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Chromatographic method involving inductively coupled plasma atomic emission spectrometric detection for the study of metal–protein complexes

Agnès Sandier[a], Catherine Amiel[a,*], Bernard Sébille[a], Jean-Claude Rouchaud[b], Michel Fedoroff[b], Ladislas Soltes[c]

[a]*Laboratoire de Physico-Chimie des Biopolymères, UMR 27 CNRS, 2–8 Rue Henri Dunant, Thiais, France*
[b]*Centre d'Etudes de Chimie Métallurgique, 15 Rue Georges Urbain, 94400 Vitry-sur-Seine, France*
[c]*Slovak Academy of Sciences, Institute of Experimental Pharmacology, 84216 Bratislava, Slovakia*

Abstract

A chromatographic method has been used to study metal ion–protein complexes. It involves successively a gel filtration technique to separate and distinguish the complexed from the free metallic ions, and a spectrometric technique, inductively coupled plasma atomic emission spectrometry (ICP-AES), which allows us to calculate accurately the concentration of ionic metallic species in solution. In the chromatographic step, we applied a large-zone Hummel and Dreyer method. Thus, fractions can be collected throughout the chromatographic experiment and their metal concentration measured by ICP-AES, at constant and known protein concentration. This method has been tested on the copper complex of bovine serum albumin. Results of our study are in good agreement with previous studies on this complex. © 1997 Elsevier Science B.V.

Keywords: Metal complexes; Proteins

1. Introduction

Since the early 1950s, binding of metallic ions to proteins has been extensively studied, for a better knowledge and understanding of biological systems in which metals are involved: as a matter of fact, metals can stabilize macromolecular structures [1], participate in cross-linking, affect the binding of small molecules [2] and catalyse their reactions. They may also induce conformational changes in enzymes or in other proteins which may themselves enhance or inhibit enzyme activity [3]. Therefore, a wide variety of experimental methods has been employed to characterize metal–protein complexes. They fall into two main categories: those requiring a separation of free and bound species, and those for which the bound ligand is measured in presence of the free one. In the latter case, spectroscopic methods are mostly involved, such as UV absorption [4–9] or techniques dedicated to a given metal–protein complex which have been recently developed. They provide accurate information on metal binding sites and on conformational changes of proteins. Among them, ^{1}H NMR [10–12], metal NMR [13,14], IR [15], fluorescence and ESR spectroscopy [16,17] give interesting results.

Other methods measuring changes in the behaviour of the protein, such as amperometry [18] or changes in the properties of the metallic ions, like polarography [19–21], have also been employed. Among methods involving a separation, equilibrium dialysis [22–26] remains the most commonly used

*Corresponding author.

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one to determine the thermodynamics of metal–protein complexes. However, other well-known techniques such as electrophoresis [27], ultrafiltration [28,29] or partition analysis [30] have been successfully used in the study of metal–protein complexes.

The use of liquid chromatography has considerably increased in the study of metal–protein interactions. As a dynamic method, it allows equilibrium to be reached in a short time, which prevents denaturation of protein, or complete break-up of the molecule into subunits.

In the last 20 years, most studies involved the use of immobilized metal ion affinity chromatography (IMAC). Although this method is mainly used to purify a variety of biomolecules on an analytical or preparative scale [31], it also permits the determination of metal–protein interaction constants and estimates the type and number of amino acid residues involved in the complexation [32–37]. However, this technique does not allow a direct observation of the metal–protein complex and cannot be applied to a wide variety of metallic ions. Moreover, a metal ion transfer phenomenon has already been observed [36]. The high affinity binding site of a protein may be able to extract metallic ions from their immobilized chelates. Under these conditions, the protein is not retained on the column, which can paradoxically be interpreted as a lack of affinity for the immobilized metal ion.

Gel filtration is another chromatographic procedure often employed to study protein–ligand interactions, for instance peptide–protein complexes [38]. In this technique, the protein can be maintained in conditions very close to those encountered in natural media. However, it has rarely been used to characterize metal–protein complexes [39]. The main difficulty lies in the way to detect metal ions and therefore to quantify precisely metal–protein interactions.

This paper shows a new way to identify and characterize metal–protein complexes by a combination of gel chromatography and inductively coupled plasma atomic emission spectrometry (ICP-AES). In order to test our method, it seemed advantageous to study an already well-known metal–protein complex: the copper complex of bovine serum albumin (BSA), whose characteristics have been evaluated by several authors, using various methods [20–22,24,28,33,39]. According to the technique used and to the experimental conditions chosen (particularly the pH value, varying from 4.83 to 8.5), those studies describe high affinity binding sites, for which the equilibrium constant K is found to be 10^{11}–10^{13} M^{-1} on $n=1$ site, or low affinity sites, with $K=10^{3}$–10^{5} M^{-1} on $n=16$–17 sites.

2. Experimental

2.1. Materials

Bovine serum albumin (BSA) was a product of Sigma (St. Quentin Fallavier, France), 96–99% purity, used without further purification. Copper nitrate $Cu(NO_3)_2 \cdot 3H_2O$ was purchased from Janssen (Noisy-le-Grand, France). All solutions were made in an acetate buffer 10^{-2} M, pH 5. This pH value was chosen in order to avoid copper hydroxide precipitation, occurring above pH 6.

2.2. Apparatus

For all gel filtration experiments, a peristaltic pump (Minipuls 2, Gilson, Villiers-le-Bel, France), an injector (Altex–Beckman, Gagny, France) and two detectors were needed: a UV detector (ISCO, Lincoln, NE, USA) showing the protein signal at 280 nm, and a conductimetric detector (Vydac–Alltech, Deerfield, USA) showing both protein and metallic ion responses. A fraction collector (Gilson) was placed after the detectors.

The column, 200×5 mm, from Pharmacia (Uppsala, Sweden) was packed with a soft gel: Sephadex G15 (Pharmacia). For this purpose, the gel was first allowed to swell in the acetate buffer for 3 h at room temperature, and degassed under vacuum. It was then poured into the column, and the elution was started at a flow-rate of 0.5 ml/min. It was necessary to pass about 3 column volumes of the buffer to stabilize and equilibrate the gel bed. To check the packing, a 2 g/l solution of Blue Dextran (Pharmacia) was run through the column. The appearance of a horizontal band travelling through the column ensured that it was homogeneously packed.

Protein concentration was checked independently by spectrophotometric measurements at 280 nm. A different chromatographic system was used, which

gave higher flow-rates. Small volumes (20 μl) of protein solutions were injected on an empty column, using an HPLC pump (Kontron, St. Quentin en Yvelines, France), a Rheodyne injector (7125, Berkeley, USA) and a UV detector (Spectra-Physics, San Jose, CA, USA). The peak heights were compared to the one of a protein standard solution (BSA 2 g/l), and the protein concentration in each fraction injected was then deduced.

ICP-AES measurements were made on a Thermo-Jarrel Ash spectrophotometer, Model Atomscan 25, equipped with a Thermospec software. The conditions used were: radio frequency power 1150 W; pump rate 100 rpm; nebulizer pressure 39 p.s.i. (1 p.s.i. = 6894.76 Pa); observation height 20 mm; two emission wavelengths were chosen to determine copper concentration: 224.7 nm and 324.7 nm.

2.3. Chromatographic experiments

The gel filtration method was based on the Hummel and Dreyer experiments [40], and modified for our purpose. In the original Hummel and Dreyer method, the size-exclusion column is equilibrated with a ligand solution. A small amount of protein dissolved in the same ligand solution is injected onto the column and then eluted by the ligand solution used to equilibrate the column. With an appropriate ligand detection, the chromatogram obtained shows a positive peak followed by a negative one. The positive peak corresponds to the metal–protein complex first excluded from the column: it appears at the retention time of the protein. The negative peak, at the retention time of the ligand, represents the local decrease of the ligand concentration in the eluent, and therefore the amount of ligand bound to the protein. The bound ligand quantity is usually deduced by measuring the area of the above-mentioned negative peak. It can also be measured from the positive peak, provided that the participation of the free protein to the detector response is known or is negligible.

The advantage of this method is that the complex does not dissociate during the chromatographic experiment, since the macromolecular complex is always in equilibrium with the free ligand. However, to be able to detect a wide variety of metal ions in a large range of concentrations, especially in the very low concentration domain where the ICP-AES method is still reliable, we chose to modify the Hummel and Dreyer method.

In our experiments, the column was saturated with a solution of copper nitrate at the desired free ligand concentration, and a large volume (4.5 ml) of a copper nitrate–protein mixture was injected, so that a plateau in protein concentration is maintained at the excess ligand binding peak. In the protein–metal mixture, the concentration of copper nitrate was adjusted to be identical with that in the column buffer before injection of the sample, while protein concentration was held constant (3.10^{-5} *M* in all experiments, whereas [Cu^{2+}] was varied from 10^{-7} *M* to 10^{-3} *M*). The bound copper was measured directly in the protein plateau, rather than by measurement of the deficit in the ligand concentration following the protein plateau, as described by Hummel and Dreyer [40]. Nevertheless, this deficit was observed on the chromatogram profile (see Section 3). For this purpose, fractions (volume = 0.4 ml) were collected along the positive plateau at a flow-rate of 0.4 ml/min and their metal concentration analyzed by ICP-AES.

This method was previously used by Brumbaugh and Ackers [41], but the detection system was completely different: it was based upon the direct optical scanning of a Sephadex G-100 chromatographic column previously saturated with a solution containing the ligand and the macromolecule. They injected a sample large enough to ensure a plateau in the excess ligand binding peak. Binding ratios were thus determined at each point along this plateau.

2.4. ICP-AES experiments

Before each set of ICP-AES experiments, standard copper solutions were prepared in an acetate buffer (10^{-2} *M*/l, pH 5), so that a calibration curve could be drawn. This standardization was necessary to prevent any deviation of the emission signal during the analysis of the successive samples. A solution volume of approximately 5 ml was necessary for each ICP-AES measurement: thus, each fraction collected during the chromatographic step was suitably diluted prior to its analysis. Furthermore, at the beginning of the study, it had been checked that neither the protein nor the buffer solution would emit

at those wavelengths chosen for copper analysis, i.e., 224.7 and 324.7 nm.

3. Results and discussion

Before starting any large zone Hummel and Dreyer experiments, the elution volumes of the protein and the metallic ions were measured separately on the Sephadex column, to ensure that a sufficient separation could occur. It appeared that $V_{BSA}=4$ ml and $V_{Cu^{2+}}=11$ ml, those elution volumes being different enough to allow a distinction between the positive plateau and the negative one. Thus large zone Hummel and Dreyer experiments could be carried out.

Fig. 1a–c show the chromatograms obtained simultaneously with the UV and conductimetric detections. The shift in elution volume observed between UV and conductimetric signals can be partly explained by the longer tubing necessary to reach the conductimetric detector, so that protein and copper ions signals appear later on this detector.

At low Cu^{2+} concentrations ($c<10^{-4}$ M), the conductimetric signal of metallic ions is identical to the signal obtained without any copper ions in solution (Fig. 1a and b), and no trough can be detected. However, when Cu^{2+} concentration is increased (Fig. 1c), the presence of a trough after the protein positive peak demonstrates the existence of metal binding by the protein.

Fig. 2 shows the graph obtained from the ICP-AES analysis of eluate fractions collected all throughout a chromatographic experiment. Again, the positive and negative zones can clearly be seen, as well as the baseline recovered at large eluted volumes ensuring that equilibrium between the free metallic species and the complex is reached. As large eluate fractions were collected (1 ml), the presence of a plateau on the positive peak is not easily seen on Fig. 2. We measured the respective areas of the positive and the negative zones by integration. From the example shown in Fig. 2, the bound quantity was found to be $9\cdot10^{-5}$ M in the positive peak, and $7\cdot10^{-5}$ M in the negative one. The difference between those values probably comes from experimental uncertainty on lower copper concentrations as ICP-AES detection limits were reached.

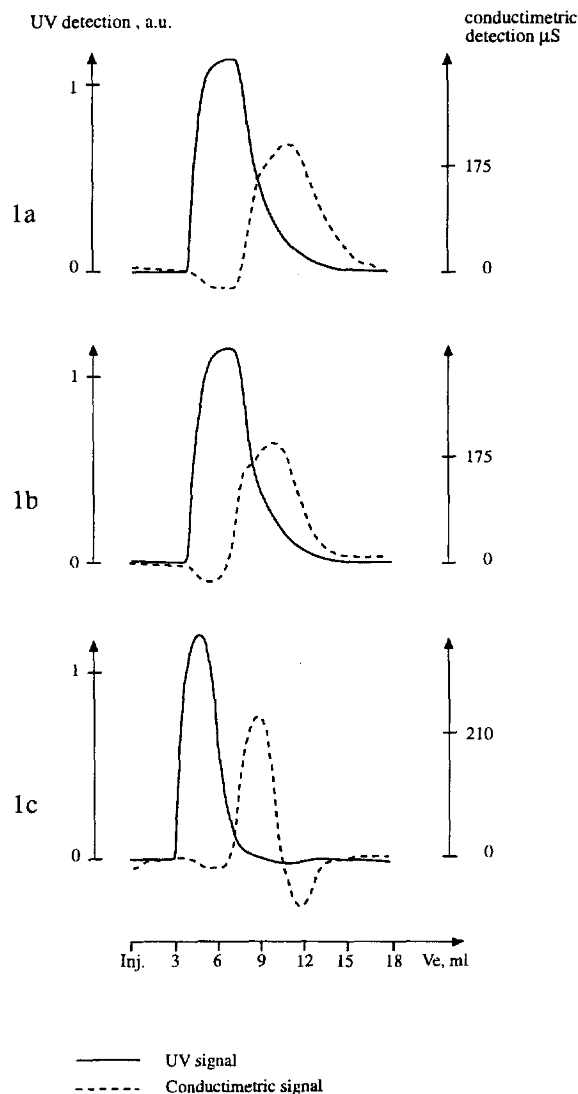

Fig. 1. Hummel and Dreyer method. Chromatograms obtained simultaneously with UV (full line) and conductimetric (dotted line) detection. The UV detector shows BSA signal (280 nm), and the conductimetric detector shows both BSA and Cu^{2+} ion responses. Experiments have been done at pH 5, in 10^{-2} M acetate buffer, flow-rate = 0.3 ml/min. (a) [BSA] = 2 g/l, $[Cu^{2+}]=0$; (b) [BSA] = 2 g/l, $[Cu^{2+}]=10^{-5}$ M; (c) [BSA] = 2 g/l, $[Cu^{2+}]=10^{-3}$ M.

Thus, in order to measure the amount of bound Cu^{2+} at a known protein concentration, it appeared that only the positive zone could be taken into account. It was then necessary to reach a plateau on the positive peak, provided that protein concentration was con-

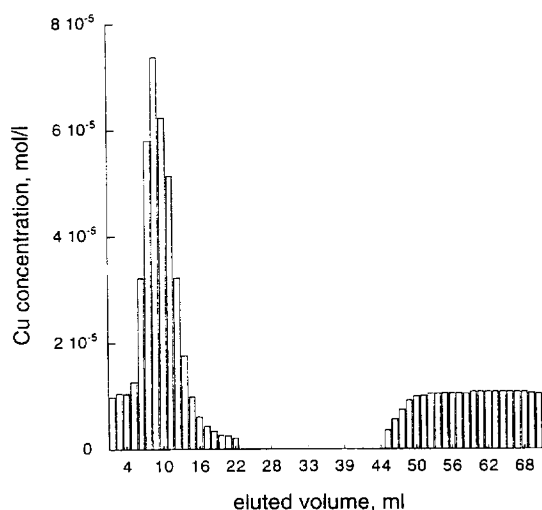

Fig. 2. ICP-AES analysis of fractions (1 ml) collected throughout a chromatographic experiment. [BSA]=2 g/l, $[Cu^{2+}]=10^{-5}$ M, 10^{-2} M acetate buffer pH 5, flow-rate=0.3 ml/min.

stant in this zone. For this purpose, lower eluate fractions were collected (0.4 ml).

A plateau in copper concentration was then clearly obtained, as can be seen in Fig. 3. Independent absorbance measurements at 280 nm (see Section 2.3) confirmed that the positive peak did correspond also to a plateau in protein concentration.

As shown on Fig. 4, bound copper concentration is

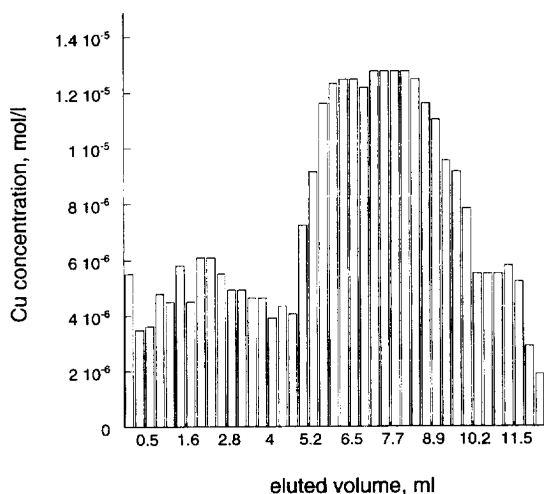

Fig. 3. ICP-AES analysis of fractions (0.4 ml) collected in the first half of a chromatographic experiment. [BSA]=2 g/l, $[Cu^{2+}]=5\cdot10^{-6}$ M, 10^{-2} M acetate buffer, pH 5, flow-rate=0.3 ml/min.

deduced from the positive zone, by measuring the difference between the plateau and the baseline corresponding to free Cu concentration, which was fixed by the eluent. This experiment was repeated for several free Cu concentrations and a given protein concentration (2 g/l) at pH 5.

Fig. 5 reports these results, as the percentage of bound copper versus free copper concentration. This graph represents the copper binding capacity of BSA, at constant protein concentration (2 g/l). It shows that maximum binding (98%) occurs when copper concentration around the protein is low, typically 10^{-7} M in our set of experiments.

The binding profile can also be represented as a Scatchard plot (Fig. 6). For this purpose, we consider the equilibrium

$$P + n\mathrm{Cu} \Leftrightarrow P\text{-}\mathrm{Cu}_n$$

It is then assumed that the protein has n identical binding sites for the ligand (copper), and that the sites do not interact in any way. It can be shown that the complexation constant K (M^{-1}) is related to the binding ratio r, defined as the ratio of the bound copper concentration to the BSA concentration, by the following relationship:

$$\frac{r}{[\mathrm{Cu}]_{\mathrm{free}}} = nK - Kr$$

The plot $r/[\mathrm{Cu}]_{\mathrm{free}}$ versus r allows us to determine the thermodynamic parameters of the copper–BSA complex: complexation constant K and number of affinity sites n.

Results of former studies suggested the presence of two sets of binding sites [23,24]. Our analysis clearly brings out 2 different slopes which indicate the presence of high and low affinity sites. An estimation of K, the complexation constant, and n, the number of binding sites, gives for the high affinity sites $K_1=3.5\cdot10^6$ M^{-1} and $n_1=1$ and, for the low affinity sites, $K_2=3\cdot10^3$ M^{-1} and $n_2=17$ sites. These results correlate well with the literature data [20–22,24,28,33,39], although the high affinity constant measured has a lower value than those commonly found. This comes from our inability to carry out experiments in the high affinity domain (very low copper concentrations) as the copper detection limits were reached. The complexation

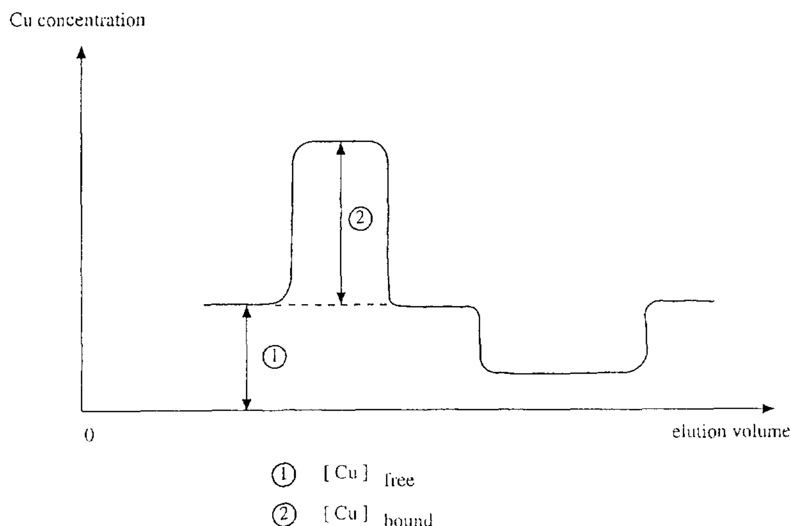

Fig. 4. From ICP-AES data, bound copper concentration is deduced by measuring the difference between the positive plateau and the baseline. The baseline represents free Cu concentration, which is kept constant throughout each chromatographic experiment.

constant measured could thus be underestimated. Nevertheless, the finding of one high affinity site and seventeen low affinity sites is in good agreement with the known structure of BSA: it has now been clearly established that the first Cu(II) is strongly bound to the N-terminal square-planar site on albumin, formed by the terminal amino-group, two deprotonated peptide amino nitrogens and the imidazole nitrogen from His 3 (sequence Asp–Thr–His) [11,43,44].

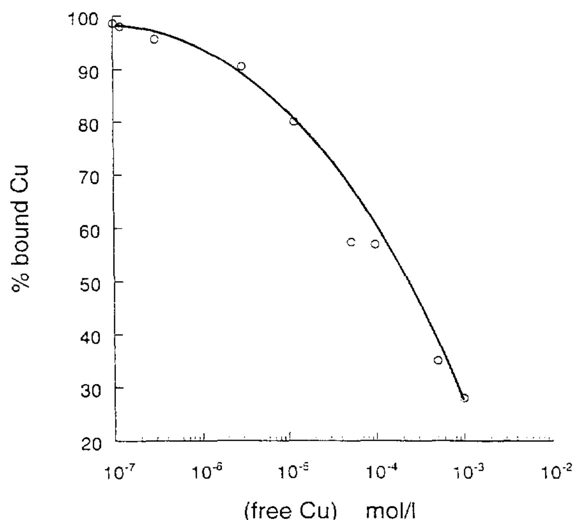

Fig. 5. Percentage of bound copper versus free copper concentration. [BSA]=2 g/l, 10^{-2} *M* acetate buffer, pH 5.

The seventeen low affinity sites have not been so fully characterized; however, imidazole residues of histidine in BSA seem to be mainly involved in the formation of those less stable chelates [20,22], although some neighbouring groups may also interact with copper ions. Thus, our experimental results prove the validity of this method to characterize metal–protein complexes, provided a number of conditions are fulfilled.

Firstly, the pH value of the solution must be under the pH of onset of precipitation [42]. Some previous investigators avoided this problem by presenting the metal in the form of a well-characterized chelate [24,25].

Moreover, the large zone Hummel and Dreyer method can only be applied if the complex and the free species have elution volumes V_e different enough to distinguish the positive plateau and the negative one ($V_{e_{BSA}}$ =4 ml and $V_{e_{Cu^{2-}}}$ = 11 ml in our study).

The choice of the stationary phase is another determining factor for a good resolution of the metal–protein complex. As a matter of fact, strong adsorption of metallic species onto the gel would lead to erroneous results. The metal concentration measured in the first eluate fractions, if similar to the

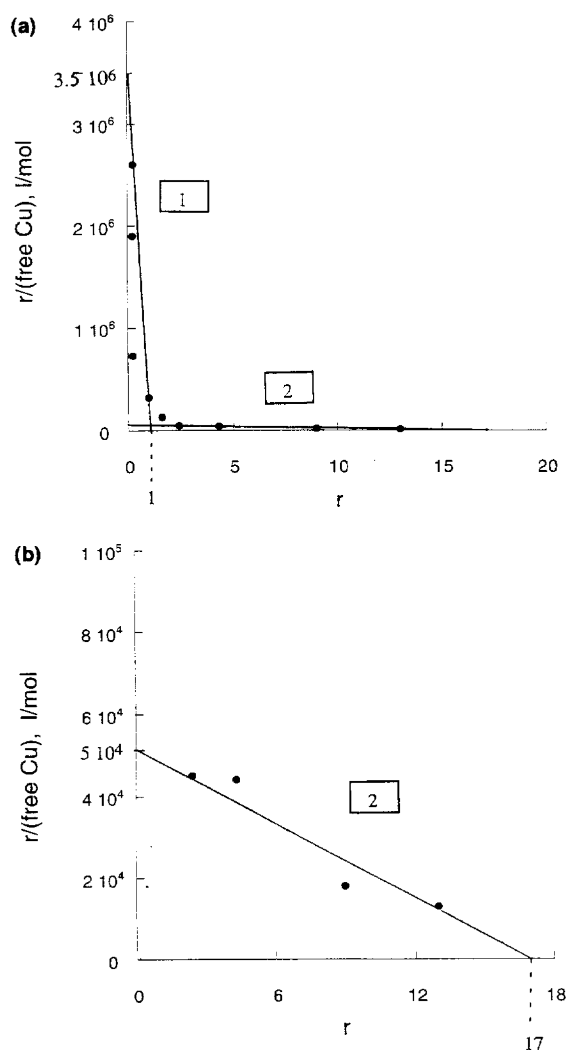

Fig. 6. Scatchard plot of Cu(II) binding to BSA. (a) Plot over the entire $r/[Cu]_{free}$ range. Regions (1) and (2) where linear fits have been drawn correspond respectively, to high and low affinity zones. (b) Extension of the low affinity zone.

metal concentration in the injected solution, ensures that no irreversible gel adsorption has occurred.

Besides those chromatographic constraints, the detection limits of the ICP-AES technique must be taken into account. Although a few metallic species cannot be precisely measured, this method allows detection of low concentrations (1 μg/l for copper) of a wide variety of metals, whose signal would not be measurable on usual chromatographic detectors, particularly when associated with a protein.

4. Conclusions

From the results reported here, it is apparent that gel chromatography followed by ICP-AES provides a simple and valuable method for the detection and characterization of metal–protein complexes.

This method has been tested on the copper complex of serum albumin, which had already been studied by other experimental techniques. Results of our study are found to be in good agreement with previous studies on this complex.

The chromatographic procedure involves the use of a modified Hummel and Dreyer method, which precisely locates the elution zone of the metal–protein complex at a known protein concentration. The metal content of appropriate fractions is then analysed by ICP-AES. This large-zone Hummel and Dreyer method determines, by maintaining protein concentration constant, the binding capacity of the protein. This was not possible for the original Hummel and Dreyer method, as a small injection volume does not ensure that protein concentration remains constant when metal binding occurs.

The metal binding capacity of a protein is therefore accurately determined by the combination of gel chromatography and atomic emission spectrometry, whereas the thermodynamic parameters describing the high affinity domain can be underestimated. This is due to the detection limits of the ICP-AES technique.

This method can be applied to a wide variety of metal–protein complexes, as the use of ICP-AES allows the detection of numerous metallic ions.

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