

Binding of Metal Ions to Wool Keratin. I. Comparison of Binding Data by Gel Filtration and Equilibrium Dialysis

Satoru KADOKURA, Takeaki MIYAMOTO, Hiraku ITO, and Hiroshi INAGAKI

*Institute for Chemical Research, Kyoto University,
Uji, Kyoto 611, Japan.*

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ABSTRACT: The binding of Cu(II) by keratin proteins extracted from wool in aqueous solution has been investigated by gel filtration and equilibrium dialysis in order to compare the binding data obtained by different methods. The gel filtration binding experiments were carried out on a Sephadex G-25 gel column using 0.05M acetate buffer as eluent in a pH range of 4.5—6.5. The elution profiles of the Cu(II)-keratin complexes were classical at equilibrium Cu(II)-concentrations from 1 to 25 ppm in the range of pH from 4.5 to 5.5. It was found that if the elution profile is classical, reliable binding data can be obtained by gel filtration with a simple technique. On the other hand, at a higher pH of 6.5, anomalous elution profiles were observed; in addition to the classical peaks, a ghost peak with respect to the Cu(II)-concentration appeared. Different mechanisms proposed for the appearance of the ghost peak by other authors were tested but failed to explain the present data. However, it was experimentally proved that the gel filtration data agreed with those of equilibrium dialysis if the Cu(II) exhibiting the ghost peak was independent of the binding by protein.

KEY WORDS Gel Filtration / Equilibrium Dialysis / Ghost Peak / Wool
Keratin / Keratin-Cu(II) Complex /

A simple method for investigating the interaction between a small ligand* molecule and a macromolecule by gel filtration chromatography was first proposed by Hummel and Dreyer,¹ and has been applied to studies on the binding of metal ions or peptides with proteins.²⁻¹¹ This method may, in principle, be identified with equilibrium dialysis using a double-compartment system. Its merits are that the binding data can be obtained (i) without differentiating the free and bound ligands, (ii) with a small amount of sample, (iii) in a relatively short time, and (iv) with a simple technique, when certain experimental requirements are met. However, some anomalous phenomena in the elution profiles have been frequently reported: ghost peaks with respect to the ligand concentration.^{5,9,10,12,13}

During the course of gel filtration studies on the binding of heavy metal ions by various keratin

* The term "ligand" in this paper is used for a small molecule which has positive interaction with the macromolecule in the system under study.

derivatives from wool, we also observed a ghost peak in the elution profile of Cu(II)-keratin systems under certain experimental conditions. For systems which exhibit *nonclassical*¹³ elution profiles, it is desirable to compare the results obtained by gel filtration with those by the conventional equilibrium dialysis in order to investigate the advantages and limitations of the former method. However, most available data on the binding of ligands by macromolecules have been derived from either of these two methods, and no detailed comparison has so far been made between the experimental binding data obtained by gel filtration and equilibrium dialysis.

This paper deals with chromatographic anomalies observed in the elution profiles of Cu(II)-keratin systems and a comparison of the binding data obtained by these two methods. The results on the binding parameters of the Cu(II)-keratin complexes and the structure of these complexes will be reported in subsequent papers.

GEL FILTRATION PROCEDURE OF HUMMEL AND DREYER

There are two principal requirements for gels to be used in studying interaction between ligand and macromolecule. One is that the gel pores be freely accessible to unbound ligands but able to exclude macromolecule species. The other is that there must be no adsorption of macromolecules or their complexes onto the gel. The interaction of the ligand with the gel does not interfere with the evaluation of results, according to the theories for the ligand binding by macromolecules.¹⁴⁻¹⁶

The gel filtration experiment involves the following three processes: (i) the column is equilibrated with an eluent containing the ligand of known concentration, (ii) the sample macromolecule dissolved in the eluent is injected into the column, and (iii) the elution is continued until expected peaks appear. As the macromolecules flow through the column, the ligands bind to the macromolecules until equilibrium is reached with respect to the ligand concentration in the eluent. Subsequently, the macromolecule and its complexes emerge from the void volume of the column, giving a positive peak both in the plots of ligand and macromolecule concentrations *versus* elution volume, and then a negative peak in the elution profile of the ligand, *i.e.*, a trough reflecting the amount of ligand removed from the eluent. Usually, the trough is relatively broad, because it spreads out as a result of axial dispersion in the column. However, the amount of ligand bound to the macromolecule, indicated by the positive peak, should be equal to that of ligand removed from the eluent. If the experimental conditions are appropriate, both positive and negative peaks of the ligand should be observed separately. In addition, the two base-line concentrations in the elution curve just after the positive peak and the trough should be identical and correspond to the original ligand concentration in the eluent. The appearance of a plateau between the peak and the trough provides a simple criterion for the attainment of equilibrium in the column.²

EXPERIMENTAL

Materials

Salts and buffer materials were all of analytical grade and used without further purification.

Deionized water was used throughout. A standard aqueous solution of CuCl_2 with a concentration of 1000 ppm was obtained from Nakarai Chemical Co., Kyoto. A standard aqueous solution of $\text{Cu}(\text{ClO}_4)_2$ was prepared from $\text{Cu}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$.

The high-sulfur protein fraction, which is one of the major constituents of soluble proteins from wool,¹⁷ was used for the present study. Its *S*-carboxymethylated derivative was prepared from reduced wool (Merino 64's), according to the procedure of Dowling and Crewther,¹⁸ and is hereafter denoted as WCOOH. This procedure includes extraction of the wool with 2-mercaptoethanol at pH 10.5 in the presence of 8 M urea, alkylation of the extracted proteins with iodoacetic acid, and isolation from the low-sulfur protein fraction at pH 4.4 in the presence of 0.5 M KCl. The sample WCOOH dissolved in a potassium acetate buffer was dialyzed against deionized water and then freeze-dried.

The γ -keratose (WSO_3H), a high-sulfur protein fraction from oxidized wool, was prepared from performic acid-oxidized wool (Merino 64's), according to the method of Corfield *et al.*¹⁹

Gel Filtration Binding Experiments

A series of gel filtration binding experiments were carried out in a pH range of 4.5–6.5, using a Sephadex G-25 column (1.1 × 50 cm) equipped with a thermostated water jacket. Unless otherwise stated, the column was equilibrated with 0.05 M acetate buffer containing the desired concentration of Cu(II) and 0.1 M KCl. Some experiments were performed in 0.05 M acetate buffer containing 0.1 M NaClO_4 , instead of 0.1 M KCl. The sample keratin was dissolved in the buffer used for equilibration of the column. One ml of 1–2% sample solution was applied to the column and eluted at 20°C with the same buffer as that used for equilibration. The effluent was collected by a fraction collector on a weight basis to obtain 3 g for each fraction. The elution was carried out by gravitational force at a flow rate of *ca.* 90 ml h⁻¹. The time required for each chromatographic run was approximately 3 h.

The pH was adjusted with a relatively concentrated solution of KCl or HCl to prevent appreciable volume change. The keratin concentration of the fraction was estimated from the absorbance at 278 nm by a Shimadzu UV-200S spectrophotometer, and the concentration of Cu(II) by a

Shimadzu Model AA-610S atomic absorption spectrometer. The equilibrium Cu(II)-concentration was not corrected for the formation of the Cu(II)-acetate complex in the acetate buffer,⁵ so that the total concentration in the elution buffer was equated to the equilibrium Cu(II)-concentration.

Equilibrium Dialysis

A visking cellophane tubing was thoroughly rinsed with deionized water and immersed in the buffer used for equilibrium dialysis for 24 h. A cellophane tubing sack containing 10 ml of the sample solution was equilibrated with 90 ml of the external solution at 20°C for 2 days. Preliminary experiments showed that it took 2 days for the attainment of dialysis equilibrium and that the amount of Cu(II) bound to the cellophane was negligible.

RESULTS AND DISCUSSION

Elution Profiles at pH 4.5 and 5.5

Figure 1 shows a typical elution profile obtained for the system Cu(II)-WCOOH on a Sephadex G-25 column at pH 5.5 and 20°C. As can be seen from the figure, a positive peak for Cu(II) eluted immediately after the void volume of the column appear in the same retention range as that for a UV absorption peak associated with the protein component. This indicates that the positive peak for Cu(II) is caused by the WCOOH-bound Cu(II). Consequently, the negative peak for Cu(II), *i.e.*, a relative broad trough, reflects the amount of Cu(II) removed from the eluent. In fact, the areas of both

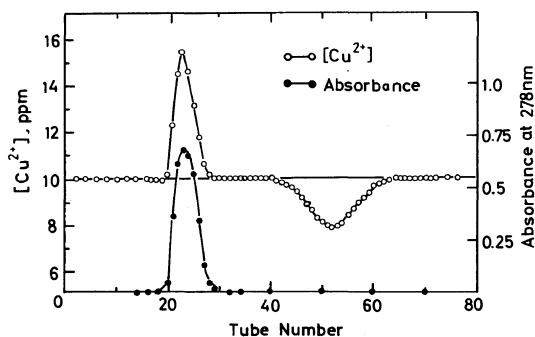


Figure 1. A typical elution profile of the system Cu(II)-WCOOH on a Sephadex G-25 column at pH 5.5 and 20°C. Eluent, 0.05 M acetate buffer containing 10 ppm of Cu(II) and 0.1 M KCl.

peaks were equal within experimental error, which suggests that there occurred no adsorption of keratin and its Cu(II)-complexes onto the Sephadex gel used. Moreover, the UV absorption peak convinced us that the sample recovery was approximately 100%. From these findings, we drew the following conclusions. The presence of protein little interferes with the determination of Cu(II)-concentration by atomic absorption spectrometry, and the equilibrium is attained throughout the eluting system as can be confirmed by the base-lines in Figure 1, so that the rate of complex formation of keratin with Cu(II) is rapid enough in comparison with that of sample transport through the column.

Similar *classical*¹³ elution profiles were obtained at equilibrium Cu(II)-concentrations from 1 to 25 ppm and at pH from 4.5 to 5.5. At equilibrium Cu(II)-concentrations lower than 1 ppm, the negative peaks were too broad to determine their areas accurately. However, it was possible to calculate the

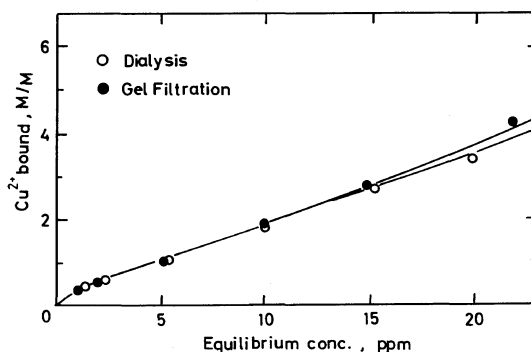


Figure 2. Comparison between the binding isotherms obtained by equilibrium dialysis (○) and gel filtration (●) for the system Cu(II)-WCOOH at pH 5.5.

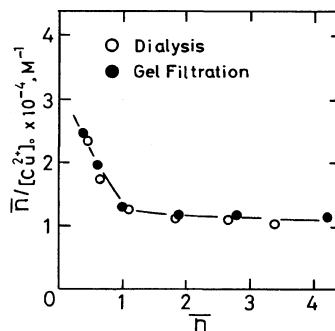


Figure 3. Scatchard plots of the binding data in Figure 2.

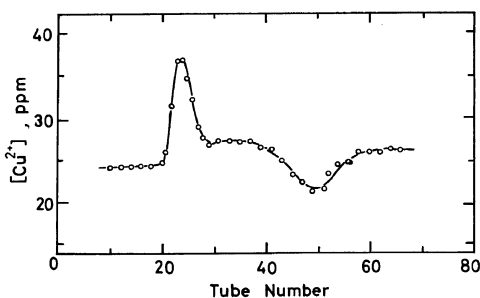


Figure 4. Elution profile of the system Cu(II)-WCOOH on a Sephadex G-25 column at pH 5.5 and 20°C. Eluent, 0.05 M acetate buffer containing 27 ppm of Cu(II) and 0.1M KCl.

amount of keratin-bound Cu(II) from the area under the sharp positive peak of Cu(II).

Figure 2 compares the binding isotherm for the system Cu(II)-WCOOH at pH 5.5 obtained by gel filtration with that by equilibrium dialysis. In Figure 3, these binding data are examined according to the relation proposed by Scatchard,¹⁴

$$\frac{\bar{n}}{[M]_0} = K(n - \bar{n})$$

where \bar{n} is the amount in mol of the bound ligand per mol of macromolecule, n the number of binding sites, K the binding constant and $[M]_0$ the concentration of the free ligand. The two Scatchard plots derived from the different methods are quantitatively in good agreement with each other. The data points in the figure cannot be fitted by a straight line but follow a curve characteristic of a macromolecular species having more than two classes of independent binding sites with different binding constants. The binding parameters, *i.e.*, the binding constant and the number of binding sites, may be estimated from such a binding curve using a procedure similar to that described by Price,⁶ and will be reported elsewhere.²¹

Differing from those reported so far, the elution profiles we observed were unusual at equilibrium Cu(II)-concentrations higher than 25 ppm; the plateau between the positive and negative peaks appeared on a level different from the original baseline, as shown in Figure 4. However, the reason for this is still unclear.

Chromatographic Anomalies at pH 6.5

At a high pH value of 6.5, we obtained anom-

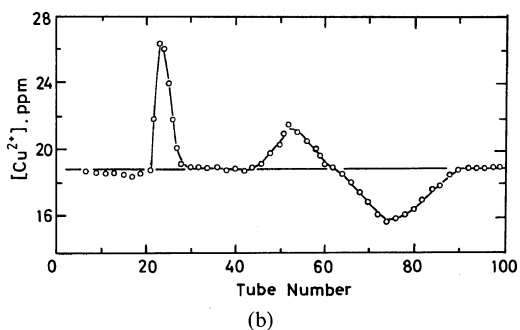
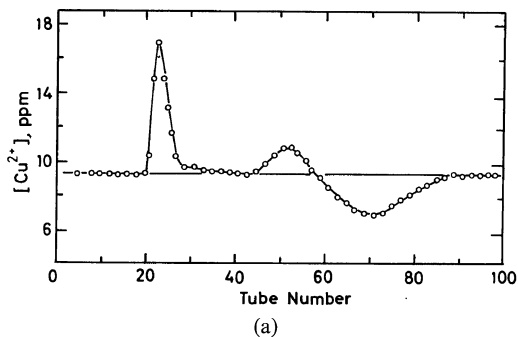


Figure 5. Anomalous elution profiles observed for the system Cu(II)-WSO₃H on a Sephadex G-25 column at pH 6.5. Eluent, 0.05 M acetate buffer containing 0.1 M KCl. The equilibrium Cu(II)-concentration are 9.5 (a) and 18.6 ppm (b), respectively.

alous profiles distinctly different from those at pH 4.5 and 5.5. Figures 5a and 5b show two examples of such elution profiles obtained for the system Cu(II)-WSO₃H. We can see that, in addition to the normal positive peak, an additional ghost peak with respect to the Cu(II) concentration appeared just prior to the trough of Cu(II). These nonclassical elution profiles were observed even in a Cu(II)-concentration range in which classical elution profiles had been obtained at pH 4.5 and 5.5. The area of the ghost peak increased with increasing concentration of Cu(II). It was found that the sum of two positive peak areas was almost the same as that of the trough. No difference in UV/visible absorption was observed between the plateau and the ghost peak.

In the gel filtration, the free Cu(II)-concentration in the buffer in the early stage of a chromatographic run was less than that required to equilibrate the column. However, there is no doubt that equilibrium was attained, as has already been mentioned, and the appearance of the ghost peak should not be

attributed to the variation in free Cu(II)-concentration.

In the present system, the binding experiments were carried out in an acetate buffer to prevent the change in pH as the sample traversed the column. Hence, the ghost peak may possibly be affected by the buffer anion concentration, since it is a known fact that Cu(II) interacts with acetate anion to form Cu(II)-acetate complex.⁵ However, our experiments showed that the ghost peak becomes more distinctive as the acetate concentration in the buffer decreases from 0.05 to 0.01 M. Furthermore, the same ghost peak as in the acetate buffer was observed in an *N*-ethylmorpholine buffer (0.05 M *N*-ethylmorpholine-HCl), for which *N*-ethylmorpholine interacts but little with Cu(II).⁵ These results suggest that the appearance of the ghost peak cannot be interpreted in terms of the attractive interaction between the acetate anion and Cu(II).

On the other hand, the buffers used contained KCl at a relatively high ionic strength, namely 0.1 M, in order to reduce the Donnan equilibrium effect. Thus, we examined the effects of the ionic strength and the kind of anion in the buffer on the appearance of the ghost peak by changing the concentration of KCl from 0.1 to 0.01M and by replacing Cl⁻ by ClO₄⁻.²⁰ We found that the KCl concentration and the type of anion had little effect on the appearance of the ghost peak.

The appearance of the ghost peak has been interpreted in two ways.^{5,13} One is that for a system containing two ligands, the peak appears when a fraction of either ligand attached to the macromolecule in advance is replaced by the other ligand during the elution.⁵ The other is that for a system in which some ligand-mediated association of macromolecule occurs, the ghost peak is expected to appear when a fraction of the ligand is released from the macromolecule by a decrease in the macromolecule concentration during the elution.¹³ This prediction was made by computer simulation.

Now, it is obvious that the ghost peak found in our system cannot be explained in the former way, since the system contains no competitor ligand to interact negatively with Cu(II). On the other hand, the latter way appears to be pertinent for our observation since our profile including the ghost peak is quite similar to that predicted by the aforementioned computer simulation.¹³ However, we found no symptom for any ligand-mediated

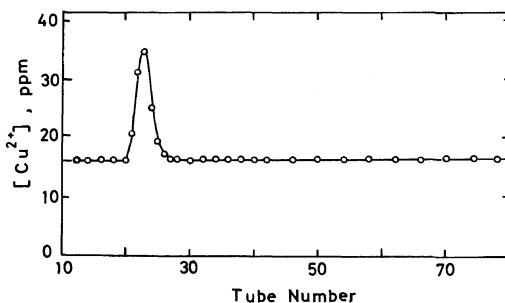


Figure 6. Elution profile of the system Cu(II)-WSO₃H on a Sephadex G-25 column at pH 6.5 and 20°C. Eluent, 0.05M acetate buffer containing 16.5 ppm of Cu(II) and 0.1M KCl. Column loaded with sample solution previously dialyzed against the eluent.

association of the macromolecule in our system.

Further, to discuss the appearance of ghost peak we did another experiment described below. Before the elution experiment, we dialyzed a sample keratin solution, whose concentration and pH were the same as those for the elution experiment, against a large amount of the elution buffer for two days. The keratin solution was then subjected to the elution experiment using the above elution buffer. It was found that the ghost peak disappeared even for the system adjusted at pH 6.5, as shown in Figure 6. This result shows that the appearance of the ghost peak cannot be attributed to a decrease in the concentration of keratin and its complexes with Cu(II) during the elution; our system is not a ligand-mediated associating one. Note that the negative peak also disappeared as a result of the dialysis.

Before we go into a detailed discussion of the above result, we will now analyze two series of binding data obtained by the different methods. Figure 7 shows the binding isotherms found for the system Cu(II)-WSO₃H at pH 6.5, which exhibit nonclassical elution profiles. Curve-A in the figure was obtained from the binding data obtained by gel filtration by estimating the amount of bound Cu(II) as the sum of the areas of the first and the ghost peaks. On the other hand, Curve-B was constructed from the data obtained by equilibrium dialysis and distinctly differs from Curve-A. However, we found that the gel filtration data gave Curve-B when the amount of bound Cu(II) was estimated only from the first peak, as shown by the filled circles in Figure 7. In this connection, it should be mentioned that

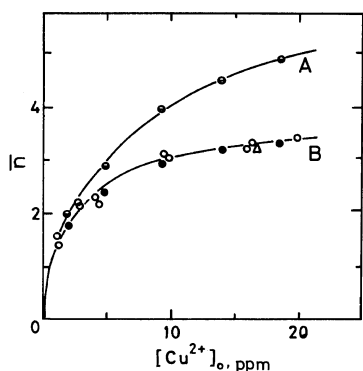


Figure 7. Binding isotherms obtained for the system Cu(II)-WSO₃H at pH 6.5: ○, equilibrium dialysis; ⊙, calculated from the sum of two positive peaks; ●, calculated from only the first peak; △, derived from the experiment shown in Figure 6.

the data point shown by a triangle derived from the curve shown in Figure 6 falls on Curve-B.

From these findings, we may conclude that the binding parameters derived from gel filtration can be identified with those from equilibrium dialysis even for elution systems showing the ghost peak. However, we are as yet unable to explain the reason why the ghost peak appears. Finally we point out that the ghost peak was much more pronounced for WSO₃H than for WCOOH; these are polyelectrolytes strong and weak, respectively.

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