On the aggregation of bovine serum albumin

Pedro P. Madeira*, Inês L. D. Rocha, Marguerita E. Rosa, Mara G. Freire, João A. P. Coutinho

CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

*Corresponding author: Tel: +351 234401507; Fax: +351-234370084; E-mail address: p.madeira@ua.pt

Abstract

In an attempt to elucidate the aggregation behaviour of bovine serum albumin and its modulation by salt ions, size-exclusion high-performance liquid chromatography was used and complemented by dynamic light scattering. The influence of the protein concentration, and type and concentration of inorganic salt on the aggregation of albumin in solution have been analyzed. Based on the observations herein reported an aggregation mechanism is proposed, according to which (i) some functional groups of albumin dissociate in solution forming macromolecular ions; (ii) the macromolecular ions bind with each other into large aggregates; and (iii) the salt ions establish chemical equilibria with the charges of opposing character present in the macromolecular ions' backbone, promoting or preventing the aggregation. The present work shows that the aggregation behaviour of bovine serum albumin in solution and its modulation by salt ions can be explained by chemical concepts, with chemical equilibrium playing an essential role.

Introduction

Inorganic salts, like sodium chloride, modify to a considerable extent the proteins' physical, chemical, and biological properties[1-9]. The gradual addition of an inorganic salt to an initially salt-free protein solution can bring about changes in the protein behaviour of monotonic or non-monotonic contour[2-3, 7]. Furthermore, the protein properties and the quantity of added salt usually vary in distinct proportions[10-14]. For instance, Medda *et. al.*[14] reported that the amount of added salt increases more rapidly than the molecular motion of albumin decreases. If the protein quantity is allowed to change, the role of salt ions becomes even more intricate[10-13]. To result in analogous effects, typically, the salt and the protein vary in opposite ways[10-13]. For example, it is required to increase the concentration of salt to precipitate a protein as its concentration decreases[10-11, 15].

The molecular mechanism underlying the salt-induced effects in the proteins' properties remains unclear. According to the prevailing ideas, certain salt ions specificities, such as their kosmotropic or chaotropic behaviour[16], their strength of hydration[17-18] or their ability to specifically interact with certain proteins' functional groups[19-23], amongst many others[24-30], underlie the effects under consideration. The rise of the ionic specificity concept is intrinsically connected with the Hofmeister precipitation experiments[10-11, 15-16, 21-22, 26, 31]. Hofmeister observed, more than one century ago, that ions display distinct effectiveness in precipitating a protein out of solution[10-11, 15]. In a modern version of the Hofmeister series, anions could be arranged according to their increasing efficacy in salting a protein out of solution in the following way[32]:

$$SCN^- < ClO_4^- < I^- < NO_3^- < Cl^- < SO_4^{2-},$$

While cations could be ordered in the following incremental order of salting-out efficacy[32]:

$$Ca^{2+} < Mg^{2+} < Li^+ < Na^+ < K^+ < NH_4^+.$$

Slight modifications, such as the switch between ions in the series[17-19, 31-34], or considerable variations, such as its complete reversal[17-19, 31-36], are also often reported. The Hofmeister series shows no relation to the stoichiometric properties of the ions, which have led to the general acceptance that ions, in addition to electrostatic forces, must exhibit certain specificities in their interaction with the solvent (chaotropicity and kosmotropicity) rather than with the solute.

Recent works[12-13] dealing with the precipitation of electropositive albumin, however, gave strong arguments to consider that the solute, i.e. the protein, must play an important, while often neglected, role upon the phenomena under consideration[12-13]. More specifically, at pH values considerably below 3, cations were shown to display none or a negligible role in the precipitation of albumin[12-13]. An anion series, reflecting their ability to precipitate electropositive albumin, was therefore unambiguously established[12]. The

increasing efficacy of anions in salting albumin out of solution observed was as follows[12]: $F^- < Cl^- < Br^- < NO_3^- < I^- < SCN^- \sim ClO_4^- < SO_4^{2-}$. The ordering of anions, which was also reported for distinct proteins and using other experimental techniques[37-43], harmonize with the purely electrostatic forces as follows:[12-13] (i) divalent anions are the strongest precipitating agents, which is understandable in connection with Coulomb's law;[44] and (ii) the trend displayed by halogens is what could be anticipated by taking into account Pauling's electronegativity scales[45]. This explains the tendency observed for the whole range of the monovalent anions studied[12-13].

Our previous studies[12-13] raised important questions on this matter: Why does the anions' role harmonize with the electrostatic forces only under those circumstances where the counterion does not play any role? Why have different Hofmeister series been reported?[33] The first, and widely advocated possible explanation is that the anions (and cations) specificities change considerably, whether direct or indirectly, on varying the experimental conditions[10-11, 15-16, 21-22, 26, 31]. The hypothesis, which has been both experimentally and theoretically tested for over one century[10-11, 15-16, 21-22, 26, 31], has not yet provided a framework of compelling acceptance which allows clarification of the manifold Hofmeister series. Another possible explanation, for which previous studies by us[12-13] and others[3-4, 6, 8], gave some support, is that anions (and cations) harmonize with the purely electrostatic forces, and the Hofmeister series is the result of comparing the effects of ions in experimental conditions in which no direct comparison is possible.

In a typical Hofmeister study, the effect of some anions (or cations) holding a common counterion upon a certain protein property is analyzed. The increasing, or decreasing, efficacy of the ions in inducing that property is measured, from which the series is obtained. Since the counterion also plays a role in the phenomena, any comparison between ions of distinct valence, or amongst ions of identical valence in which their

effects are determined at distinct concentrations, may obviously lead to erroneous assumptions[12-13]. Most Hofmeister studies, however, compare the effect of ions of identical valence at the same concentration. The approach relies on the assumption that the quantity of active ions in solution does not depend on the inorganic salt from which they dissociate. The theory of electrolytes suggests that this hypothesis does not hold in all cases. Whether analyzed in light of the classical electrolytic dissociation of Arrhenius[46-47], according to which strong electrolytes do not dissociate completely, or in light of the Bjerrum concepts of ion association[48], according to which ions associate in pairs, triplets, etc. as contact, solvent-shared or solvent-separated entities, it could be assumed that the quantity of active counterion is different if dissociated from equal molar quantities of distinct salts. The disparity, which should be greater the higher the salt concentration, holds likewise true for the quantity of the ion under comparison. Therefore, in most Hofmeister studies both ions are exerting their influence at distinct degrees, rendering the comparison between them doubtful. These concepts, which could provide a rationale for the manifold distinct Hofmeister series reported[33], is only conceivable if rather small differences in the concentration of ions would have pronounced effects upon the protein properties.

The term microheterogeneity was first introduced in connection with proteins by Colvin, *et al.* [1] over 60 years ago, according to which native proteins should be regarded as populations of closely related but non-identical molecules. In the same line of reasoning is the more recent and widely advocated intrinsically disordered concept[49-56], according to which proteins exist in solution as dynamic ensembles of interconverting structures[49]. More specifically, proteins in solution would behave as populations of closely related but non-identical macromolecular ions in chemical equilibria between them. Therefore, macromolecular ions differing slightly in structure or charge would display distinct

biological activities, physical and chemical properties. Salt ions, at even extremely low concentrations, would act as perturbation agents, which, according to Le Chatelier's principle[57], would shift the chemical equilibrium in different directions.

Due to the relevance of the matter under consideration for scientific domains where proteins play a role, it seems justifiable to investigate in more detail the protein aggregation/dissociation to get a better understanding of the role of salt ions upon it. By analyzing how the relative frequency of the aggregates in solution changes in response to the presence of ions introduced thereto, it is hoped to throw some light into the matter under scrutiny. Rather than making use of distinct salts, the effect of sodium chloride upon the equilibrium between the albumin forms will be first analyzed. In an attempt to understand the role of the individual ions, higher valence ions will be gradually used to replace one or both ions of sodium chloride. The experimental technique used to probe the presence of protein forms of distinct size is size-exclusion high-performance liquid chromatography (SE-HPLC). Dynamic light scattering (DLS) data complement the information provided.

Experimental

Materials:

Reagents: The salts used were NaCl (from Fisher, 99.5%), $CaCl_2$ (from Panreac, 95 %), Na_2SO_4 (from Sigma-Aldrich, 99.9%), $MgSO_4$ (from Panreac, 98 %), $InCl_3$ (from Sigma, 98%) and $Na_3PO_4.12H_2O$ (from Sigma, 98%). The bovine serum albumin - BSA (Mw = 66.5 kDa) used was fatty acid free (< 0.02 %) from Fisher Scientific, lot 66-1384, with purity > 98 %, ash content below 3 % (heavy metals < 10 ppm) and an isoelectric point (pI) of ~ 4.7. The water used was ultra-pure water, double distilled, passed by a reverse osmosis system and further treated with a Mili-Q plus 185 water purification apparatus.

Methods:

Preliminary experiments: In a preliminary set of experiments, the protein solution was submitted to a filtration step (micron filter of 45 μm). The filtration step proved to be unnecessary since no changes in the experimental results derive from this approach. Both the protein and the salts were likewise submitted to previous purification steps[12-13], which were all proved to be unnecessary. Accordingly, the results herein reported correspond to the use of salts and BSA as received.

In all the experiments here reported, protein solutions were prepared in glass flasks. The required quantities of stock solutions of all components and water were dispensed with a Multipette Xstream pipette (Eppendorf, Hamburg, Germany). The flasks were gently shaken (3-4 times). About 1 mL was then transferred to proper vials for HPLC analysis. All the solutions were kept in an incubator (protected from light) at 25 ºC for 24 h. After this period, SE-HPLC or DLS measurements were undertaken.

SE-HPLC experiments: A Chromaster HPLC system (VWR Hitachi) equipped with a binary pump, column oven, temperature controlled auto-sampler and DAD detector was used as the main technique to probe the presence of protein forms of distinct size. SE-HPLC was performed using analytical columns Shodex Protein KW-802.5 (8 mm × 300 mm). For the detection of the protein, Mili-Q water was used as eluent at a flow rate of 1 $mL \cdot min^{-1}$. The temperature of the column and auto-sampler were kept constant at 25 ºC. The injection volume was 20 μL. The wavelength was set at 214 nm, which, according to preliminary tests proved to display higher sensitivity towards the protein aggregates, in conformity with earlier observations[58]. The performance of SE-HPLC varies with the column batch and with elapsed time. The results herein reported were obtained with the same column which operated for a period of five weeks. Replicas with the same column

and two distinct ones were obtained, with qualitatively similar results to those here reported.

DLS measurements: For light scattering measurements a commercial instrument Zetasizer Nano ZSP (Malvern instruments) was used, with a He-Ne laser (633 nm, 4 mW) as a light source. Analysis was performed using the light scattering software DTS application. The scattering light was collected at a 173º backscattering angle. At least three scans of 15 seconds each were performed at the studied conditions. The sixth power dependence of the scattered light intensity on the size of the scatters, renders DLS extremely sensitive towards the presence of large aggregates. DLS is also reported to overestimate the mean size of the clusters, rendering a qualitative interpretation of the same often doubtful[59]. On an exhaustive examination of DLS, experiments upon BSA solutions in which the concentration varied between 40 $g.L^{-1}$ and 0.00625 $g.L^{-1}$, were undertaken. Aggregates of similar and reproducible radius were observed, which are herein reported. Qualitatively similar results to those reported below in Figure 1b were also systematically observed. That is, the relative fraction of aggregates increase upon dilution, which is unmistakable if the successive dilutions vary considerably (*e.g.* 10 X). Mention should be made to the fact that both techniques, that is SE-HPLC and DLS, do not perturb the system significantly.

pH measurements: The pH must be controlled since it affects considerably the protein properties in solution. The pH was measured with a Metter Toledo Seven Excellence pH meter with temperature control in the following manner: Solutions with the required quantity of protein were prepared and left to equilibrate for 3 h at 25 ºC. After calibration according to manufacturer instructions, the electrode was inserted in a sample for at least

5 min at constant temperature (25 ± 0.1 ºC), after which the pH was measured (at least three measurements have been carried out). No significant changes were observed and in the reported experiments the value of the pH is 6.5 ± 0.3.

Results and Discussion

Protein concentration

The influence of the quantity of protein upon its aggregation behaviour was firstly addressed. The chromatograms of albumin at different concentrations are shown in Figure 1a. Here, it can be seen that, at a concentration of 5 $g.L^{-1}$, the albumin solution is eluted

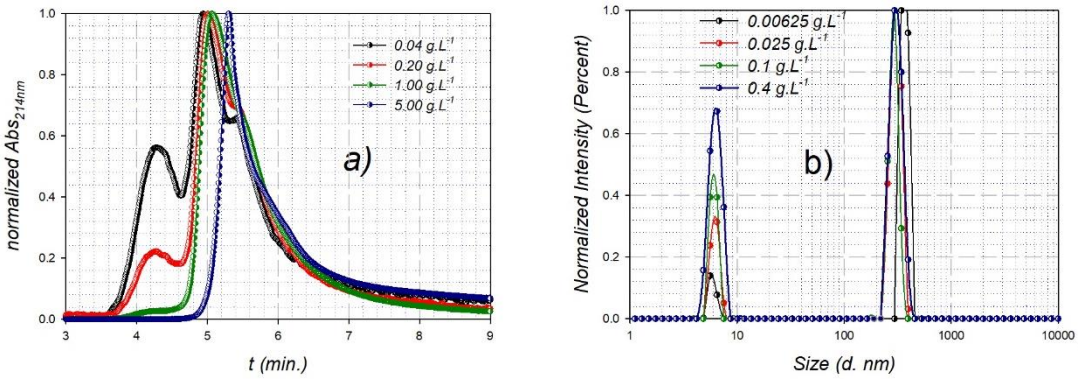

Figure 1. Influence of the quantity of protein upon the relative frequency of the aggregated albumin at 25 ºC as probed by a) SE-HPLC and b) DLS. In the SE-HPLC experiments, the eluent is water at a flow rate of 1 $mL.min^{-1}$. The protein solution was kept at 25 ºC for 24 h before the measurements have been undertaken.

from the column into a single peak of large width with a retention time slightly above 5 min. Upon dilution, the retention time decreases, and at the same time, the single peak is gradually resolved into distinct zones. Likewise noteworthy is that the relative frequency of the protein aggregates, which are retained in the column to a lesser extent, increases upon dilution. In other words: the propensity of albumin to aggregate seems to be

intensified by increasing the distance between the protein molecules, or rather, by decreasing their probability to collide.

The validity of the preceding figures was verified on further dilution of the protein. Since SE-HPLC does not display the required sensitivity, DLS took its place. A mention should be made to the fact that in the DLS experiments three or even four peaks were often detectable rendering a comparison between distinct experimental conditions sometimes doubtful (see experimental section). However, stable aggregates which allowed an unambiguous comparison were detected and are shown in Figure 1b. Therein, if the area below the curves is taken as the relative measure of its occurrence, then the frequency of the aggregates increases from about 60% up to 90%, while the protein concentration decreases sixty-four times, thus supporting the SE-HPLC observations.

If all the albumin monomers displayed a natural tendency to aggregate, we would expect different results. Upon increasing the quantity of protein in the solution, one would expect that the frequency of the aggregates would likewise increase or, conceivably, remain constant. However, from the observations, the following hypothesis could be suggested: Albumin, when dissolved in water, reveals the presence of two distinct forms of monomers. One, which does not display a natural tendency to aggregate, and another one, which is active in this regard. The relative frequency of the latter increases on dilution, and as a result, as does the propensity of albumin to aggregate.

This hypothesis could be formalized in the following way: Albumin, similarly to electrolytes, when in solution undergoes a dissociation process. Therefore, a number of molecules remain undissociated in solution, below represented by P_U, and part of them dissociate into active monomers, that is, into macromolecular ions, beneath as $P_M^{\pm}$, according to the following simplified equation:

$$P_U \leftrightarrow P_M^{\pm} \qquad (1).$$

The symbol ±, means that the macromolecular ion contains both positive and negative charges on its backbone. The counter ion, which is H^+, is intentionally omitted since, depending on the pH, the process carries with it the consumption or release of H^+.

The active monomers, that is, those which display a natural propensity to bind with each other, react according to equation 2, bringing into the solution large protein aggregates (P_A):

$$P_M^{\pm} \leftrightarrow P_A \qquad (2).$$

Whether this process carries with it, or not, the release of H^+ remains an open question.

It should be emphasized that the simplification in the general chemistry of proteins implied by this formalism is considerable. Notwithstanding, the proposed equations will be helpful for the discussion.

The degree of the dissociation of albumin like that of electrolytes increases upon dilution. Therefore, on decreasing the protein concentration, equation 1 is shifted in the forward direction. As a result, equation 2 is likewise shifted in the forward direction, with a subsequent increase upon the relative frequency of the aggregates, giving therefore a rationale for the observations reported in Figure 1.

The fact that the propensity of albumin to aggregate increases upon dilution does not mean that the quantity of aggregates increases in the same direction. On the contrary, what does increase upon dilution is the relative occurrence of active monomers, and consequently, the frequency of aggregated albumin (see equations 1 and 2).

Concentration of inorganic salt

The influence of the addition of sodium chloride to the protein solution on the relative frequency of its aggregates is shown in Figure 2. Thereinafter, for better visualization of

the graphs, only the section of the chromatogram corresponding to aggregated albumin is shown.

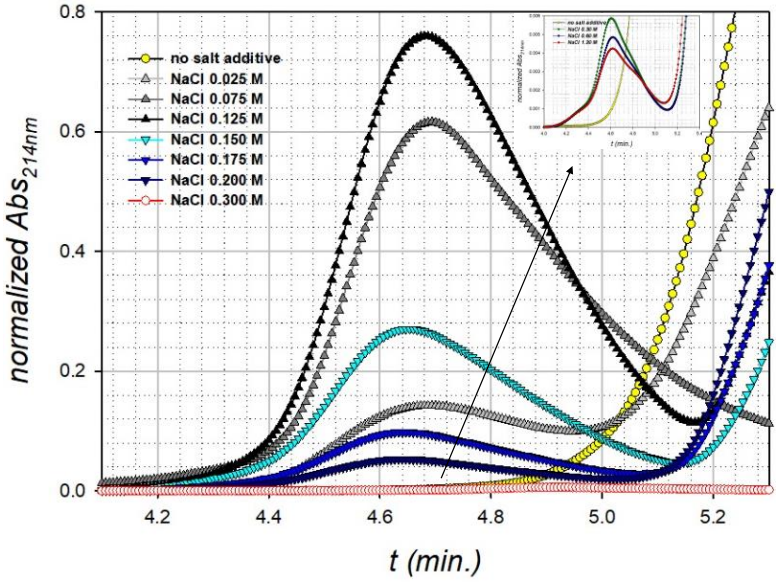

Figure 2. SE-HPLC chromatogram, illustrating the influence of sodium chloride upon the relative frequency of the albumin aggregates. The protein solution at a concentration of 5 $g.L^{-1}$ was kept at 25 ºC for 24 h before the SE-HPLC measurements have been undertaken. The eluent is water at a flow rate of 1 $mL.min^{-1}$.

The observations illustrated in Figure 2 show that the albumin solution at a concentration of 5 $g.L^{-1}$ does not display a peak characteristic of aggregate formation. The gradual addition of sodium chloride to the originally salt-free albumin solution first induces an increase in the aggregated population up to a concentration of 0.125 M, followed by a decrease in the aggregates for higher salt concentrations. These figures are emphasized by the insert in Figure 2. Herein, it could be seen that on adding further salt up to 1.2 M, the aggregates are not completely suppressed, but rather inhibited to a large extent.

To get further insights into the role of sodium chloride upon the aggregation behaviour of albumin, further experiments, in which the quantity of protein was allowed to vary, have been undertaken. The results are shown in Figure 3.

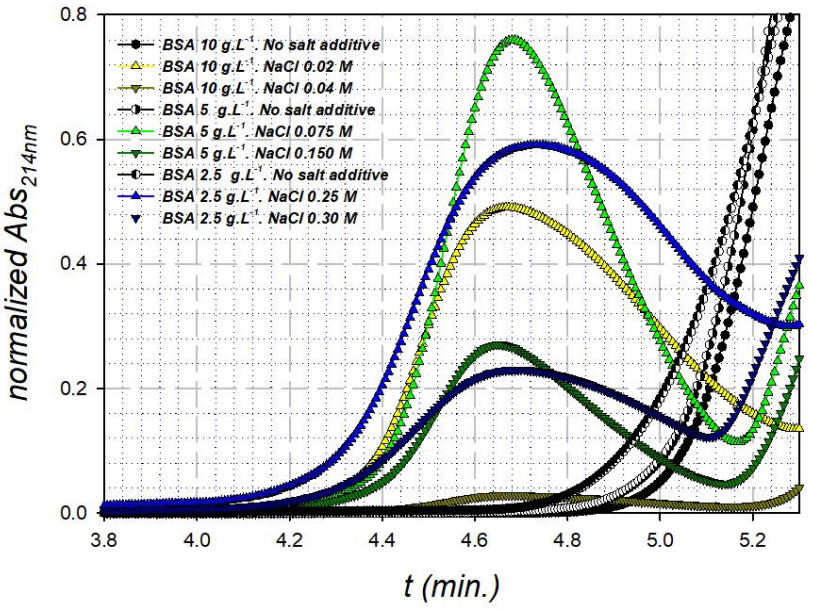

Figure 3. SE-HPLC chromatogram illustrating the influence of sodium chloride on the aggregation behaviour of albumin for varying protein concentration. The protein solution was kept at 25 ºC for 24 h before the SE-HPLC measurements have been undertaken. The eluent is water at a flow rate of 1 mL.min^{-1}.

Perhaps the chief observation from the results reported in Figure 3 concerns the fact, often found in the general chemistry of proteins, that, to result in analogous effects, the quantities of salt and protein must be varied in opposite directions and distinct proportions.

The results shown in Figures 2 and 3 are hard to reconcile with the concepts of ions' specificities. Not just because their specificities would have to change considerably in the presence of albumin at 5 g.L^{-1}. That is, between NaCl 0.025 M and NaCl 0.125 M, their specificities would have to be of a certain type. Thereafter, up to 1.20 M, they would need to be of opposing character (see Figure 2). Moreover, the salt specificities, upon slightly varying the protein concentration, would have to change drastically (see Figure 3). That is, their specificities would have to reverse between 0.02 M and 0.04 M, and between 0.075 M and 0.150 M and between 0.25 M and 0.30 M if in the presence of albumin at 10 g.L^{-1}, 5 g.L^{-1} or 2.5 g.L^{-1}, respectively. But above all, due to the fact that the effects are created by the presence of sodium chloride. According to the prevailing ideas, the

Hofmeister series divide ions into two types of opposing character: kosmotropic or chaotropic[16], strongly or weakly hydrated[17-18], salting-out or salting-in agents[16-30], protein stabilizers or destabilizers[16-30] and so forth. Na^+ and Cl^- are ions typically positioned in the middle of the Hofmeister series[16-30]. They are "neutral ions" that separate those of opposing specificity. That is, they are of neither type.

The results here reported can only be explained in the light of electrolyte chemistry with the protein macromolecular ions reacting with those of opposing charge. Due to their amphoteric nature, proteins contain both types of charges. Therefore they can react with cations (C^+) and anions (A^-), bringing into the solution other protein forms, below represented by P_{MCA}:

$$P_M^{\pm} + C^+A^- \leftrightarrow P_{MCA} \qquad (3).$$

It should be emphasized once again that the simplification in the general chemistry of proteins implied by this formalism is considerable. Notwithstanding, the experimental observation here reported can be explained by equations 1-3, as discussed hereafter.

Two processes, with opposing effects, must be considered when sodium chloride is added to the protein solution. One, in which further dissociation of the protein must take place (equations 1 and 3), and another one, in which reaction 2 is shifted towards the reverse direction. The first process induces an increase in the aggregated albumin population, whereas the second one a decrease. At low concentration of sodium chloride the first chemical equilibrium prevails. When the forces opposing the dissociation equilibrates those which favours it, the second process dominates. Therefore, on gradual addition of sodium chloride, the aggregated protein population firstly increases and then decreases, explaining the observations reported in Figure 2. The fact that the addition of sodium

chloride brings about further dissociation of electrolytes is a phenomena often found in general chemistry[46-47]. For example, as Arrhenius pointed out[46-47], when sodium chloride is gradually added to hydrochloric acid, it brings about, initially, further dissociation of the acid.

If a mass action indeed takes place between the protein and the inorganic salt, according to equation 3, then, the equilibrium constant depends chiefly, amongst other factors which are not relevant to this discussion such as the temperature and pressure, upon the concentration of the reactants. Therefore, the quantities of salt and protein must vary in opposite and most likely in distinct proportions to induce similar effects, which explains the results reported in Figure 3.

Type of inorganic salt

To get further insights into the role of the individual ions upon the phenomena under study, further experiments with ions of higher valence replacing those of sodium chloride, have been undertaken. The results of the addition of calcium chloride, indium chloride, sodium sulphate, sodium phosphate and magnesium sulphate upon the aggregation behaviour of albumin are shown in Figure 4. Since calcium sulphate is not sufficiently soluble, $MgSO_4$ which, for the reasons discussed below suits our purposes, was used instead.

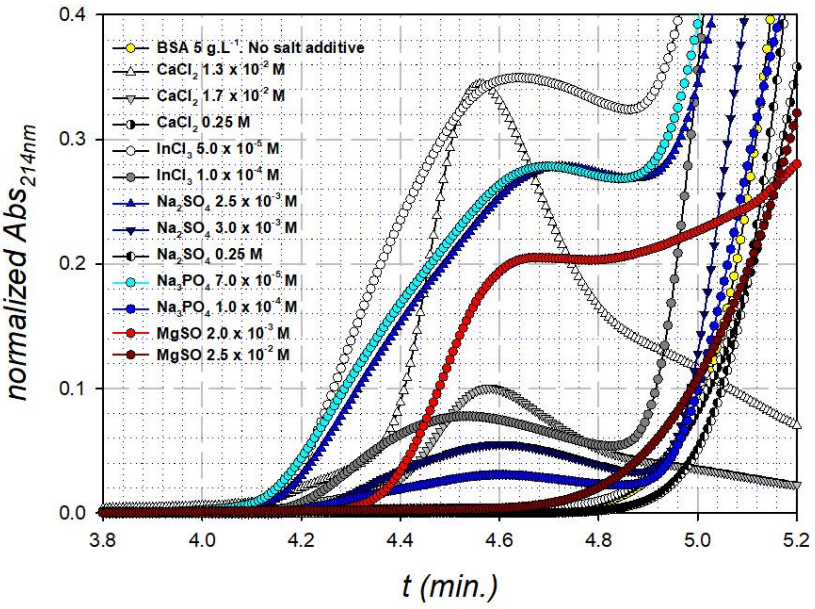

Figure 4. SE-HPLC chromatogram illustrating the influence of salts containing higher valence ions upon the aggregation behaviour of albumin. The protein solution at a concentration of 5 $g.L^{-1}$ was kept at 25 ^{o}C for 24 h before the SE-HPLC measurements have been undertaken. The eluent is water at a flow rate of 1 $mL.min^{-1}$.

If a comparison is made between these figures and those illustrated in Figure 3, there are some important conclusions to be drawn. In the first place, the replacement of any of the ions of NaCl by an ion of higher valence brings about a pronounced influence upon the aggregation behaviour of albumin, similarly, to a certain extent, to that observed in the presence of NaCl. Secondly, the effect under consideration depends much more pronouncedly upon the ion's valence than on its ionic character. Thirdly, the required quantity of salt to bring about the effects under observation is lower the higher the valence of at least one of its ions. And, in connection with the previous observation, the quantity of salt follows, to a certain extent, the proportion x, x^2, x^3 for monovalent, divalent and trivalent ions, respectively. Considered together, these results suggest that the supposedly mass action that takes place between the protein and the salt ions is of electrostatic nature. The previous observations could find an explanation under the light of the following proposed mechanism. The reaction between the salt and the macromolecular ions, according to equation 3, is a phenomenon of electrostatic nature. Therefore, to bring about

a similar effect, two or three times of a monovalent ion ($M^{\pm}$, where the symbol ± represent cations or anions) are required by a divalent ($D^{2\pm}$) or a trivalent one ($T^{3\pm}$), respectively. In terms of chemical equilibria concepts, it follows that[13]:

$$[M^{\pm}] = [D^{2\pm}]^{1/2} = [T^{3\pm}]^{1/3} \qquad (4).$$

Therefore, the effect brought about by sodium chloride between 0.025 M and 0.20 M finds a theoretical correspondence of between 6.25×10^{-4} M and 4.0×10^{-2} M for divalent ions and of between 1.56×10^{-5} M and 8.0×10^{-3} M for trivalent ions, which explains, to a certain extent, the observations reported in Figure 4, and in particular the proportion x, x^2, x^3 for monovalent, divalent and trivalent ions, respectively. Mention should be made to the fact that the observed effects holds both for the ion that has charge opposite to the protein surface, and for the ion that has the same sign of the protein surface.

There is an experimental observation that calls for further scrutiny. The fact that the required quantity of salt to suppress the aggregates is much lower (*i.e.* about ten times lower) if it contains two divalent ions ($MgSO_4$) rather than just one ($CaCl_2$ or Na_2SO_4). This observation could also be explained in the light of the herein proposed aggregation mechanism as follows: According to equation 3, and illustrated in Figure 4, both salt ions react with the macromolecular ions. Therefore, the reaction which brings about the suppression of the aggregates is limited by the concentration of the less active ion in this regard, or rather, by the concentration of the ion of lower valence. In both $CaCl_2$ and Na_2SO_4, the suppression of the aggregates is therefore limited by the required concentration of the monovalent ions to create this effect. However, in $MgSO_4$, both ions are of divalent character. Therefore, they should exert their full influence at much lower concentrations, as is indeed observed.

The results here presented and discussed suggest that the changes induced by salt ions upon the behavior of proteins are the result of their ability to participate in chemical

equilibria with the protein macromolecular ions in solution. Therefore, salt ions introduce perturbations upon the chemical equilibria established between the monomeric and aggregated protein populations, according to which they can promote or inhibit the aggregation, and whereby the protein properties change accordingly.

Salt ions influence on proteins' properties

Salt ions influence proteins' properties in many distinct and complex ways[1-9]. The fact that they can induce either monotonic or non-monotonic changes in the protein behaviour or that the quantity of added salt and the protein property vary in opposite proportions show a remarkable parallelism with their effect upon the protein aggregation behavior, as here reported. The molecular mechanism of aggregation here proposed could be used as a guide for the interpretation of the diffusion coefficient (D) of BSA, which increases with the increasing concentration of BSA[14, 60]. This is apparently counterintuitive inasmuch, as due to a higher viscosity, a slower diffusion would be expected. This could be explained if, as observed, the frequency of the aggregated albumin increases on dilution (Figure 1 and equations 1 and 2). Therefore, on average, the hydrodynamic radius of the albumin molecules increases upon dilution, which, according to the Stokes-Einstein concepts[61], brings about a decrease in its Brownian movement and thus on the diffusion coefficient.

On adding sodium chloride - 0, 50, 100 and 300 mM - to a albumin solution at 40 $g.L^{-1}$ the diffusion coefficient of BSA decreases[14]. Firstly, about 22%, then 10%, and finally less than 5%[14]. These observations could be explained based on the results reported in Figure 2, according to which at a NaCl concentration of 50 mM, the frequency of the BSA aggregates should increase considerably. Therefore, its molecular movement should decrease significantly, as observed[14]. On gradually adding further salt, the aggregates

begin, first to be slightly suppressed, and thereafter suppressed to a larger extent. Therefore, D should decrease more and more slowly, as the experiments corroborate[14]. The behaviour of the diffusion coefficient of albumin in solution is in good agreement with our observations of the aggregate formation and could thus be explained by the proposed mechanism according to which the protein acts as a weak electrolyte, in which the emerging protein macromolecular ions in solution display a natural propensity to bind with each other into large aggregates.

Conclusions

The aggregation behaviour of bovine serum albumin in solution and its modulation by means of salt ions was analysed in the present work. The main observations were the following: (i) The propensity of albumin to aggregate increases upon dilution; (ii) Inorganic salts, like sodium chloride, induce a non-monotonic effect upon the aggregation behaviour of albumin; (iii) The required quantity of sodium chloride to bring about the preceding effect is higher for lower protein amount; (iv) The effect brought about by inorganic salts containing one or two ions of higher valence is similar to that induced by sodium chloride; and (v) The required quantity of salt to induce the effect is much lower, and follows the proportion x, x^2, x^3 for monovalent, divalent and trivalent ions, respectively, regardless of its ionic character.

The observations were perfectly accounted for by classical chemical concepts. Moreover, albumin´s properties in solution such as its molecular motion or the non-monotonic

behaviour induced by salt ions, were qualitatively explained in light of the proposed molecular mechanism of aggregation.

On its whole, the facts herein reported are a strong support for the chemical behaviour of albumin in solution, according to which chemical equilibrium play an essential role on its behaviour.

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