Biophysical Chemistry 14 (1981) 293–300
Elsevier/North-Holland Biomedical Press

DILATOMETRIC, REFRACTOMETRIC AND VISCOMETRIC STUDY OF LYSOZYME-CATION INTERACTION

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Received 20 November 1980

The interaction between hen egg-white lysozyme and Cu(II) or Co(II) cations has been studied by dilatometry, equilibrium dialysis-differential refractometry and viscometry at different metal cation concentrations. δV isotherms in copper and cobalt solutions have been obtained from dilatometry. Preferential adsorption parameters and specific viscosity have been determined from refractometric and viscosimetric measurements. It has been observed that this interaction produces structural alterations in lysozyme. The magnitude of these conformational changes depends on the metal ion and protein concentration. The results obtained using the three techniques are in good agreement.

1. Introduction

Hen egg-white lysozyme (EC 3.2.1.17) is a low molecular weight enzyme with a known amino acid sequence [1,2]; its structure has been determined by X-ray crystallographic analysis [3–6], and it has been extensively studied from other points of view [7–10]. The mechanism of its enzymatic activity has been also established [11–14]. In solution, lysozyme has been shown to bind polyvalent cations [15–20]. Metal ions seem to bind carboxyl groups of Glu-35 and Asp-52 in the active site of the enzyme [6,18,21,22], and they often act as inhibitors [18,20,21,23]. Conformational studies on the photooxidized derivatives of lysozyme demonstrated that the inhibitory action occurs almost throughout the entire protein molecule and caused no appreciable perturbation of the protein spatial geometry [24]. On the other hand, X-ray data indicate coformational perturbations associated with metal binding apparently confined to the immediate vicinity of the metal-binding site [22]. Conversely, the results of Agresti et al. [25] and Lenkinski et al. [26] which provided a quantitative method for determining the site of metal complexation provide evidence that such complexation did not alter significantly the overall tertiary structure of the macromolecule.

The study of volume changes associated with the reaction of proteins with metal ions may yield information concerning the solvation and conformation of these molecules. With polyelectrolytes the volume changes may also reflect local conformational changes [27]. Changes in solvation are reflected in changes in the partial molar volumes [28,29].

The aim of the present study is to demonstrate that Cu(II) and Co(II) cations produce conformational alterations in lysozyme and that this effect can be determined by volume change (δV), specific viscosity (η_{sp}/c), and preferential adsorption parameter (λ) measurements.

0301-4622/81/0000–0000/$02.50

2. Materials and methods

Lysozyme used in this study was three-times recrystallized hen egg-white lysozyme L-6876, lot No. 57C-8025 obtained from Sigma Chemical Co., and was used without further purification; the purity of the preparation was checked by polyacrylamide gel electrophoresis.

A molecular weight of 14000 for lysozyme was used for calculations. Analytical reagent grade metal nitrates and KCl were obtained from Merck and Darmstadt. All solutions were carefully degassed before use.

A dilatometer was constructed according to Komiyama et al. [30]. The respective volumes of the lower and upper compartments were 8.92 and 9.72 ml. An additional inlet was provided, with a Quickfit stopper. The capillary tube was 0.3 ± 0.01 mm I.D.

Volume changes at 30.0 ± 0.01°C were measured according to the following procedure. Solution I, containing Cu(II) or Co(II) in concentrations ranging from 0.05 to 0.40 M in the lower compartment, and solution II, containing a 1 or 2% (w/v) solution of lysozyme in distilled water in the upper compartment, were mixed by opening the internal stopper and stirring for 2 min. The capillary meniscus height was read by means of a cathetometer with an accuracy of 0.01 mm.

The volume change, in microliters, produced by mixing both components, is referred to as ΔV. ΔV values were measured 5–15 min after mixing. Values reported here are averages of two or three experiments and could be reproduced to ± 0.07 µl, which leads to an uncertainty of ± 5 ml/10^5 g protein.

Blank experiments were run in which the Cu(II) or Co(II) solutions were mixed with water. Volume changes observed in these experiments were substracted from those obtained by adding the protein under the same conditions [31].

Differences in refractive index were measured with a Brice-Phoenix differential refractometer model BP-2000 V at 546 nm and 30°C, and employing a sealed-type differential cell with ground-glass stoppers to prevent solvent losses. Calibration was made with aqueous solutions of highly purified KCl taking the data of Kruis [32] as reference. The temperature difference between the bath and the cell did not exceed 0.02°C. Values of $(dn/dc)_\pi$ were determined by the method of Hertz and Strazielle [33] as described previously [34]. Values of $(dn/dc)_\mu$ were obtained from the refractive index differences between protein solutions and their dialysates.

The membranes used for equilibrium dialysis were Visking tubing type from Union carbide Corp., Chicago, and were pretreated as described by Cooper and Wood [35].

For determining total metal binding to protein, changes in concentration between the original metal solutions and the same solutions after dialysis equilibrium (approx. 3 h) were determined with a Beckman spectrophotometer model DB-GT at 722 nm for Cu(II) and 510 nm for Co(II) at 30°C.

Viscometric measurements were made using a modified Ubbelohde suspended level viscometer thermostatically maintained at 30°C. Solutions were subjected to dialysis equilibrium and experimental measurements were carried out comparing flow times of each dialyzed solution and the corresponding dialyzed solvent.

A Phillips pH meter model PR-9403-01 with a semi-micro combination electrode was used for pH measurements.

3. Results

Volume changes produced by the addition of Cu(II) or Co(II) to proteins are the sum of two major processes: cation-protein and cation-water interactions.

The volume effects due to cation-protein interaction are defined as follows:

$$\delta V = \frac{\Delta V_3 - \Delta V_2}{w_3} \times 10^5$$

where ΔV_3 is the volume change produced when a protein solution of defined volume and concentration (component 3), is mixed with an equally defined metal ion solution (component 2); ΔV_2 is the volume change due to cation-water interactions; w_3 is the protein weight in grams. The factor 10^5 refers this parameter to 10^5 g protein. Justification for using this equation was given by Katz and

Ferris [36] and the procedure used for analyzing the data is practically identical to that of Katz and Roberson [31].

Volume effects for lysozyme-Cu(II) and lysozyme-Co(II) interactions at protein concentrations 0.50% (3.57×10^{-4} M) and 0.25% (1.79×10^{-4} M) are shown in fig. 1. Volume effects are always positive and are a function of metal concentration, being higher for the lysozyme-Cu(II) system. It is observed that δV increases with increasing metal concentrations until a maximum is attained at 0.0334 M metal, which corresponds to 305 ml/10^5 g protein for 0.50% lysozyme-Cu(II) and 325 ml/10^5 g for 0.25% lysozyme-Cu(II). In the case lysozyme-Co(II) the maximum δV values are 99 and 144 ml/10^5 g for protein concentrations of 0.50 and 0.25%, respectively. For higher metal ion concentrations δV decreases, reaching a minimum at 0.0478 M. These changes are more pronounced as protein concentration decreases.

Volume effects determined at 2% protein concentration and 0.05 M Cu(II) or Co(II) are 362 and 125 ml/10^5 g, respectively. These values are in good agreement with those published by Katz and Roberson [31].

pH values after mixing range from 5.0 to 3.9 for lysozyme-Cu(II) systems and from 5.4 to 5.1 for lysozyme-Co(II) systems.

By means of absorption spectrophotometry the number of moles of ligand, copper or cobalt, bound to protein have been determined at 722 and 510 nm, respectively. The obtained values are expressed as $\bar{\nu}$ mol ligand bound/10^5 g protein. The results are shown in figs. 2 and 3 at different protein concentrations, as a function of total metal ion concentration (free + bound).

It can be seen that for lysozyme-Cu(II) (fig. 2) there is a steady increase in $\bar{\nu}$ up to 0.024 M, after this a plateau occurs, between 0.024 and 0.0334 M, increasing again markedly at the lower protein concentrations. This effect is higher at the lower protein concentrations. The increment of $\bar{\nu}$ between 0.033 and 0.048 M is coincident with the decrease in δV detected by dilatometry (fig. 1). For lysozyme-Co(II), $\bar{\nu}$ values are smaller, but in this case there is also a sharp incrase between 0.033

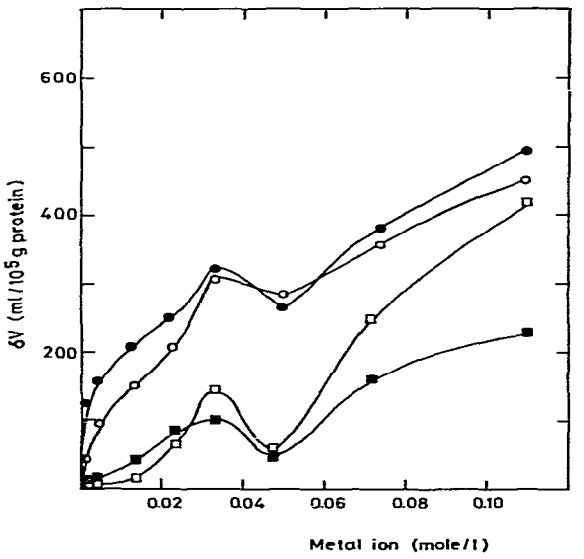

Fig. 1. δV isotherm produced by the reaction of lysozyme with Cu(II) (●, 0.25%; ○, 0.50%) and Co(II) (□, 0.25%; ■, 0.50%). Data are referred to 10^5 g protein.

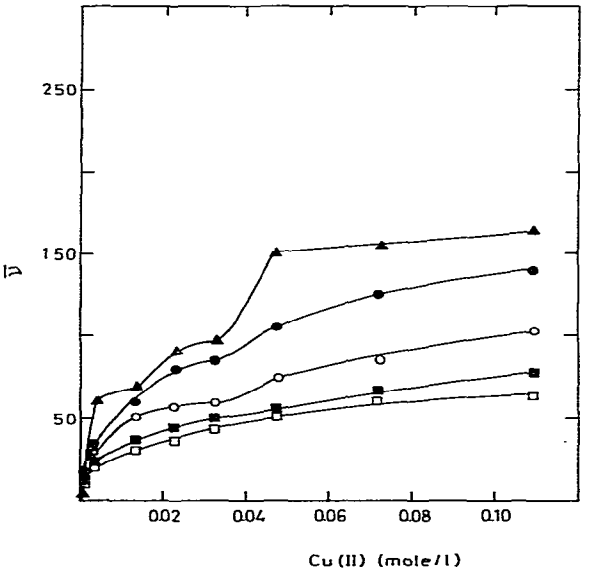

Fig. 2. Plot of $\bar{\nu}$ against total Cu(II) concentration. $\bar{\nu}$ values indicate mol Cu(II) bound to lysozyme/10^5 g protein. (▲) 0.125%, (●) 0.25%, (○) 0.50%, (■) 1%, (□) 2%.

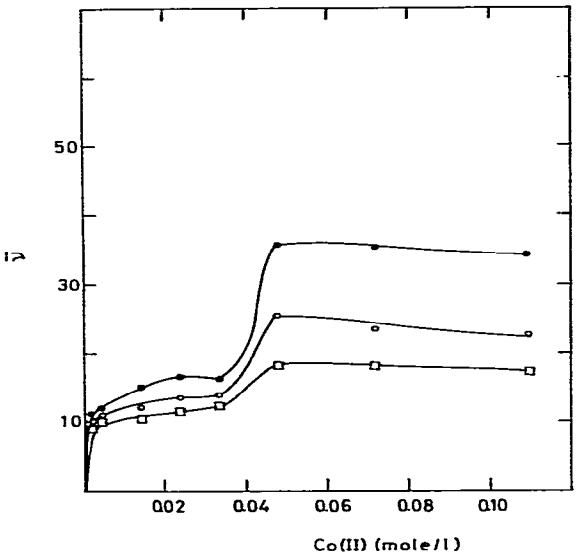

Fig. 3. Plot of $\bar{\nu}$ against total Co(II) concentration. $\bar{\nu}$ values indicate mol Co(II) bound to lysozyme/10 g protein. (●) 0.5%, (○) 1%, (□) 2%.

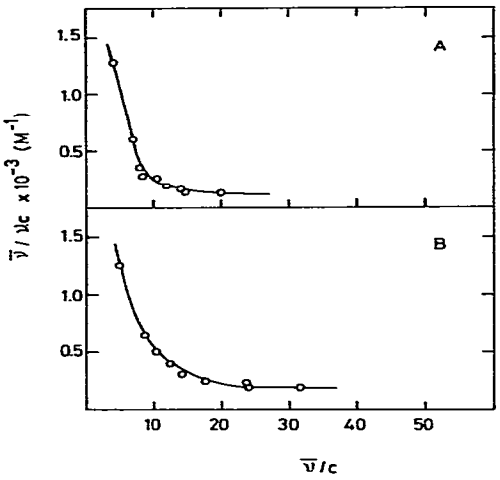

Fig. 4. Scatchard plots for lysozyme-Cu(II) systems. (A) 3.75×10^{-4} M. (B) 1.79×10^{-4} M.

and 0.048 M, correlated with a decrease in δV (fig. 1).

From $\bar{\nu}$ results and following the Scatchard procedure [37], the number of Cu(II)-binding sites, n, and the association constant, K_a, have been evaluated plotting $\bar{\nu}/\nu c$ versus $\bar{\nu}/c$, where ν is the free copper concentration and c the protein concentration, both expressed as molarities. Fig. 4 presents the results for 3.57×10^{-4} M (0.50%) and 1.79×10^{-4} M (0.25%) lysozyme. These results indicate that there are two types of sites: high-affinity sites equivalent and independent at low copper concentrations and low-affinity ones at higher Cu(II) concentrations. In table 1 n and K_a values for both protein concentrations are summarized; K_a values are referred to as high-affinity sites.

Association constants determined in this way are similar to those described by Teichberg et al. [18].

Dilatometric results have been compared to those obtained by the viscometric technique. Intrinsic viscosities of macromolecules (η) are proportional to their corresponding hydrodynamic volumes, therefore, an evaluation of their viscosity will give a qualitative idea of their macromolecular volume for different metal concentrations as have been used in this work.

Intrinsic viscosity is defined as:

$$(\eta) = \lim_{c\to 0} (\eta_{sp})/c$$

where (η_{sp}) is the specific viscosity of the macromolecule and c its concentration. The customary practice when employing protein is to employ the factor (η_{sp}/c) in view of difficulties intrinsic in the extrapolation procedure [38]. It should be noted (figs. 5 and 6) that $\eta_{sp})/c$ is calculated taking into account the observations of Reynolds et al. [38]. c is the concentration of protein + bound metal in g/ml. At high values of $\bar{\nu}$, the concentration in g/ml of protein plus bound metal is actually

Table 1
Values of the number of Cu(II)-binding sites and the association constants.

	Lysozyme (M)	
	3.75×10^{-4}	1.79×10^{-4}
n	10	11
K_a (M^{-1})	2.4×10^{2}	2.0×10^{2}

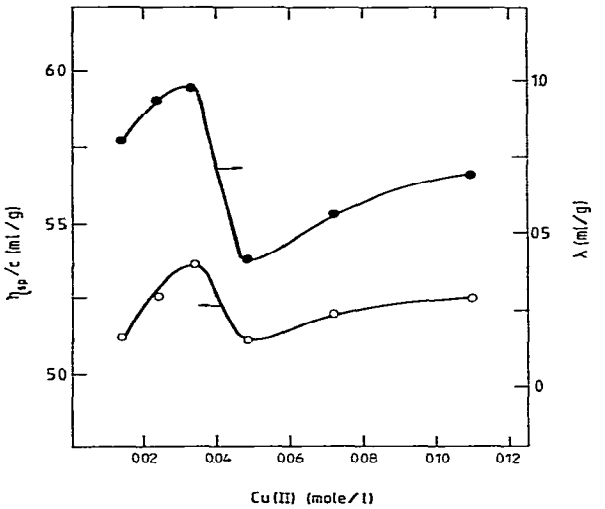

Fig. 5. Plot of $(\eta_{sp})/c$ and λ against Cu(II) concentration. (○) $(\eta_{sp})/c$ is indicated with an arrow towards the left ordinate; (●) λ is the preferential adsorption parameter and is indicated with an arrow towards the right ordinate. Protein concentration is 3.57×10^{-4} M.

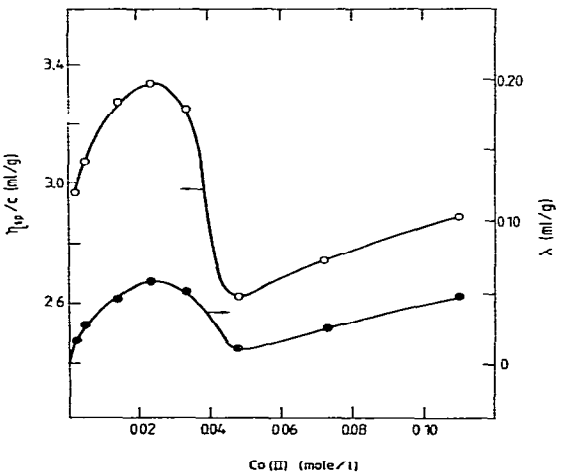

Fig. 6. Plot of $(\eta_{sp})/c$ and λ against Co(II) concentration. (○) $(\eta_{sp})/c$ is indicated with an arrow towards the left ordinate; (●) λ is the preferential adsorption parameter and is indicated with an arrow towards the right ordinate. Protein concentration is 3.57×10^{-4} M.

higher. For example, at protein concentration 5×10^{-3} g/ml and total metal concentration 0.0718 M, $\bar{v}\simeq 86$ for a Cu(II)-lysozyme complex (fig. 2); 0.0050 g/ml of protein is converted to 0.00604 g/ml of protein-bound metal. For low v values, the bound metal contribution will be small. Therefore, it will be necessary to take into account $\bar{v}$ values when calculating the complex protein-metal concentrations in order to evaluate the real viscosity $(\eta_{sp})/c$. Relative errors calculated for η_{sp}/c values are about 1–2%.

When comparing figs. 5 and 6 with fig. 1 it can be stated that $(\eta_{sp})/c$, from a qualitative point of view, is practically coincident with the variation of δV in the dilatometric measurement. At a total metal concentration of 0.0344 M a maximum for lysozyme-Cu(II) in $(\eta_{sp})/c$ (fig. 5) as well as in δV (fig. 1) is observed. The same phenomenon occurs when comparing both parameters for the system lysozyme-cobalt.

Similarly, we observe the existence of a minimum in $(\eta_{sp})/c$ (figs. 5 and 6) and δV (fig. 1) for a total metal concentration of 0.0478 M and for both systems lysozyme-Cu(II) and lysozyme-Co(II).

By means of dialysis equilibrium and differential refractometry, the preferential adsorption (or solvation) parameter λ has been obtained for both systems at a protein concentration of 3.57×10^{-4} M (figs. 5 and 6). It is well known that when thermodynamic equilibrium is attained between a ternary system solvent 1-solvent 2-macromolecule 3 and a binary system consisting of two solvents, there is a redistribution of solvent components due to a selective adsorption of one of the solvent components by the macromolecule [39,40]. This technique has been used by other authors [28,41–44] and recently by us in studies on proteins in dilute urea solutions [34]. The evaluation of λ has been explained in detail previously [34]. Positive λ values represent the preferential solvation of component 1 (water) by protein (component 3) and negative values, the preferential solvation of component 2 (metal ion).

Tables 2 and 3 summarize the experimental data needed for λ determination. u_{02} is the volume fraction of component 2 in the solvent mixture,

Table 2
Values obtained for lysozyme-cobalt system from equilibrium dialysis-differential refractometric measurements. $dn_0/du_2 = 0.024$; $\rho_{Co(NO_3)_2} = 1.8725$ (g/ml).

Equilibrium concentration (M)	u_{02} ($\times 10^{-2}$)	$(dn/dc)_\kappa$ (ml/g)	$(dn/dc)_\mu$ (ml/g)	λ (ml/g)
0.0024	0.004	0.195	0.192	0.103
0.0048	0.007	0.198	0.192	0.231
0.0143	0.022	0.197	0.189	0.326
0.0239	0.037	0.203	0.194	0.343
0.0334	0.052	0.196	0.188	0.334
0.0478	0.074	0.199	0.198	0.025
0.0718	0.112	0.195	0.192	0.142
0.1100	0.171	0.197	0.190	0.283

without protein and before dialysis. $(dn/dc)_\kappa$ and $(dn/dc)_\mu$ are variations of refractive index with protein concentration before and after dialysis respectively; dn/du_2 is the refractive index variation with the volume fraction of component 2 without protein. Correction methods as described by Noelken [43] and Span et al. [28] have not been used because under our experimental conditions significant changes between molar and molal scales have not been observed except for high cobalt concentrations but without qualitative changes. An absolute error of ± 0.03, for all the measures of λ, was estimated.

For all metal concentrations (or volume fraction of component 2) λ values are always positive (tables 2 and 3), this indicates that lysozyme is preferentially solvated by water (preferential hydration) in the ternary system water 1-metal 2-lysozyme 3. For lysozyme-Cu(II) the maximum water solvation occurs at 0.033 M total Cu(II), and the minimum at 0.048 M Cu(II). The same occurs for lysozyme-Co(II) system, but λ values are clearly lower. When comparing these results with δV values in fig. 1 we can see that δV variations are coincident with λ variations in the whole range of concentrations studied. The increases and decreases in δV seem to be related to the higher or lower preferential solvation by water.

Table 3
Values obtained for lysozyme-copper system from equilibrium dialysis-differential refractometric measurements. $dn_0/du_2 = 0.205$; $\rho_{Cu(NO_3)_2} = 2.3225$ (g/ml).

Equilibrium concentration (M)	u_{02} ($\times 10^{-2}$)	$(dn/dc)_\kappa$ (ml/g)	$(dn/dc)_\mu$ (ml/g)	λ (ml/g)
0.0143	0.148	0.203	0.039	0.80
0.0239	0.248	0.206	0.013	0.94
0.0334	0.347	0.207	0.006	0.98
0.0478	0.497	0.212	0.128	0.41
0.0718	0.746	0.217	0.102	0.56
0.1100	1.144	0.196	0.054	0.69

4. Discussion

Unlike protein-anion systems which produce positive or negative volume effects [45,46] the reaction of Cu(II) or Co(II) cations with lysozyme produces always positive volume effects (fig. 1). The slope and sign of the δV isotherm change as a function of cation concentration and the most pronounced effects are produced in the range 0.0334–0.048 M.

The protein contribution in the volume change is basically determined by both (a) the release of water from the binding site upon complex formation, and (b) structural changes of the protein. In principle, the volume rise δV can be explained in terms of Cu(II) or Co(II) bound to high-affinity sites through electrostatic interactions between metal and protein anionic groups with displacement of hydration water initially located around the ionic groups [31,47,48]. As described in previous works for protein-anion [45,49], protein-denaturing agent [29,48] and protein-cation systems [50], the change of sign in the slope of the δV isotherm could be due to a structural alteration of the protein; this change being more pronounced for the lowest protein concentrations (0.25%).

The observed changes in pH solutions are not related to structural changes since lysozyme is a protein exceptionally stable to pH variations as has been demonstrated by Aune and Tanford [51], Rasper and Kauzmann [52] and Katz and Miller [53]. pH contribution to the observed volume change can result in a small quantitative correction of the parameter evaluated for the protein-metal interaction. The NO_3^- contribution to the volume effects is negligible as shown by the low δV values

produced by KNO_3-protein systems [31] and by studies on protein-anion interactions [45,46].

When comparing n, $\bar{\nu}$ (expressed in mol bound metal/mol protein) and δV values for lysozyme-copper (figs. 1 and 2) the following correlations were observed. for the maximum δV value at protein concentration 3.57×10^{-4} M and metal concentration 0.033 M, $\bar{\nu} = 8.5$ mol bound metal/mol protein. Taking into account that lysozyme has eleven acid residues and that eight of them can be titrated in 0.2 M KCl [54], they may be in principle considered as the high-affinity sites to which metal ion is bound.

Up till now, the volume change has been assumed to be entirely due to the release of the water molecules from the ionic groups of the protein upon complex formation. This may not be strictly correct. It has been described that interactions between positive and negative charged residues produce volume rises between 10 and 20 ml/mol reacting ionic pair [53], and also, that Cu(II) interaction with propionate and acetate produces volume changes between 7 and 13 ml/mol [55]. If we take a mean value of 10 ml/mol reacting Cu(II), for lysozyme 0.5% of the δV maximum value, i.e., 46 ml/mol protein, it will imply 4 or 6 reacting ionic pairs. This result does not agree with the value of $\simeq 8$ mol bound Cu(II). This leads us to suppose that for the maximum value (fig. 1) not all Cu(II) ions are bound to ionic groups of the protein.

On the other hand, a preferential solvation by water is observed in all cases in lysozyme-Cu or -Co(II) systems in contrast to the protein-urea [34,44] and protein-guanidine hydrochloride systems [28]. The preferential solvation is a measure of the difference between solvent composition in the protein domain and outside it. If this is attributed to the selective binding of one of the components of the solvent [43] the observed decrease in the preferential solvation by water in the metal concentration range between 0.033 and 0.048 M (figs. 5 and 6) implies a selective binding of the other component to the protein, as observed in figs. 2 and 3. Again this change could be related to any type of structural change, since preferential hydration is characteristic of aqueous systems such as NaCl and KCl, in which the native structure is maintained whereas denaturation is characterized by a preferential salt binding [43], and the existing correlation between preferential solvation and protein unfolding [28,44].

However, the denaturation mechanism by neutral salts is complicated and implies the reciprocal interaction of the salt, macromolecules and water. A salt can produce structural alteration or denaturation by direct interaction with the macromolecule, or indirectly, by altering solvent properties. Therefore, from these results and taking into account other works [56–60] alternative explanations may be suggested. It is known that lysozyme in solution undergoes structural changes as a function of temperature [8,56–58]: the existence of D_1 and D_2 forms or A and B has been shown in solution [8] and in crystalline state [56,58]. Besides, under physiological conditions, lysozyme molecules have been found to exist in two forms N (native) and D (denatured) and an alteration in the net charge by acetylation causes no gross conformational changes but instead shifts the N $\rightarrow$ D transition to the right [59,60]. All of the foregoing evidence seems to indicate that lysozyme undergoes some conformational transition in the metal range from 0.0334 to 0.048 M. It means that lysozyme in solution could be in two or more conformational states coexisting in equilibrium each one having a characteristic specific volume, which may or may not be dependent on the number of cations bound. Cation binding would affect the relative free energies of each state in a different manner and therefore lead to altered equilibrium between the states and changes in average specific volume.

Acknowledgment

A part of this work was supported by Comision Asesora de Investigación Científica y Técnica, Grant No. 3795/79.

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