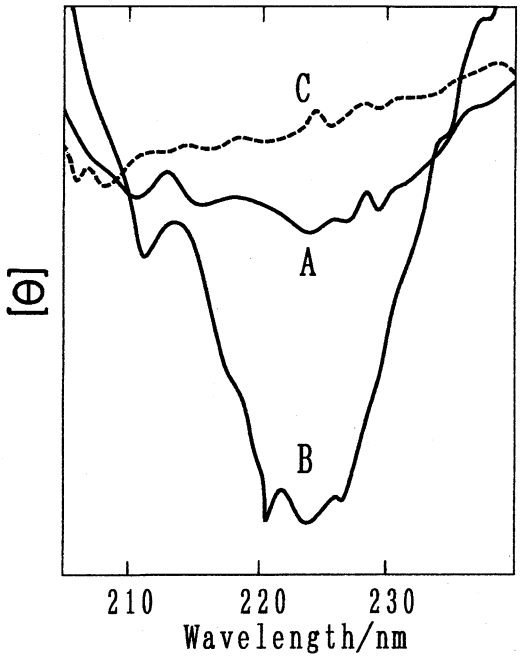

Fig. 5. Change in the CD spectrum of PLL with addition of Cu^{2+} ion in phosphate buffer (pH 9.3) at 25 °C. curve a, PLL alone ([PLL]=0.27 mM); b, Addition of 1/40 (ion/monomer) amount of $CuCl_2$ to a; c, Control (buffer). Ordinate; 5200 deg cm^2/decimole (of monomer unit of PLL) for the full scale.

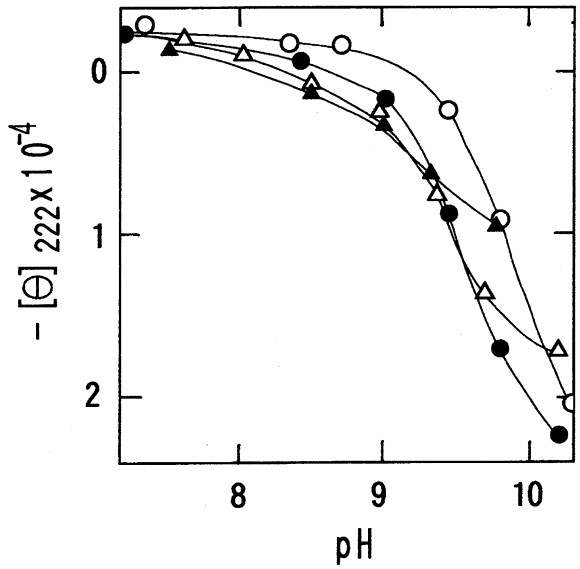

Fig. 6. Influence of Cu(II) addition on the pH dependence of molar ellipticity of PLL at 222 nm. Measured at 25 °C, [PLL]=1 mM in phosphate buffer. [Cu(II)] in mM: ○, 0; ●, 0.05; △, 0.1; ▲, 0.2. Ordinate in deg cm^2/decimole.

dence of the catalytic property in Fig. 4, we see that the partly ordered structure of PLL backbone is favorable to exhibit excess activity on uric acid oxidation. Inferiority of PDLL at lower pH shows the requirement of some ordered structure and the inferiority of PLL at higher pH indicates that the completely ordered structure is unfavorable. Though the visible absorbance spectrum at around 600 nm tells that Cu(II) is certainly coordinated with PLL even in the fully ordered state, its catalytic activity does not exceed that of the free Cu(II) ion. This kind of consideration should include the flexibility or dynamism required for the expression of full catalytic activity of natural enzymes.

References

1) G. G. Overberger and J. C. Salamone, *Acc. Chem. Res.*, **2**, 217 (1969).

2) J. H. Fendler and E. J. Fendler, in "Catalysis in Micellar and Macromolecular Systems," Academic Press, New York (1975).

3) "Bioorganic Chemistry," ed by E. E. v. Tamelen, Academic Press, New York (1977), Vol. 1.

4) T. Ohkubo and N. Ise, *Adv. Polym. Sci.*, **25**, 136 (1977).

5) N. Ise, S. Kunugi, and T. Ohkubo, *Acc. Chem. Res.*, **15**, 171 (1982).

6) "Bioinorganic Chemistry," ed by R. Dessey, J. Dillard, and L. Taylor, Am. Chem. Soc., Washington (1971).

7) G. L. Eichhorn, "Inorganic Biochemistry," Elsevier Sci. Pub., Amsterdam (1973), Vols. 1 and 2.

8) "Metal Ions in Biological Systems," ed by H. Siegel, Marcel Dekker, New York (1976), Vol. 5.

9) M. Kaneko and E. Tsuchida, *J. Polym. Sci., Macromol. Rev.*, **16**, 397 (1981).

10) T. D. Tullius, *ACS Symp. Ser.*, **402**, 1 (1989).
11) Y. Moriguchi, *Bull. Chem. Soc. Jpn.*, **39**, 2656 (1966).
12) M. Hatano and T. Nozawa, in "Metal Ions in Biological Systems," ed by H. Siegel, Marcel Dekker, New York (1976), Vol. 5, p. 245.
13) H. Mahler, *Enzymes*, **8**, 285 (1963).
14) I. Okumura, K. Hondo, Y. Miyake, K. Itaya, and T. Yamamoto, *J. Biochem.*, **79**, 1013 (1976).
15) G. P. A. Bongaerts and G. D. Vogels, *Biochim. Biophys. Acta*, **567**, 295 (1975).
16) D. G. Priest and O. M. Pitts, *Anal. Biochem.*, **50**, 195 (1972).
17) O. M. Pitts and D. G. Priest, *Biochemistry*, **12**, 1358 (1973).
18) O. M. Pitts and D. G. Priest, *Arch. Biochem. Biophys.*, **163**, 359 (1974).
19) A. H. Schein and A. S. Kunin, *FEBS Lett.*, **2**, 339 (1969).
20) M. Poje and L. Sokolic-Maravic, *Tetrahedron*, **42**, 747 (1986).
21) N. Modric, A. E. Derome, S. J. H. Ashcroff, and M. Poje, *Tetrahedron Lett.*, **33**, 6691 (1992).
22) E. M. Grindle, *Clin. Chem. (Winston–Salem, NC)*, **16**, 536 (1970).
23) G. Lum and S. R. Gambino, *Clin. Chem. (Winston–Salem, NC)*, **19**, 1184 (1973).
24) T. Okubo and N. Ise, *Proc. R. Soc. London, Ser. A*, **327**, 413 (1972).
25) S. Kunugi and N. Ise, *Z. Phys. Chem. (N. F.)*, **92**, 69 (1974).
26) S. Kunugi, T. Okubo, and N. Ise, *J. Am. Chem. Soc.*, **98**, 2282 (1976).
27) T. Ueda, S. Harada, and N. Ise, *Polym. J.*, **3**, 476 (1972).
28) D. H. Gold and H. P. Gregor, *J. Phys. Chem.*, **64**, 1464 (1960).
29) K. Mita, S. Kunugi, T. Okubo, and N. Ise, *J. Chem. Soc., Faraday Trans. 1*, **1974**, 936.
30) H. Baum, H. Hübscher, and H. R. Mahler, *Biochim. Biophys. Acta*, **22**, 514 (1956).
31) J. L. Mahler, *Anal. Biochem.*, **38**, 65 (1970).
32) K. Arima and K. Nose, *Biochim. Biophys. Acta*, **161**, 54 (1968).
33) P. H. Stahl, *Biochemistry*, **8**, 733 (1969).
34) M. Hatano, T. Nozawa, S. Ikeda, and T. Yamamoto, *Makromol. Chem.*, **141**, 1 and 21 (1971).
35) M. Palumbo, A. Cosani, M. Terbojevich, and E. Peggion, *J. Am. Chem. Soc.*, **99**, 939 (1979).
36) M. Palumbo, A. Cosani, M. Terbojevich, and E. Peggion, *Macromolecules*, **10**, 813 (1977).
37) A. Garnier and L. Tosi, *Biochem. Biophys. Res. Commun.*, **74**, 1280 (1977).
38) M. Palumbo, A. Cosani, M. Terbojevich, and E. Peggion, *Biopolymers*, **17**, 243 (1978).