Copper Ion Complex Formation of a Dicarboxylic Acid-Containing Polypeptide

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ABSTRACT: A side-chain dicarboxylic acid-containing polypeptide was prepared by introduction of L-glutamic acid to poly(L-glutamic acid) in the side chains. The polypeptide showed extremely high ability for copper ion complex formation. Complex formation induced the stabilization of α-helix structure of the polypeptide, which enabled Cu–Cu interactions in the side-chain region.
KEY WORDS Biomimetic System / Polypeptide / Dicarboxylic Acid / Cu^{2+} Ion Complex / Random Ciol to α-Helix Transition / Cu–Cu Interaction / dimeric Structure of Cu^{2+}–Acetate Type /

Biological activity of metalloproteins is induced by specific complexation with metal ions.[1-11] For example, osteocalcin, a dicarboxlic acid-containing protein of bone, contains a series of γ-carboxyglutamic acid (Gla) residues along the "Gla helix" as specific binding sites for Ca^{2+} ions.[12,13] The transition to the α-helical structure, essential for biological activity, of osteocalcin can be regulated by millimolar levels of Ca^{2+} ion.[12] In this study, therefore, an artificial model polypeptide containing dicarboxylic acid residues similar to Gla was prepared and complex formation of the polypeptide with metal ion and induced conformational changes of the polypeptide were investigated by absorption and circular dichroism (CD) methods.

First, the copper ion complex formation was studied because complex formation behavior between Cu^{2+} ion and carboxylic acid-containing polypeptide such as poly(L-glutamic
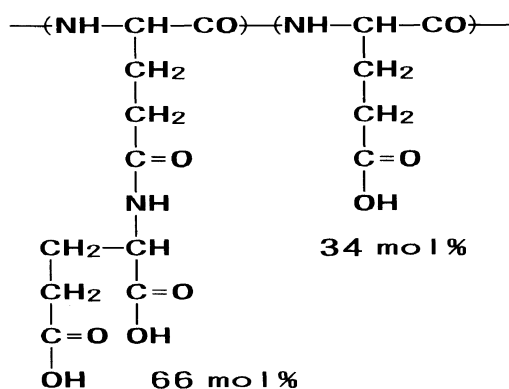
acid) (PGA) has been well characterized.[14-21] The present paper indicates that the complex formation ability of the dicarboxylic acid containing polypeptide to Cu^{2+} is 50 times that of PGA. The induced transition to the α-helical structure may be explained as due to the neutralization effect of Cu^{2+} and Cu–Cu dimeric linkage on the periphery of the polypeptide backbone.

EXPERIMENTAL

Materials

A side-chain dicarboxylic acid-containing polypeptide, poly(γ-L-glutamyl-L-glutamic acid-*co*-L-glutamic acid) (GGA/GA, Scheme 1), was prepared by introduction of L-glutamic acid to poly(L-glutamic acid) (PGA, $M_v = 1.19 \times 10^5$) in the side chains.[22] PGA was obtained by the saponification of poly(γ-methyl L-glutamate) (PMG, $M_v = 1.50 \times 10^5$)

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—(NH—CH—CO)—(NH—CH—CO)—
　　　　|　　　　　　　　|
　　　CH₂　　　　　　　CH₂
　　　　|　　　　　　　　|
　　　CH₂　　　　　　　CH₂
　　　　|　　　　　　　　|
　　　C=O　　　　　　　C=O
　　　　|　　　　　　　　|
　　　NH　　　　　　　　OH
　　　　|
　CH₂—CH　　　　　34 mol%
　　|　　|
　CH₂　C=O
　　|　　|
　C=O　OH
　　|
　OH　　　66 mol%

Scheme 1. GGA/GA.

(kindly provided by Ajinomoto Co., Ltd.).[23] PGA (1 g) was dissolved in N,N-dimethylform-amide (DMF, 50 ml) at 0°C with equimolar L-glutamic acid diethyl ester (1.9 g), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.5 g), and 1-hydroxy-1H-benzotriazole (1.2 g). The reaction mixture was stirred for 216 h at room temperature. The dimethylformamide (DMF) solution was poured into HCl aqueous solution (≈pH 2.0). The residues obtained were washed with acidic solution 3 or 4 times until unreacted monomer, L-glutamic acid diethyl ester, could not be detected spectroscopically by analysis of amino group of the monomer with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, DOJINDO Lab.),[24] fluorescent labeling reagent. The content of dicarboxylic acid diethyl ester residue determined by the high-resolution ^1H NMR analysis (Varian XL-200 spectromeater) was 66 mol%. Protecting ethyl esters of the dicalboxylates (1 g) in DMF (25 ml) solution were hydrolyzed by KOH (0.46 g) in mixed solution of methanol (18 ml), 2-propanol (18 ml) and H_2O (9 ml) at 25°C.[23] Complete deprotection was confirmed by ^1H NMR analysis.

CuCl₂·2H₂O (Nacarai Tesque Co., Ltd.) was guaranteed reagent.

Measurement

The absorption and circular dichroism spectra of GGA/GA in aqueous solution were measured with spectrophotometer (Jasco, UVIDEC-670) and spectropolarimeter (Jasco, J-600) at room temperature, respectively. Solutions of 7.2×10^{-4} base molar GGA/GA and PGA in aqueous solution containing 1.0×10^{-3} M KCl were used.

## RESULTS AND DISCUSSION

Conformation of GGA/GA in Aqueous Solution

Poly(γ-L-glutamyl-L-glutamic acid), the 100% modified polymer, could be obtained by the above procedure. However, the complete introduction of dicarboxylic acid residues prevented the satisfactory formation of the α-helix in aqueous solution even at extremely low pH.

We selected poly(γ-L-glutamyl-L-glutamic acid-co-L-glutamic acid) (GGA/GA) containing 66 mol% dicarboxylic acid (GGA) residues. The reasons are as follows:

(i) The 66% GGA copolypeptide conserved the typical α-helical-random coil transition induced by environmental pH change.

(ii) The probability of GGA residues between jth and (j+3)rd or (j+4)th units, i.e., the close-neighboring side chain positions in the α-helix conformation, is approximately 90% even when GGA content is 66%. These characteristics mean that the 66% GGA copolypeptide may be useful for the dicarboxylic acid containing proteins.[12,13]

Figure 1 shows the circular dichroism (CD) spectra of GGA/GA at various pHs. It is apparent that GGA/GA exhibited a typical pH-induced α-helix-random coil transition. The double minimums at 222 and 208 nm in the CD spectra at pH 3.5 imply that no intermolecular association of α-helical GGA/GA occurs.[25] The midpoint pH of transition about 4.7 was evaluated by pH dependence of $[\theta]_{222}$ shown in Figure 2. The α-helix structure of GGA/GA was somewhat unstable compared with that of PGA (the broken line in Firue 2) owing to the larger electrostatic
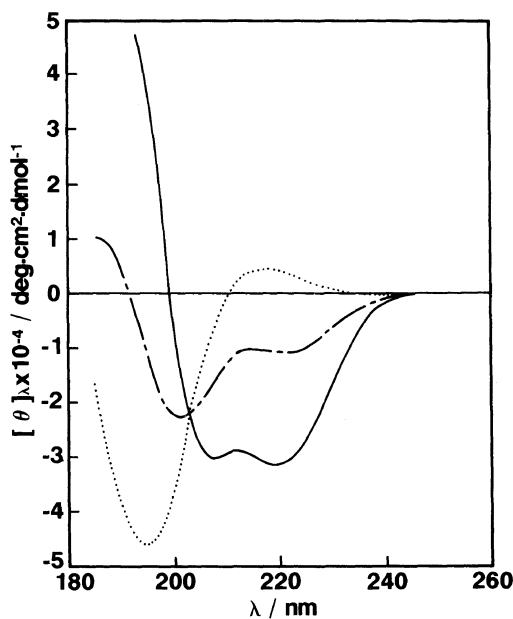
Figure 1. Circular dichroism spectra of GGA/GA in aqueous solution at various pH. (——), pH 3.5; (- - -), pH 5.0; (· · · ·), pH 7.0.
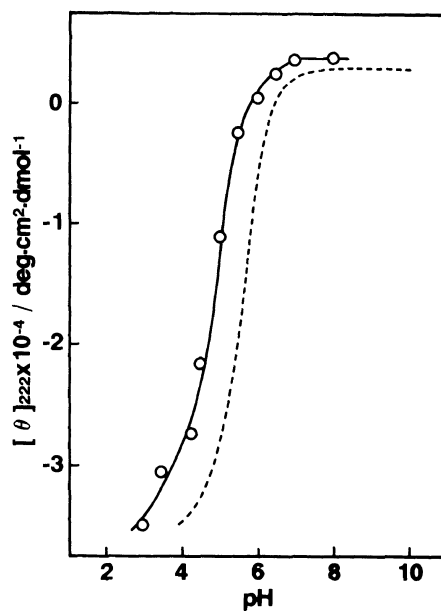

Figure 2. pH dependence of $[\theta]_{222}$ of GGA/GA and PGA in aqueous solutions. (—O—), GGA/GA; (- - - - -), PGA.

repulsion force between dicarboxylic acid residues.

Complex Formation of GGA/GA with Cu^{2+} Ion

When Cu^{2+} ion was added to GGA/GA aqueous solution of various pH, new absorption bands appeared at about 250 nm and 710 nm (Figure 3). The absorption band at 250 nm corresponds to the band at 250 nm of Cu–acetate.[26] The band could be assigned to the charge transfer (CT) from $RCOO^-$ to Cu^{2+}. This CT band was observed with the PGA–Cu system at 250 nm.[17,19,20] The latter band at 710 nm could be asigned to the d–d transition of Cu^{2+} coordinated by carboxylates.[17,19,20] Both wavelengths, 250 and 710 nm, were not dependent on pH between 4.5—6.0. However, their intensities varied with pH, indicating that the concentration and/or stability of the complex decreased with decreasing pH, keeping the coordination structure almost uncharged.[19] Furthermore, a shoulder band also appeared near 370 nm.
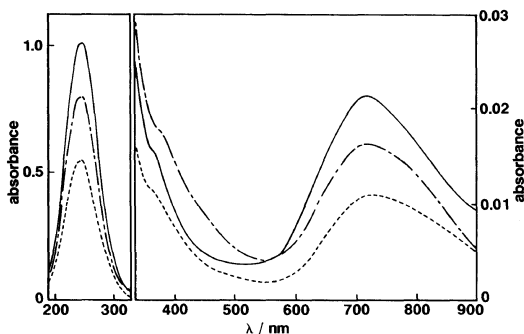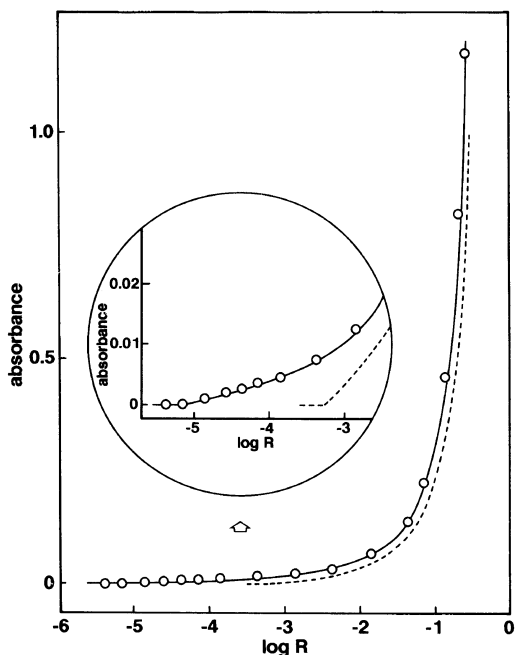

Figure 3. Absorption spectra of GGA/GA–Cu^{2+} in aqueous solution at various pH. (——), pH 6.0; (- - -), pH 5.0; (- - - - -), pH 4.5. $R=[Cu]/[COOH]=0.25$.

This band may be associated with a characteristic dimeric structure of the Cu^{2+}–acetate type.[26-29] The shoulder band intensity at pH 5.0 was larger than that of pH 6.0 even though the lower the pH, the lower was complex stability. This difference is considered to be related to the backbone structure of the polypeptide as described below.

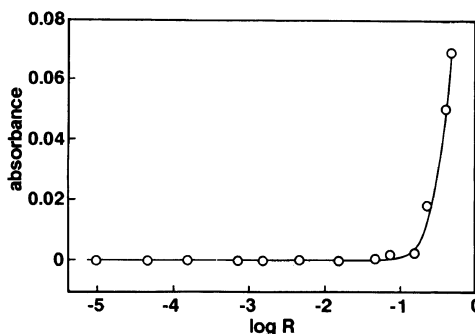Figure 4 shows the absorption spectra of
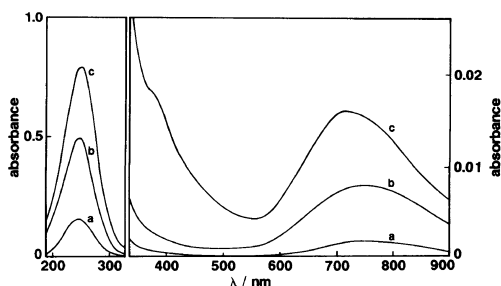
Figure 4. Absorption spectra of GGA/GA–Cu^{2+} in aqueous solutions at pH 5.0. a, $R=0.05$; b, $R=0.17$; c, $R=0.25$. $R=[Cu]/[COOH]$.

Figure 6. Changes in absorbance at 370 nm of GGA/-GA–Cu^{2+} in aqueous solutions as a function of the mixing ratio, R.

Figure 5. Changes in absorbance at 250 nm of GGA/-GA–Cu^{2+} (—○—) and PGA–Cu^{2+} (- - - -) in aqueous solution as a function of the mixing ratio, R.

GGA/GA–Cu^{2+} aqueous solutions at various mixing ratios of Cu^{2+} ion to carboxylic acid group in GGA/GA, $R=[Cu]/[COOH]$ at pH 5.0. The miximum wavelengths of the bands, 250 and 710 nm, were almost independent of Cu^{2+} ion concentration. Figure 5 shows changes in absorbance at 250 nm as a function of R with the results of PGA at pH 5.0. It is clear that the absorption intensity at

250 nm was larger in GGA/GA–Cu^{2+} than in PGA–Cu^{2+} at the same R. The dicarboxylic acid-containing polypeptide, GGA/GA, thus has higher complex formation ability with Cu^{2+} ion than PGA. As seen in the insert, the minimum Cu^{2+} concentration required to induce formation of the GGA/GA–Cu^{2+} complex, 0.01 μM, was lower than of PGA–Cu^{2+}, 0.5 μM. Thus the sensitivity of the dicarboxylic acid to Cu^{2+} ion is 50 times that of the monocarboxylic type. Furthermore, when the Cu^{2+} ion concentration exceeded $R=0.25$, a shoulder band appeared near 370 nm associated with the dimeric Cu^{2+} structure. Changes in the aborbance at 370 nm as a function of the mixing ratio, R, are shown in Figure 6. Beyond the threshold value about $R=0.16$, intensity increased significantly. To examine the relation between backbone structure and shoulder band intensity at 370 nm, changes in α-helix content estimated from the molecular ellipticity at 222 nm of GGA/GA in aqueous solution were plotted as a function of the mixing ratio, R (Figure 7). It is apparent that conformational change of GGA/GA was also induced above $R=0.16$; that is, Cu–Cu dimerization may be synchronized with the conformational transition to the α-helical structure. When the mixing ratio of Cu^{2+} ion to carboxylic acid group in GGA/GA exceeds 0.16, complexing Cu^{2+} ions compensate for the
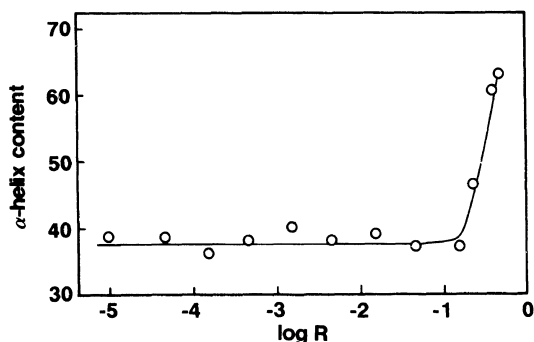
Figure 7. Changes in α-helix content of GGA/GA in aqueous solution as a function of R.

α-helix

Figure 8. Schematic presentation of anticipated Cu^{2+}–GGA/GA complexes.

anioinc repulsion between dicarboxylic acid side chains, which results in the random coil to α-helix transition of the polypeptide. The transition to the compact structure, α-helical structure, brought the side chains between jth and $(j+3)$rd or $(j+4)$th residues so close together, that Cu–Cu interactions became possible. The characteristic dimeric structure of Cu^{2+} may thus be produced in the side-chain region (Figure 8). Though PGA was in the α-helix conformation (ca. 92%) at pH 5.0, the absorbance of the shoulder at 370 nm was only about 0.012 at $R=0.26$, corresponding to the maximal mixing ratio in Figure 6. Therefore, the remarkable increase of the band in Figure 6 was effectively caused by dimeric Cu^{2+} complexation formed between adjacent side-chain dicarboxylic acid groups. That the dicarboxylic acid residues were appreciably present on the peripheryl of the α-helix domain in GGA/GA was thus experimentally confirmed again. The difference in the shoulder bands intensity between pH 5.0 and 6.0 at $R=0.25$ (Figure 3) is also accounted for by α-helix content of GGA/GA at pH 5.0, 47%, being higher than that at pH 6.0, 26%. Moreover, cross-links of side chains based on the dimeric structure of Cu^{2+} may increasingly stabilize the α-helix structure of the polypeptide.[30] The steep rise of helix content in Figure 6 supports the idea that the in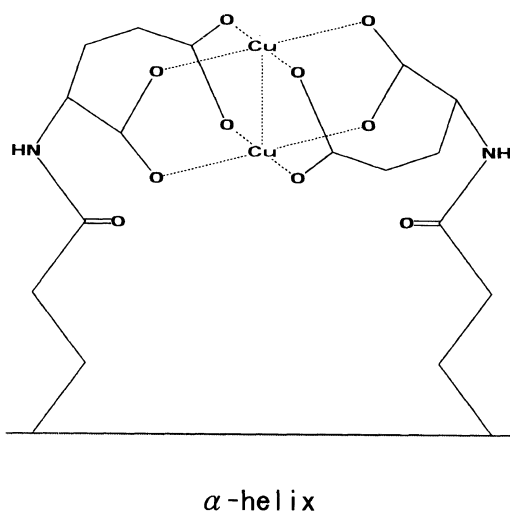duced α-helical structure may be explained as due to the co-operative effect between the neutralization of Cu^{2+} for GGA residues and the Cu–Cu dimeric linkage.

In conclusion, complex formation between Cu^{2+} ion and dicarboxylic acid-containing polypeptide in aqueous solution could be observed even at extremely low Cu^{2+} concentration by the absorption method. Furthermore, the backbone structure of the polypeptide is remarkably affected by copper ion complexation. Such specific complex formation accompanying conformational change may also be useful for constructing bio-mimetic macromolecular systems possessing metal ions.

REFERENCES

1. J. Stenflo, P. Fernlund, W. Egan, and P. Roepstorff, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2730 (1974).

2. P. A. Price, A. S. Otsuka, J. W. Poser, J. Kristaponis, and N. Raman, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1447 (1976).

3. B. C. Furie, M. Blumenstein, and B. Furie, *J. Biol. Chem.*, **254**, 12521 (1979).

4. R. W. Romberg, P. G. Werness, B. L. Riggs, and K. G. Mann, *Biochemistry*, **25**, 1176 (1986).

5. B. Furie and B. C. Furie, *Cell*, **53**, 505 (1988).

6. J. S. Pollock, A. J. Shepard, D. J. Weber, D. L. Olson, D. G. Klapper, L. G. Pedersen, and R. G. Hiskey, *J. Biol. Chem.*, **263**, 14216 (1988).

7. M. Soriano-Garcia, C. H. Park, A. Tulinsky, K. G. Ravichandran, and E. Skrzypczak-Jankun, *Biochemistry*, **28**, 6805 (1989).

8. A.-K. Öhlin, I. Björk, and J. Stenflo, *Biochemistry*, **29**, 644 (1990).

9. M. Hessing, *Biochem. J.*, **277**, 581 (1991).

10. E. Persson, I. Björk, and J. Stenflo, *J. Biol. Chem.*, **266**, 2444 (1991).

11. M. Soriano-Garcia, K. Padmanabhan, A. M. de Vos, and A. Tulinsky, *Biochemistry*, **31**, 2554 (1992).

12. P. V. Hauschka and S. A. Carr, *Biochemistry*, **21**, 2538 (1982).

13. M. H. J. Knapen, K. Hamulyák, C. Vermeer, *Ann. Int. Med.*, **111**, 1001 (1989).

14. S. Inoue, K. Yamaoka, and M. Miura, *Bull. Chem. Soc. Jpn.*, **44**, 1443 (1971).

15. S. Inoue, K. Yamaoka, and M. Miura, *Bull. Chem. Soc. Jpn.*, **45**, 1314 (1972).

16. S. Noji and K. Yamaoka, *Macromolecules*, **12**, 1110 (1979).

17. K. Yamaoka and T. Masujima, *Bull. Chem. Soc. Jpn.*, **52**, 1286 (1979).

18. T. Hiraoki, M. Kaneko, and K. Hikichi, *Polym. J.*, **11**, 397 (1979).

19. T. Masujima and K. Yamaoka, *Biopolymers*, **19**, 477 (1980).

20. M. Koide, E. Tsuchida, and Y. Kurimura, *Macromol. Chem.*, **182**, 359 (1981).

21. H. Maeda, T. Hiramatsu, and S. Ikeda, *Bull. Chem. Soc. Jpn.*, **59**, 587 (1986).

22. M. Sato, T. Kinoshita, A. Takizawa, and Y. Tsujita, *Macromolecules*, **21**, 3419 (1988).

23. T. Kinoshita, T. Yamashita, T. Iwata, A. Takizawa, and Y. Tsujita, *J. Macromol. Sci.*, **B22**, 1 (1983).

24. P. B. Ghosh and M. W. Whitehouse, *Biochem. J.*, **108**, 155 (1968).

25. O. Pieroni, A. Fissi, J. L. Houben, and F. Ciardelli, *J. Am. Chem. Soc.*, **107**, 2990 (1985).

26. M. Kato, H. B. Jonassesn, and J. C. Fanning, *Chem. Rev.*, **64**, 99 (1964).

27. S. Kida, Y. Nakashima, Y. Morimoto, K. Niimi, and S. Yamada, *Bull. Chem. Soc. Jpn.*, **37**, 549 (1964).

28. A. E. Hansen and C. J. Ballhausen, *Trans. Faraday Soc.*, **61**, 631 (1965).

29. L. Dubicki and R. L. Martin, *Inorg. Chem.*, **5**, 2203 (1966).

30. F. Ruan, Y. Chen, and P. B. Hopkins, *J. Am. Chem. Soc.*, **112**, 9403 (1990).