Monolayer Studies of the Interactions between Histidyl Polypeptides and Metal Ions. I. The Effect of Cupric Ions on the Monolayer Properties of Histidyl Polypeptides

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The histidyl residue in protein is a very important binding site for metal ions because of the reactivity of its imidazole group.[1] It was found in our laboratory that bacterial-α-amylase interacts strongly with metal ions such as copper, zinc and mercury, and that the interaction of cobalt ions with this enzyme is rather weaker.[2] The combination of metal ions has been studied with imidazole[3,4] and with 4-methylimidazole[5] as model substances of the histidyl residue. Patchornik et al.[6] synthesized poly-L-histidine and found that it forms insoluble complexes with various heavy metals.

Monolayer technique appears to be a useful way to investigate the interaction of protein with metal ions, as has been shown by the monolayer studies of the mineral tanning of proteins.[7,8] Schulman and Dogan[7] have found that copper ions do not react with the carboxyl group but with imidazole in the protein monolayer. We have attempted, by using a monolayer technique, to study the interaction of poly-L-histidine with cupric and other biologically-important metal ions in order to investigate the metal binding property of the histidyl residue in protein. Poly-L-histidine, however, could not be spread as a monolayer, even on a subphase containing 40% ammonium sulfate or on subsolutions containing cupric sulfate at various concentrations, probably because of the strong hydrophility of its side chains. Thus, poly-1-benzyl-L-histidine was chosen as a model substance in the present experiments for studying the interaction of metal ions with the histidyl group, as it could form stable films at the air/water interface. The monolayer of copoly-2:2:2:1-(glycine, *O*-benzyl-DL-serine, β-benzyl-L-aspartate, 1-benzyl-L-histidine) were also studied.

Experimental

Poly-1-benzyl-L-histidine and copoly-2 : 2 : 2 : 1-(glycine, *O*-benzyl-DL-serine, β-benzyl-L-aspartate, 1-benzyl-L-histidine), which were synthesized by the polymerization of the *N*-carboxyanhydrides of their corresponding amino acids, were supplied by Professor Junzo Noguchi of Hokkaido University. The spreading solutions were made by dissolving the former in 0.05 N hydrochloric acid and the latter in a mixture of dichloroacetic acid and benzene (1 : 4, v/v). The concentration of these polymers in solutions was about 0.25 mg./ml.

The Wilhelmy hanging-plate method was found inadequate for the present experiments because the surface of the glass plate became remarkably water-repellent, probably because of the adsorption of poly-1-benzyl-L-histidine onto the plate. Therefore, the surface pressure was measured using a float-type surface balance. In order to avoid the contamination by metal ions in the trough, a trough

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1) F. R. N. Gurd and P. B. Wilcox, "Advance in Protein Chemistry," Vol. 11, Academic Press, New York (1956), p. 311.
2) K. Kakiuchi, S. Kato and T. Isemura, unpublished results.
3) J. T. Edsall, G. Felsenfeld, D. S. Goodman and F. R. N. Gurd, *J. Am. Chem. Soc.*, **76**, 3054 (1954).
4) R. B. Martin and J. T. Edsall, ibid., **80**, 5053 (1958).
5) Y. Nozaki, F. R. N. Gurd, R. F. Chen and J. T. Edsall, ibid., **79**, 2123 (1957).
6) A. Patchornik, A. Berger and E. Katchalski, ibid., **79**, 5227 (1957).
7) J. H. Schulman and M. Z. Dogan, *Discussions Faraday Soc.*, **16**, 158 (1954).
8) K. G. A. Pankhurst, "Surface Phenomena in Chemistry and Biology," Pergamon Press, London and New York (1958), p. 100.

made of poly(methyl methacrylate) was used, the rim of which was coated with purified paraffin.

The surface viscosity was measured by the rotatory oscillation of a disk on the surface of the liquid; it was calculated by the following formula;[9]

$$\eta_s = \Delta\lambda_{10}\frac{2.303I}{2\pi P}\left(\frac{1}{a^2}-\frac{1}{b^2}\right)$$

where I is the moment of inertia of the disk; P, the period of oscillation; $\Delta\lambda_{10}$, the difference between the logarithm decrements of the oscillation in the presence of film and in its absence; a, the radius of the disk, and b, the radius of the film surrounding the oscillation disk. In the apparatus used in the present experiments, I was 31.0 g. cm., P was 13.18 sec., a was 1.00 cm., and b was 3.01 cm.

The surface potential was measured by the vibrating-electrode method. The surface moment, μ, was calculated from the surface potential, ΔV, utilizing the Helmholtz formula, $\mu = \Delta VA/4\pi$, where A is the area.

The compression of the film was started 20 min. after the polypeptides had been spread. All the experiments were carried out at room temperature. The fluctuation of the temperature, however, never exceeded one degree during the course of the experiments. The pH of the subsolution was measured by a Horiba M-3 glass electrode pH-meter.

An acetate buffer consisting of 0.1 M sodium acetate and 0.1 M acetic acid in an appropriate ratio was used as the subsolution in some cases.

Results

The effects of potassium chloride, sodium sulfate and sodium acetate (0.1 M acetate buffer, pH 5.6) on the surface pressure-area (Π-A) relationship of the monolayer of poly-1-benzyl-L-histidine are shown in Fig. 1.

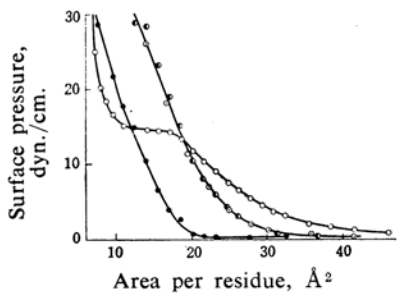


Fig. 1. Surface pressure-area curves of poly-1-benzyl-L-histidine on distilled water (●), 0.1 M potassium chloride (◐), 0.01 M sodium sulfate (⊙) and 0.1 M acetate buffer (pH 5.6, ○): 20°C.

Potassium sulfate (0.01 M, not shown in the figure) gave a Π-A curve identical with that on 0.01 M sodium sulfate. The limiting area per residue, which was determined from the minimum compressibility of the film, was found to be 16.0Å² for the film on distilled water, a value similar to those for such nonionic polypeptides as poly-DL-phenylalanine (15.6Å²).[10] The presence of salts in the subsolution resulted in the expansion of the film, which suggests a more horizontal orientation of the side chains. The effect of the acetate ion is very specific. The film expands on the acetate subsolution more than on other salts, such as potassium chloride and sodium sulfate. There appears a plateau region between 12 and 18Å² per residue in the Π-A curve. The addition of 0.1 M potassium chloride (not shown in Fig. 1) to a 0.1 M acetate buffer had no influence upon the Π-A relationship on the 0.1 M buffer solution.

The effect of cupric sulfate on the poly-1-benzyl-L-histidine monolayer is shown in Fig. 2, together with those of potassium chloride and sodium sulfate. On 0.01 M cupric sulfate, the area per residue is much larger than on 0.1 M potassium chloride, though the film is somewhat more condensed than on 0.01 M cupric sulfate.

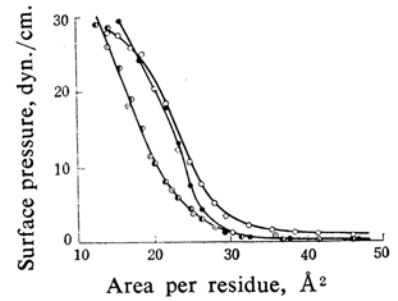


Fig. 2. Effect of cupric sulfate on poly-1-benzyl-L-histidine monolayer at 20°C: ○, 0.01 M cupric sulfate; ●, 0.1 M potassium chloride containing 0.01 M cupric sulfate; ◐, 0.1 M potassium chloride; ⊙, 0.01 M sodium sulfate.

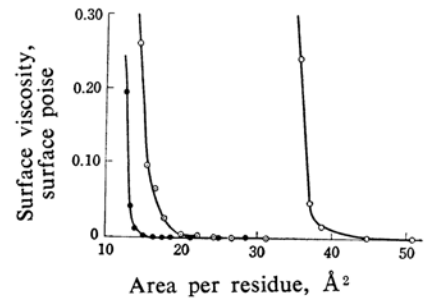


Fig. 3. Surface viscosity-area curves of poly-1-benzyl-L-histidine on distilled water (●), 0.01 M potassium sulfate (⊙) and 0.01 M potassium sulfate containing 0.01 M cupric sulfate (○): 16°C.

9) L. G. Feurt and W. D. Harkins, *J. Phys. Chem.*, **42**, 897 (1938).

10) T. Yamashita and T. Isemura, This Bulletin, **35**, 929 (1962).

Figure 3 shows the surface viscosity-area (η_s-A) curves of this polypeptide on distilled water, on 0.01 M potassium sulfate and on 0.01 M cupric sulfate containing 0.01 M potassium sulfate. The influence of cupric sulfate is markedly different from that of potassium sulfate. The area where the surface viscosity appears is quite large compared with those on distilled water and potassium sulfate. The results shown in Figs. 2 and 3 suggest that the influence of cupric ions on the monolayer properties of poly-1-benzyl-L-histidine differs from that of alkali salts.

The Π-A curves of poly-1-benzyl-L-histidine on the subsolutions at various concentrations of cupric sulfate, in the absence and in the presence of acetate ions, are shown in Figs. 4 and 5 respectively. In both cases, the Π-A relations are affected markedly by a change in the concentration. The plateau of the Π-A curve found on the acetate buffer disappeared upon the addition of cupric ions. The maximum effect of cupric ions was found to be at the concentration of 0.01 M, whether acetate ions are present or not.

The effect of the concentration of cupric

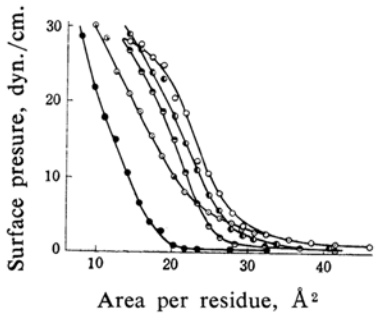


Fig. 4. Effect of the concentration of cupric sulfate on the surfrace pressure-area relation of poly-1-benzyl-L-histidine at 20°C: ●, distilled water; ◓, 0.0001 M; ◐, 0.001 M; ○, 0.01 M; ⊙, 0.05 M.

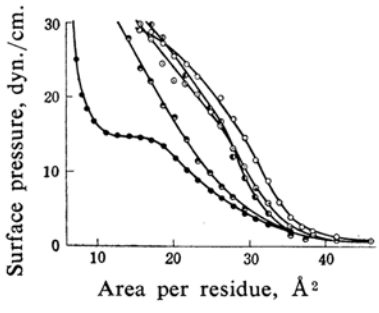


Fig. 5. Effect of the concentration of cupric sulfate dissolved in 0.1 M acetate buffer (pH 5.6) on the surface pressure-area relation of poly-1-benzyl-L-histidine at 20°C: ●, 0 M (control); ◓, 0.0001 M; ◐, 0.001 M; ○, 0.01 M; ⊙, 0.05 M.

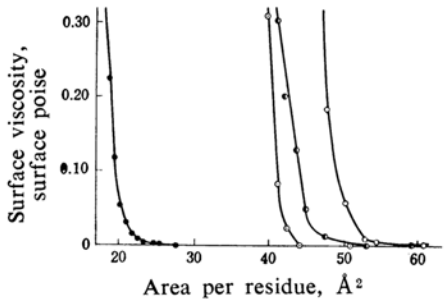


Fig. 6. Effect of the concentration of cupric sulfate dissolved in 0.1 M acetate buffer (pH 5.6) on the surface viscosity-area relation of poly-1-benzyl-L-histidine at 13.5°C: ●, 0 M (control); ◐, 0.001 M; ○, 0.01 M; ⊙, 0.05 M.

sulfate in the subphase of the 0.1 M acetate buffer (pH 5.6) on the η_s-A relation of the poly-1-benzyl-L-histidine monolayer is represented in Fig. 6. The surface viscosity was detected over a much larger area where the surface pressure is considerably lower than in the absence of the cupric ion. The maximum effect of cupric sulfate was found at the concentration of 0.01 M, just as in the case of the Π-A relation.

The surface potential-area (ΔV-A) and the surface moment-area (μ-A) curves of poly-1-benzyl-L-histidine on a 0.1 M acetate buffer (pH 5.6) containing 0.01 M cupric sulfate are compared with those on a copper-free subsolution in Fig. 7.

In the presence of the cupric ion, the surface potential and the surface moment increased more than in its absence in the larger-area region. The surface moment of the film on the subsolution containing the copper ion was higher by about 50 mD in the horizontal portion of the μ-A curve than that on the acetate buffer without the cupric ion.

The effect of the cupric ion on the film properties of this polypeptide is summarized in Table I. It is evident that the interaction

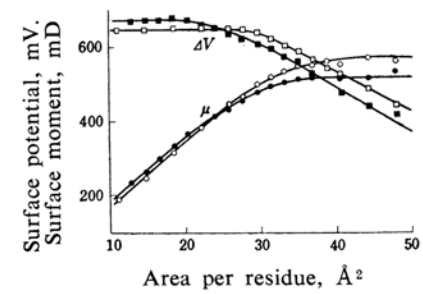


Fig. 7. Surface potential-area and surface moment-area curves of poly-1-benzyl-L-histidine on 0.1 M acetate buffer (pH 5.6; ■, ●) and on 0.1 M acetate buffer containing 0.01 M cupric sulfate (pH 5.6; □, ○): 12°C.

TABLE I. THE EFFECT OF THE CUPRIC ION ON THE SURFACE VISCOSITY, THE SURFACE POTENTIAL AND THE SURFACE MOMENT OF POLY-1-BENZYL-L-HISTIDINE

Subphase	Area at 0.05 surface poise Å²/res.	Π at 0.05 surface poise dyn./cm.	ΔV 35Å²/res. mV.	ΔV 45Å²/res. mV.	μ** mD
Distilled water	13.0	13.6			
0.01 M K_2SO_4	16.6	26.1			
0.01 M $CuSO_4$ in 0.01 M K_2SO_4	37.0				
0.1 M acetate (pH 5.6)	20.4	12.0	546	428	516
$CuSO_4$* 0.001 M	45.0	0.85			
$CuSO_4$* 0.01 M	50.6	0.86	580	484	568
$CuSO_4$* 0.05 M	41.9	1.0			

* Dissolved in 0.1 M acetate buffer (pH 5.6).
** Values at the linear portion of Π-A curves.

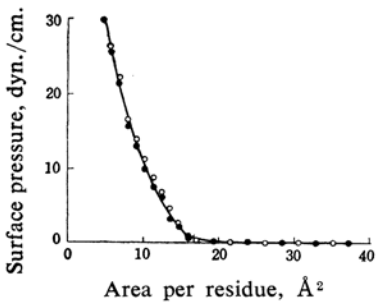


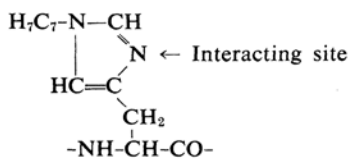
Fig. 8. Surface pressure-area curves of copoly-2 : 2 : 2 : 1-(glycine, *O*-benzyl-DL-serine, β-benzyl-L-aspartate, 1-benzyl-L-histidine) on distilled water (○) and on 0.01 M cupric sulfate (●) at 16°C.

of cupric sulfate with poly-1-benzyl-L-histidine differs from that of alkali salts.

The Π-A curves of copoly-2 : 2 : 2 : 1-(glycine, *O*-benzyl-DL-serine, β-benzyl-L-aspartate, 1-benzyl-L-histidine) on distilled water and on 0.01 M cupric sulfate are shown in Fig. 8. No effects were found with cupric sulfate. 0.05 M potassium chloride and 0.01 N hydrochloric acid also did not affect the Π-A relation of this copolypeptide.

Discussion

As has been described in the preceeding section, cupric sulfate and univalent alkali salts differ significantly in the nature of their interaction with a histidine-containing polypeptide, poly-1-benzyl-L-histidine, although in both cases the interacting site is at the nitrogen atom in the 3-position of the benzylimidazole group,



When cupric sulfate is present in the subsolution, the film expands much more than in the presence of alkali salts. The difference between the effects of alkali salts and cupric sulfate is pronounced in the surface viscosity-area relations. If the cupric ion is present in the subsolution, surface viscosities appear over larger areas and, accordingly, at lower surface pressures than in their absence. Therefore, there might be two types of interaction between poly-1-benzyl-L-histidine and salts: (1) interaction with alkali salts, and (2) interaction with divalent metal salts.

Interaction with Alkali Salts.—On the basis of his study of the properties of long chain amines on aqueous solutions at different pH values, Adam[11] pointed out that the pH's of the subsolution seem to be of little importance in the acid subsolution, and that the films were very much affected by anions in buffer solutions. He concluded that the amines form salts with anions. A similar conclusion was also reached by Hoffman et al.[12]

Patchornik et al.[6] found that a dilute aqueous solution of poly-1-benzyl-L-histidine containing hydrochloric acid gave precipitates of polyvalent salts with various alkali salts. This fact suggests a probable combination of the poly-1-benzyl-L-histidine monolayer with the chloride, sulfate and acetate ions, as in the case of long chain amine monolayers. The salts would be formed between positively-charged benzylimidazolium groups of this polypeptide and anions. Consequently, the film expands on the alkali salt subsolutions more than on distilled water, because the side chains probably take a more horizontal orientation because of the decrease in their hydrophility.

The effect of acetate ions is anomalous.

11) N. K. Adam, *Proc. Roy. Soc.*, **A 126**, 526 (1930).
12) E. J. Hoffman, G. E. Boyd and A. W. Ralston, *J. Am. Chem. Soc.*, **64**, 498 (1942).

Adam[11] found that the monolayer of long chain amine acetate has much less lateral adhesion than that of amine chloride. The monolayer of the salt of poly-1-benzyl-L-histidine with the acetate ion might have much less lateral adhesion than that with chloride or sulfate, which causes a greater expansion and a reorientation of its side chains resulting in the plateau in the Π-A curve.

Interaction with Divalent Metal Salts. — The pronounced effect of the cupric ion on the film properties of poly-1-benzyl-L-histidine can hardly be interpreted in terms of mere interaction between film and anion. A strong interaction exists between the monolayer and the cupric ion. It has been found that the zinc, cobaltous and calcium ions also interact with the poly-1-benzyl-L-histidine monolayer. The results will be reported on in a subsequent paper.

Schulman and Dogan[7] have found a stronger interaction of the copper ion with methemoglobin than with serum albumin in the monolayer, the former having more histidyl groups in its molecule. Patchornik et al.[6] reported that poly-L-histidine forms insoluble complexes with copper, silver, magnesium, mercury, zinc and cobalt. It thus seems reasonable to assume a formation of complexes of poly-1-benzyl-L-histidine with the cupric ions through the nitrogen atoms in the 3-position of benzylimidazole groups. The assumption could possibly be supported by the fact that the surface viscosity of the film appears over a much larger area and at a lower surface pressure in the presence of the cupric ion than in its absence.

It has been found in the mineral or vegetable tanning of protein monolayers that the cross-linkage structure is formed between molecules at the surface, accompanying a remarkable increase in surface viscosity.[13] A similar structure can be considered between poly-1-benzyl-L-histidine molecules as a result of complex formation, which is responsible for the large area over which surface viscosity is first detected. The formation of cross-linkage has been reported also for a protein monolayer reacting with silicic acid.[14][15]

The fact that the surface potential and the surface moment increase more in the presence of the cupric ion than in its absence seems to be another evidence for the formation of complexes. The cationic groups are revealed in the interaction of cupric ions with the poly-1-benzyl-L-histidine monolayer. A similar fact is found to be true in a gelatin monolayer tanned with basic chromium sulfate.[13]

The maximum effect of the cupric ion was found at 0.01 M, above which the area of the monolayer decreased whether acetate ions were present or not, as Figs. 4 and 5 show. It has been found that the coordination ratio of imidazole[3] or 4-methylimidazole[5] to copper decreases from the maximum (4 : 1) with an increasing concentration of the cupric ion. By analogy with this, the number of benzylimidazole groups which associate with a copper ion in subsolution seems to be four at its maximum value and to decrease with an increase in the ion concentration. The decrease may result in the weakening of the cross-linkage structure and may account for the decreases in the areas in Π-A and η_s-A relations.

In contrast with poly-1-benzyl-L-histidine, the monolayer of copoly-2 : 2 : 2 : 1-(glycine, *O*-benzyl-DL-serine, β-benzyl-L-aspartate, 1-benzyl-L-histidine) is not affected by the presence of cupric sulfate in the subsolution. This copolymer contains only 14% of a benzylhistidyl residue, so the polynuclear complexes with copper can be formed only with difficulty.

Summary

The effects of salts on the monolayer properties of poly-1-benzyl-L-histidine and copoly-2 : 2 : 2 : 1-(glycine, *O*-benzyl-DL-serine, β-benzyl-L-aspartate, 1-benzyl-L-histidine) have been investigated.

In the presence of potassium chloride, sodium sulfate, and sodium acetate (pH 5.6), the film expansion has been observed; this has been interpreted in terms of the salt formation between side chains and anions. The addition of cupric sulfate to a subsolution causes the film to expand to increase in surface viscosity and potential, for which cross linkage by complex formation between side chains and metal ions might be responsible.

On the other hand, the monolayer of the copolymer is not affected by the presence of cupric sulfate because of the smaller content of histidyl residue.

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13) S. C. Ellis and K. G. A. Pankhurst, *Discussions Faraday Soc.*, **16**, 170 (1954).
14) S. G. Clark, P. F. Holt and C. W. Went *Trans. Faraday Soc.*, **53**, 1500 (1957).
15) S. G. Clark and P. F. Holt, ibid., **53**, 1509 (1957).