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Preferential Solvation of Lysozyme and Bovine Serum Albumin in Copper Salt Solutions. A Quantitative Chromatographic Study

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Abstract

Preferential solvation λ parameters for systems containing water–copper salt–protein (lysozyme or bovine serum albumin) have been determined by gel permeation chromatography. When water is preferentially adsorbed by the protein, good agreement is found between λ values determined by this method and by equilibrium dialysis-differential refractometry. The influence of the concentration and type of anion component of the copper salt, protein concentration and temperature has been investigated. The methodology used also allows direct visualization of the metal ion bound to the protein and to determine binding parameters. Apparent association constants of 2.0×10^2 M^{-1} and 1.7×10^2 M^{-1} have been obtained for the binding of copper nitrate to lysozyme at 30 and 4 °C, respectively.

Introduction

The biochemistry of copper and other metal ions has been a matter of interest in recent years, mainly due to the relationship existing between abnormally elevated levels of these ions and metabolic disorders [1–3]. Albumin is present in many biological fluids and binds a wide variety of organic and inorganic ligands [4]. Serum albumin is known to be the major copper binding protein in humans and different animal species. Complex formation between Cu(II) and serum albumin has been studied quite extensively using a variety of techniques [5–8]. Quantitative data on aluminium binding to human serum albumin [3] or cobalt binding to bovine serum albumin (BSA) have been recently published [9]. On the other hand, the interaction between Cu(II) and lysozyme, a carbohydrate hydrolitic enzyme, has been widely investigated [10–12] and has been also characterized in terms of binding parameters [13].

Gel permeation chromatography (GPC) is a very useful method for studying protein ligand interaction and, from its development by Hummel and Dreyer [14], it has been applied to the study of different systems under various experimental conditions [15, 16]. We have carried out several studies on protein–metal ion interactions by means of techniques such as dilatometry, equilibrium dialysis differential refractometery, and viscometry; these studies have demonstrated that copper(II) and cobalt(II) nitrate salts produce conformational alterations in lysozyme and BSA, depending on the metal ion and protein concentration, and that these effects can be determined by volume change, specific viscosity and preferential solvation parameter, λ, measurements [7, 9, 13].

This paper deals with the application of GPC, in Sephadex G-25 support, to the evaluation of λ parameters in ternary systems containing water **1**/copper salt **2**/protein **3** (lysozyme or BSA). Two copper salts (nitrate and chloride) have been compared and factors such as copper salt concentration, protein concentration and temperature have been also investigated. The analysis of the chromatograms obtained by using spectrophotometry with a copper salt selective wavelength allows the determination of copper binding parameters for the proteins.

Experimental

Hen egg white lysozyme (three-times recrystallized L-6876 lot No. 57C-8025) and bovine serum albumin (crystallized and lyophilized A-4378 lot No. 38C-8160) were purchased from Sigma (St. Louis, Mo, U.S.A.) and were used without further purification. Sephadex gel (G-25) fine and Blue Dextran 2000 were from Pharmacia (Uppsala, Sweden). Analytical

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reagent grade copper salts were from Merck (Darmstadt, F.R.G.).

Gel permeation chromatography was performed in a Wright 13/30 column. Samples were directly monitored at 254 nm with a LKB detector, and occasionally with a Beckman spectrophotometer model UV-5260 for visible detection. The flow rate was controlled with a LKB peristaltic pump. All solutions were carefully degassed before use.

The column was packed according to the instructions from manufacturer [17], using accurately weighted portions of Sephadex swollen for 24 h in the appropriate eluent. The column was equilibrated with each eluent according to the Hummel and Dreyer's method [14] at the desired temperature (4 or 30 °C). All the eluents contained copper salt solutions in twice-distilled water.

Before injecting protein samples, several solutions of copper salt were injected, corresponding to known excess of absolute amount of copper salt, Δm°, in mol/l. A calibration plot of Δm° *vs.* h° was obtained, h° being the height of the peak appearing in the chromatogram. A small amount of protein (5 or 10 mg) dissolved in 100 ml of the same equilibrating solution was injected onto the column and then eluted by the copper solution used for equilibration. The copper salt excess peak in the chromatogram was related to the corresponding Δm from the calibration plot. λ parameters were then calculated in a manner similar to that previously described [18, 24]. Eluent salt concentrations varied from 1.43×10^{-2} to 7.18×10^{-2} M.

Each measurement was repeated three times and the average values were calculated. The relative mean deviation was in all cases lower than 2% for Δm values and lower than 1.5% for elution volume measurements.

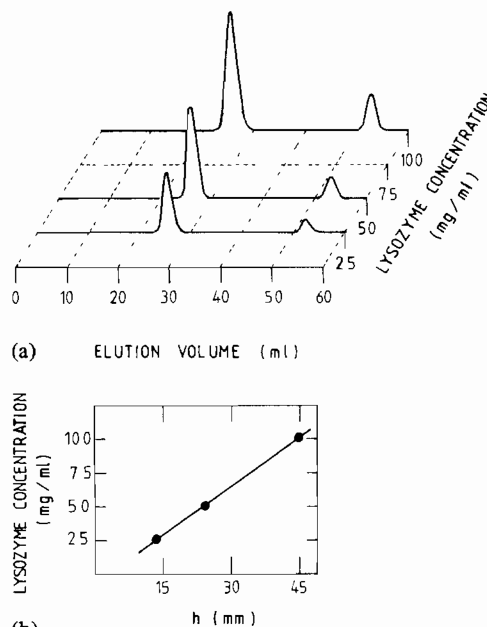

Fig. 1. (a) Chromatograms corresponding to lysozyme injections at concentrations 2.5, 5.0 and 10 mg/ml for a 1.43×10^{-2} M copper nitrate solution as eluent. (b) Dependence of the copper nitrate excess peak height, h, on lysozyme concentration.

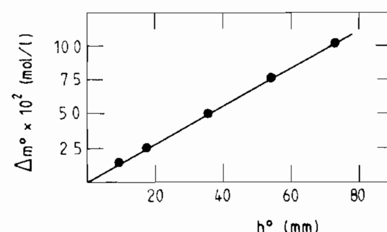

Fig. 2. Calibration curve of excess increments of copper nitrate, Δm°, in molar concentration *vs.* peak heights, h°, in mm, for a 1.43×10^{-2} M copper nitrate solution as eluent. Column temperature 30 °C

Results and Discussion

In Fig. 1a are shown as an example of the chromatograms of lysozyme at different protein concentrations, with a 1.43×10^{-2} M copper nitrate solution as eluent, monitored at 254 nm and 30 °C. The first eluting peak appearing at an elution volume (V_e) of 25 ml corresponds to the solvated protein and the second one to an excess of copper nitrate ($50 < V_e < 52$ ml). As depicted in Fig. 1b, the heights of the excess peaks are proportional to the amount of injected lysozyme. Similar patterns are obtained for BSA in the same experimental conditions.

Figure 2 shows a typical calibration curve of excess peak heights, h°, in mm, *versus* copper nitrate excess, Δm°, in molar concentration, for a 1.43×10^{-2} M copper nitrate solution as eluent at 30 °C.

The preferential solvation parameter λ has been classically measured by equilibrium dialysis differential refractometry [9, 13, 19], light scattering [20, 21] and more recently by GPC. The evaluation of λ by HPLC has been described by several authors [22, 23] and by ourselves for ternary systems solvent **1**/solvent **2**/macromolecule **3** [18] and solvent **1**/solute **2**/solute **3** [24]. According to these papers, λ is expressed as the excess or defect in volume fraction of one of the components in the domain of the macromolecule with respect to the bulk solvent, once the thermodynamic equilibrium has been attained. Negative values of λ are assigned when preferential solvation of component **3** (protein) by component **2** (copper salt) occurs. Positive λs imply preferential solvation by component **1** (water).

From the heights of the copper nitrate excess peaks in the chromatograms (Fig. 1a) and the corresponding calibration curves (Fig. 2), λ parameters can

be obtained. λ values deduced in this way are positive; this indicates, as explained above, that lysozyme is preferentially solvated by water. A density of 2.3255 g/ml was taken for copper nitrate salt in λ calculations.

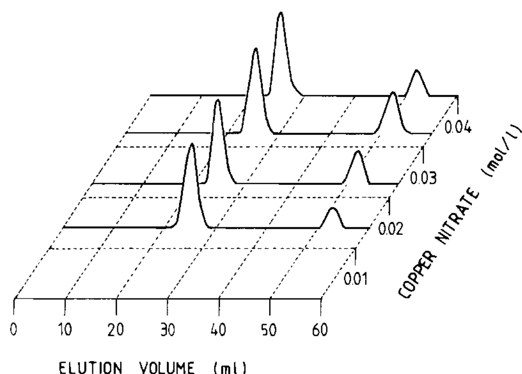

Fig. 3. Elution profiles of lysozyme injected at 5 mg/ml in eluents of copper nitrate concentration ranging from 1.43 × 10^{-2} to 4.00 × 10^{-2} M. Elution conditions are as described in the Materials and Methods Section. Column temperature: 30 °C.

Figure 3 shows the chromatograms obtained for lysozyme, injected at a concentration of 5 mg/ml, in eluents of different copper nitrate compositions at 30 °C. In order not to overcrowd the figure, only four chromatograms have been included, but more eluent compositions were assayed. The areas of copper nitrate excess peaks do not increase linearly with the amount of copper salt in the eluent. The increases and decreases in these areas with increasing salt concentration can be related to changes in the preferential solvation of lysozyme by water in the range of copper nitrate concentrations used.

In a similar manner as described above, λ parameters have been calculated for each eluent at 30 °C. The same experimental procedure produces the values of λ at 4 °C. The variation of λ is plotted in Fig. 4 as a function of eluent copper nitrate concentration at 30 °C (a) and 4 °C (b), at injected lysozyme concentrations of 5 and 10 mg/ml. It can be observed that λ is always positive at both temperatures for all eluent compositions used, which implies a preferential solvation of lysozyme by water in all cases. At 30 °C (Fig. 4a), λ increases with increasing metal concentrations until a maximum is attained at ≃3.00 × 10^{-2} M copper nitrate, and then it decreases, reaching a minimum at concentrations about 4.3 × 10^{-2} M. These changes are less pronounced as the protein concentration increases. If these data are compared with those obtained by means of equilibrium dialysis differential refractometry [13], a very good agreement both qualitatively and quantitatively can be noted. The increases or decreases in λ, that is, the higher or lower

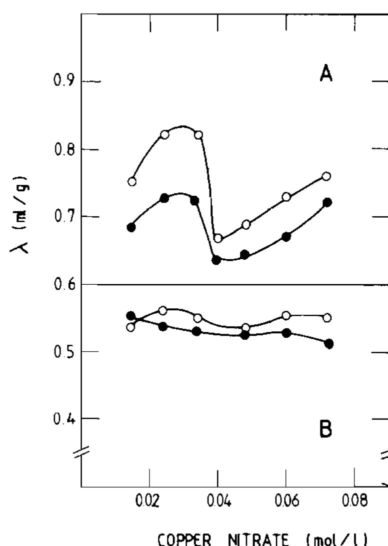

Fig. 4. Preferential solvation parameter λ *vs.* eluent copper nitrate concentration at 30 °C (a) and 4 °C (b). Lysozyme concentration: 5 mg/ml (○) and 10 mg/ml (•).

preferential solvation by water, depends on copper concentration and could be related to structural changes in the protein [9, 13, 25].

λ values at 4 °C, although positive, are smaller than those determined at 30 °C, the variations with copper concentration being less significant. This behaviour could be attributed to the existence of two different conformational states of the protein; it is known that lysozyme in solution undergoes structural changes as a function of temperature. The transition is totally reversible and affects a limited but substantial part of the molecule [26, 27].

Similar experiments with BSA as component **3** have been performed in a range of eluent copper nitrate concentrations varying from 1.43 × 10^{-2} to 6.00 × 10^{-2} M. Figure 5 shows the variation of λ as a function of eluent composition for an injected protein concentration of 10 mg/ml at 30 and 4 °C. A preferential solvation by water is also observed for this protein, λ parameters being smaller than those determined by lysozyme in all cases. On the other hand, in contrast with the above results for

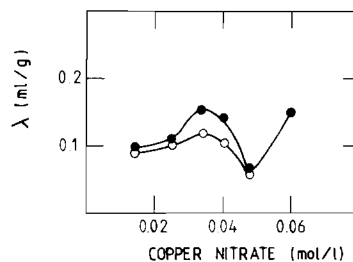

Fig. 5. λ parameter *vs.* eluent nitrate concentration at 30 °C (•) and 4 °C (○). BSA concentration was 10 mg/ml.

lysozyme, the values of λ did not vary appreciably when the temperature decreases (Fig. 5). Qualitatively the variation of λ at 30 °C as a function of copper salt concentration is similar to that previously described with cobalt nitrate, as deduced from equilibrium dialysis-differential refractometry [9].

In order to determine whether the anion component of the copper salt has some effect on the preferential solvation of these proteins, BSA or lysozyme, a parallel study was carried out with copper(II) chloride under the same experimental conditions. The λ values for both proteins for eluents of different copper chloride concentrations are summarized in Table I. These values have been deduced from the corresponding chromatograms and calibration plots as mentioned above.

TABLE I. λ Values Obtained for Water/Copper Chloride/Protein System from Chromatograms at Different Eluent Compositions at 30 and 4 °C[a]

Eluent $CuCl_2$ concentration $\times 10^2$ (mol/l)	Lysozyme			Albumin		
	C_3 (mg/ml)	$\lambda \times 10^2$ (ml/g)		C_3 (mg/ml)	$\lambda \times 10^2$ (ml/g)	
		30 °C	4 °C		30 °C	4 °C
1.43	5	10.8	3.8	10	−0.6	−0.7
	10	8.6	3.4			
2.39	5	10.7	4.7	10	−0.9	−1.8
	10	7.9	3.5			
3.34	5	8.7	4.5	10	−1.4	−2.1
	10	7.2	3.8			
4.00	5	7.7	3.6	10	−1.5	−1.8
	10	7.7	2.1			
4.78	5	7.1	2.6	10	−0.6	−1.3
	10	7.1	2.5			

[a]C_3 is the injected protein concentration. λ parameter for each eluent is the mean of the values obtained for three injected protein concentrations.

λ parameters for BSA are always negative; this means that, in contrast with lysozyme, BSA is preferentially solvated by the copper salt under these conditions. On the other hand, although λ values are positive for lysozyme in copper chloride solutions, a decrease of about 90% in their absolute value is observed relative to the corresponding copper nitrate eluent (Fig. 4). If we compare both copper salts, the experimental evidence indicates that for the same molar composition there is a higher excess of water molecules (component **1**) relative to the number of molecules of the component **2** in the solvation shell of lysozyme in the case of the copper nitrate salt, and the same is observed for BSA. These differences can be attributed to a greater difficulty for the interaction of the nitrate anion relative to the

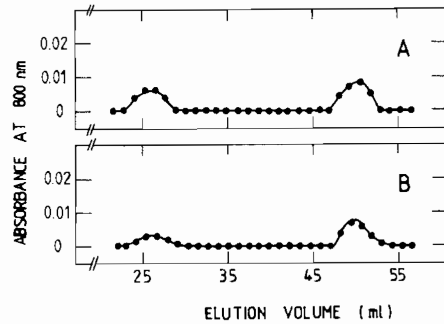

Fig. 6. Elution profile of lysozyme injected at a concentration of 10 mg/ml for a 1.43×10^{-2} M copper nitrate solution as eluent. (a) Column temperature: 30 °C. (b) Column temperature: 4 °C.

chloride one due to its larger size and to a possible lower ionic mobility.

So far, all the results have been deduced from chromatograms monitored directly at a wavelength of 254 nm. However, for lysozyme, eluate fractions were also spectrophotometrically monitored at 800 nm. Figure 6 shows, as an example, the elution pattern for lysozyme in an eluent containing copper nitrate at a concentration of 1.43×10^{-2} M, at 30 °C; at this wavelength, the protein itself does not absorb and the visible absorption maximum at 27 ml is due to the metal ion bound to the protein. The excess peak at ≃50 ml allows the determination of λ parameters using the corresponding internal calibration at this wavelength (Fig. 7). Similar experiments with copper chloride as eluent have also demonstrated the same two peaks, which are observed for all the eluent compositions assayed.

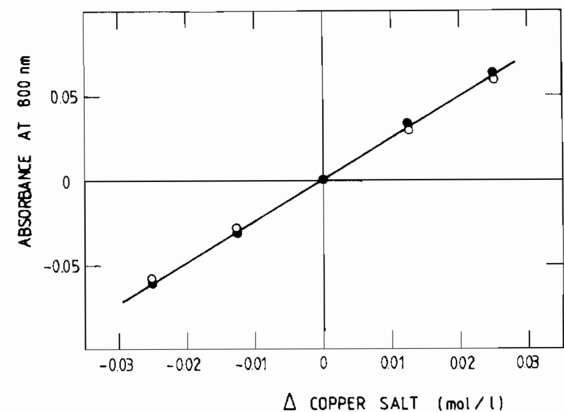

Fig. 7. Internal calibration. Absorbance at 800 nm as a function of the copper salts excess or deficiency (relative to eluent concentration) injected onto the column. Eluent salt concentration: 1.43×10^{-2} M. (•) copper nitrate; (○) copper chloride. Column temperature: 30 °C.

From the areas in the chromatograms, and knowing the molar extinction coefficient of the copper salt solutions and the molar extinction coefficient for the protein–copper complexes, it is possible to determine the binding ratios, $\bar{r}$ [16]. The molar extinction coefficient for lysozyme–copper complexes has been extrapolated from spectrophotometric measurements as a function of protein concentration at a fixed low copper salt concentration. Mean values of 13.1 M^{-1} and 12.4 M^{-1} have been obtained for lysozyme–copper nitrate complexes at 30 °C and 4 °C, respectively. The molar extinction coefficient for copper nitrate solutions was deduced from the calibration curves (Fig. 7 and results not shown). $\bar{r}$ values were then calculated from the chromatograms at different eluent salt compositions. Some of the Scatchard plots obtained are presented in Fig. 8, at 30 °C (a) and 4 °C (b). From the analysis of these plots, apparent association constants of 2.0×10^2 M^{-1} and 1.7×10^2 M^{-1} were deduced at 30 and 4 °C, respectively, for high affinity sites. *n* values varied from 10 to 5 as temperature decreased. The results at 30 °C deduced from chromatography were the same as those previously described using equilibrium dialysis-differential refractometry [13].

The Scatchard plots for each temperature with the copper chloride salt as eluent have a shape similar to those mentioned above (Fig. 8), the $\bar{r}$ values being slightly smaller in this case (results not shown). The observed differences are probably due to the fact that the pH values for copper chloride solutions are 0.50 units lower than the corresponding values for copper nitrate ones. As pH is increased, the affinity of the protein for copper(II) increases so that $\bar{r}$ is higher for the copper nitrate salt.

In summary, the results of these chromatographic experiments confirm previous data obtained for lysozyme [13] and albumin [9] deduced by means of equilibrium dialysis and differential refractometry. A preferential solvation by water is observed in all cases in lysozyme–copper(II) salt systems (Fig. 4), λ parameters at 30 °C being of the same order of magnitude as those previously described [13]. The observation that λ parameters at 4 °C differ with respect to those at 30 °C is consistent with the reported structural changes of lysozyme in solution as a function of temperature [26, 28].

On the other hand, the results for BSA in copper nitrate solutions did not show temperature dependence (Fig. 5). The decrease in λ for BSA relative to lysozyme means that BSA is less preferentially solvated by water; thus even a change in the sign of λ is observed for copper chloride solutions (Table I), indicating that the copper salt is preferentially solvated in these conditions.

The dependence of λ for both proteins upon concentration of copper nitrate salt at 30 °C also support the idea that these two proteins undergo a structural change, probably due to an indirect effect of the salt which alters solvent properties [9, 29].

Although lysozyme and BSA have shown a preferential solvation by water (Figs. 4 and 5), they also interact with the copper salt as can be seen in the elution pattern of Fig. 6. The chromatographic analysis allows, in this case, the direct visualization of the protein–copper salt complexes and the determination of binding parameters which are in agreement with those deduced indirectly using spectroscopic techniques [9, 13].

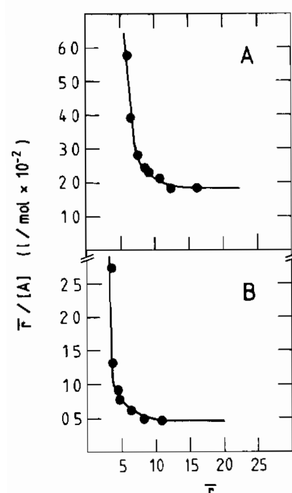

Fig. 8. Scatchard plots for lysozyme–copper nitrate system. (a): 30 °C. (b) 4 °C. Protein concentration: 7.14×10^{-4} M.

References

1 R. Milanino, E. Passerella and G. P. Velo, in G. D. Weissmann, B. Samuelsson and R. Paoletti (eds.), 'Advances in Inflammation Research', Vol. 1, Raven Press, New York, 1979, p. 281.
2 D. Shore and R. J Wyatt, *J Nerv. Ment. Dis.*, *171*, 553 (1983).
3 R. L. Bertholf, M. R. Wills and J. Savory, *Biochim. Biophys. Res. Commun.*, *125*, 1020 (1984).
4 W W. Sukow, H E. Sandberg, E. A. Lewis, D. J. Eatough and L. D. Hansen, *Biochemistry*, *19*, 912 (1980).
5 F R. N. Gurd, in 'Physical Principles and Techniques in Protein Chemistry, Part B', Academic Press, New York, 1970, p. 365.
6 D. V Naik, C. F. Jewell and S. G. Schulman, *J. Pharm. Sci.*, *64*, 1243 (1975).
7 C. Abad, M. Trueba, J. M. Macarulla and A Campos, *An. Quim.*, *76*, 171 (1980).
8 P. Mohanakrishnan and C. F. Chignell, *J. Pharm. Sci.*, *71*, 1180 (1982).
9 M. Trueba, A. Vallejo, A. Zatón and C. Abad, *Inorg. Chim. Acta*, *107*, 133 (1985)
10 V. Teichberg, N. Sharon, J. Moult, A. Smilansky and A. Yonath, *J. Mol. Biol.*, *87*, 357 (1974).
11 R. E. Lenkinski, D G Agresti, D. M. Chen and J. G. Glickson, *Biochemistry*, *17*, 1463 (1978).
12 M. Yamada, F. Vozumi, A. Ishikawa and T. Imoto, *J. Biochem.*, *95*, 503 (1984).

13 C. Abad, M. Trueba, A. Campos and J. E. Figueruelo, *Biophys. Chem.*, *14*, 293 (1981).
14 J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, *63*, 530 (1962).
15 F. Sawada, Y. Kanesaka and M. Ikie, *Biochim. Biophys. Acta*, *479*, 188 (1977).
16 B. Sebille, N. Thuaud and J. P. Tillement, *J. Chromatogr.*, *167*, 159 (1978).
17 T. C. Laurent and J. J. Killander, *J. Chromatogr.*, *2*, 317 (1964).
18 A. Campos, L. Borque and J. E. Figueruelo, *J. Chromatogr.*, *140*, 219 (1977).
19 I. Katime, A. Campos and J. M. Teijon, *Eur. Polym. J.*, *15*, 291 (1979).
20 A. Dondos and H. Benoit, *J. Polym. Sci.*, *15*, 137 (1977).
21 P. Aptel, J. Cuny, J. Jozefowiez, G. Morel, J. Neel and B. Chaufer, *Eur. Polym. J.* *14*, 595 (1978).
22 B. Chaufer, J. Lesec and C. Quivoron, *C.R. Acad. Sci. Paris*, *284*, 881 (1977).
23 B. Chaufer, J. Lesec and C. Quivoron, *J. Liq. Chromatogr.*, *2*, 633 (1979).
24 C. Abad, L. Braco, J. E. Figueruelo and F. M. Goñi, *J. Liq. Chromatogr.*, *7*, 1567 (1984).
25 J. Span, L. Lenarcic and D. Lapanje, *Biochim. Biophys. Acta*, *395*, 311 (1974).
26 J. Berthou and P. Jolles, *Biochim. Biophys. Acta*, *336*, 222 (1974).
27 P. J. Cozzone, S. J. Opella, O. Jardetzky, J. Berthou and P. Jolles, *Biochim. Biophys. Acta*, *491*, 354 (1977).