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On the role of salt type and concentration on the stability behavior of a monoclonal antibody solution

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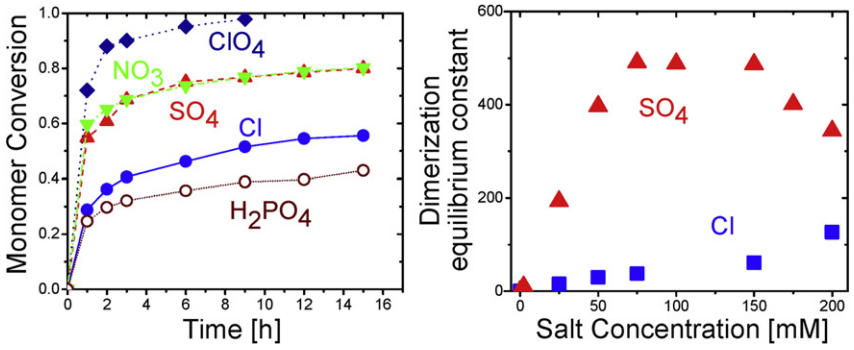
HIGHLIGHTS

- The effect of salt on the aggregation stability of an IgG is investigated.
- The salt effect is strongly ion specific and pH dependent.
- The anion ranking follows the Hofmeister series with the only exception of sulfate.
- Aggregation propensity exhibits a maximum as a function of salt concentration.
- Salt affects in a complex way both colloidal interactions and protein conformation.

GRAPHICAL ABSTRACT



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ABSTRACT

Protein–salt interactions regulate protein solubility and stability and in particular several protein related processes, such as salting-out and aggregation. Using an IgG2 monoclonal antibody as a model multi-domain therapeutic protein, we have investigated the salt effect on the reversible formation of protein clusters with small aggregation number. The oligomer formation has been quantified by size exclusion chromatography (SEC). It is found that the salt effect is strongly ion specific and pH dependent. In particular, at pH 3.0 only anions affect the aggregation propensity, while at pH 4.0 both anions and cations influence the aggregation rate. The ranking of the anion effect follows the Hofmeister series with the only exception of sulfate, while that of the cation effect does not. In addition, a maximum of the aggregation propensity as a function of salt concentration is observed (i.e., presence of re-stabilization).

By correlating the aggregation kinetics to the experimental investigation of protein structure and surface energy, it is shown that changes in pH and salt concentration induce aggregation not only through charge screening and various solvation forces, but also through the formation of protein intermediates characterized by partially ordered structures and certain degrees of hydrophobicity. The complex interaction between the solvation forces and such protein secondary structures induced by salts explains the observed experimental results relative to re-stabilization at large salt concentrations, ion specificity and the peculiar behavior of the sulfate anion.

1. Introduction

Interactions with salts affect several protein properties such as structure, solubility and stability. “Salting-out” of proteins is commonly applied in protein crystallization and separation processes [1]. Since the discovery of Franz Hofmeister in 1888 it is known that

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the propensity of salts to induce protein precipitation is ion specific [2]. Hofmeister determined an ion series, known as the Hofmeister series or lyotropic series. Originally, the specific ions effect was related to the capacity of ions in breaking or preserving the structure of water. In the Hofmeister series the ions are ranked from the smaller, tightly hydrated (kosmotropic) ions to the larger, less hydrated (chaotropic) ions:

$$SO_4^{2-} > HPO_4^{2-} > Cl^- > NO_3^- > ClO_4^-$$

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

The Hofmeister series was later observed in many chemical-physical phenomena[3] ranging from polymers and colloids [4] to biological systems [5,6], with only slight modifications in the ranking [6–8]. For example, when pH is larger than the protein isoelectric point (pI), the anion effect on salting-out follows the direct Hofmeister series, while if pH is below the pI the reversed series is observed.

In spite of the great attention given by the scientific community to salt–protein and salt mediated protein–protein interactions [9–12], the molecular origin of the Hofmeister series and of the reverse behavior below the protein pI is still unclear [8]. In addition, many other puzzling experimental observations on the behavior of protein–salt solution, including re-stabilization of protein colloids at high ionic strength [13,14], are poorly understood.

Salt-induced protein aggregation and gelation can be regarded as a special case of salting-out where the protein is assembling mainly in a non-native configurational state. The possible final products cover a wide range of sizes and can exhibit either ordered structures or amorphous morphology. Depending on the specific protein and conditions, aggregation can be both reversible and irreversible. Important examples are found in several areas including biomedical research, food technology and pharmaceutical science. A specific anion effect has been reported for the *in vitro* aggregation of several amyloidogenic proteins (e.g., A-β [15], α-synuclein [16], prion protein [17]), an important class of proteins involved in human neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's and prion diseases.

In many food processes of whey protein isolate, protein gelation is induced through salt addition, i.e. the so-called “cold-gelation” [18,19].

Salt-induced aggregation of proteins in aqueous solutions is a major problem encountered often in pharmaceutical processes [7,20]. Therapeutic peptides and proteins represent a fast-growing class of drugs in biotechnology industry. The production and purification of such proteins include multiple chromatography steps during which the product is exposed to changes in pH and salt concentration, which trigger protein aggregation and cause loss of valuable products [21]. Moreover, drug formulation must guarantee the absence of even small percentage of aggregates to avoid immunological reactions in patients [22]. Currently, the optimization of operating conditions during protein purification and formulation is mainly performed by time-consuming and expensive screening techniques. Achieving a fundamental understanding of the mechanism related to the aggregation and interactions between proteins and co-solutes is definitely desired.

In this work, we investigate the role of the type of anions and cations at various pH values on the structure and stability of a model IgG2 immunoglobulin. For this, we quantify the aggregation kinetics for different salts and salt concentrations in the range 0–200 mM at various pH values, using size exclusion chromatography (SEC). We show how the salt effect is strongly ion specific and pH dependent. Moreover, we identify the presence of a maximum of aggregation extent as a function of salt concentration. Finally, we discuss the salt effect by correlating the aggregation data to the experimental investigation of protein structure and solution surface tensions. The work provides general considerations on the interactions between ions and macromolecules, which can be applied to other systems including amyloidogenic proteins [16] and polymers [4].

2. Materials and methods

2.1. Materials

The monoclonal antibody considered in this work is an IgG2 with molecular weight ~150 kDa and with isoelectric point (pI) between 7.35 and 8.15, supplied by Merck Serono (Vevey, Switzerland). A mother antibody solution at 45 g/L in 10 mM sodium citrate, 50 mM NaCl, 150 mM sucrose at pH 6.0 was stored at 4 °C.

The solutions for aggregation studies were prepared by manually diluting the mother solution to 0.5 g/L or 1 g/L by selected buffer solutions. For each condition three repetitions were performed and average values were recorded. All buffers for aggregation studies were filtered using 0.2 μm cut-off sterile syringe filters PALL® Acrodisc® 32 mm (PALL Life Sciences, NY, USA). 0.5 g/L of sodium azide was added to all solutions to prevent formation and proliferation of bacterial growth. All chemicals were supplied by Sigma Aldrich (Buchs, Switzerland).

2.2. Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) experiments were performed on Agilent 1100 Series HPLC equipped with VWD UV–vis detector (Agilent, Santa Clara, CA, USA) combined with a Superdex 200 10/300 GL, 10 mm × 300 mm column (GE Healthcare, Uppsala, Sweden). Each sample was eluted at a flow rate of 0.5 mL/min using as mobile phase a solution of 100 mM sodium sulfate and 25 mM Na_2HPO_4 at pH 7.0, filtered with a 0.45 μm cut-off Durapore membrane filter (Millipore, Billerica, MA, USA). The absorbance was recorded at 280 nm.

2.3. Circular dichroism (CD)

Circular dichroism (CD) spectra of 0.3 g/L protein solutions were measured using a Jasco-815 CD spectrophotometer (Jasco, Easton, MD, USA). Far-UV CD spectra were recorded from 260 to 190 nm with the temperature of the cell holder controlled at 20 °C. A quartz cuvette with 0.1 cm path length was used. Spectra obtained after buffer subtraction were corrected for protein concentration and smoothed using the Savitsky–Golay function.

2.4. Intrinsic tryptophan fluorescence (Trp)

The fluorescence analysis was performed on a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian, Palo Alto, CA, USA) by exciting 0.3 g/L protein solutions at 295 nm and collecting emission spectra between 305 and 450 nm.

2.5. 8-anilino-1-naphthalenesulfonic acid (ANS) Binding

8-anilino-1-naphthalenesulfonic acid (ANS) binding was measured using a Varian Cary Eclipse Fluorescence Spectrophotometer device (Varian, Palo Alto, CA, USA) and an EnSpire 2300 Multilabel Plate Reader (Perkin Elmer, Boston, MA, USA). Emission spectra of 0.3 g/L light chain solution in several buffers with 15 μM ANS were collected at 20 °C between 420 and 600 nm using 380 nm as excitation wavelength.

2.6. Surface tension measurements

Surface tension values were measured using a DCAT 21 tensiometer with a PT11 Platinum-Iridium Wilhelmy Plate (Dataphysics, Filderstadt,

Germany). The sample temperature was fixed at 25 °C. 0.1 g/L lysozyme (Sigma Aldrich, Buchs, Switzerland) solutions were filtered using 0.1 μm cut-off sterile syringe filters PALL® Acrodisc® 32 mm (PALL Life Sciences, Port Washington, NY, USA). For each condition, 3 repetitions of 5 independent samples were performed in order to ensure experimental reproducibility.

3. Results and discussion

3.1. Aggregation kinetics: experimental evidences

Salt-induced aggregation kinetics was monitored by Size Exclusion Chromatography (SEC). A typical time evolution of SEC chromatograms is shown in Fig. 1. It is seen that, as the incubation time increases, the peaks of dimers and trimers increase at the expense of that of monomer, located at about 25 min. During the time interval monitored in this work the formed soluble oligomers are rather stable and do not further aggregate to larger clusters. The area under the SEC chromatogram of samples taken at different incubation times is constant, indicating that no insoluble large aggregates are produced or lost during the analysis. The reliability of the data has already been proven in our previous work by comparison with alternative techniques [23]. Only in the presence of perchlorate anion loss of mass during the SEC analysis is observed after few hours of incubation.

The aggregation kinetics has been investigated using different types of salts, at a fixed concentration of 150 mM in 25 mM citric acid buffer solution and at pH 3.0, 4.0 and 4.5. Note that for divalent anions (or cations), the salt concentration was fixed at 75 mM to allow comparison with equal number of monovalent cations (or anions), respectively. All antibody solutions were incubated at room temperature at a protein concentration of 1 g/L or 0.5 g/L. Examples of the reproducibility of the experimental data are reported in the Supporting Material. The monomer conversion, defined as the mass fraction of the initial amount of monomeric antibody converted to species that are different from the monomeric antibody, was evaluated from the SEC chromatograms by measuring the area under the peak of monomer at different incubation times.

Fig. 2a and b shows the effect of cation type on the monomer conversion to aggregates as a function of time at pH 3.0 and 4.0, respectively. In all cases, the corresponding anion is either chloride or sulfate. No data are reported in the case of pH 4.5, because no aggregation was observed for all the salts. In Fig. 2a at pH 3.0 and protein concentration of 1 g/L, the effect of cation type on the aggregation kinetics is insignificant. However, in Fig. 2b at pH 4.0 and protein concentration of 0.5 g/L, a substantial effect of cation type has been observed, and the aggregation rate follows the order:

$$Li^+ > K^+ > Ca^{2+} > Na^+$$

Such an order is not in agreement with neither the Hofmeister series nor the hydration radius series [24] nor the electroselectivity series [25]. To explain the difference in the effect of cation type between Fig. 2a and b, we have to consider the pH change from 3.0 to 4.0. Since the pI of the protein is between 7.35 and 8.15, positive charges are dominating on the protein surface both at pH 3.0 and 4.0. However, negative charges are still present on the surface and their amount is larger at pH 4.0 than at pH 3.0. Since the interactions of such negative charges with the cations are relatively complex (chemical, electrostatic, polarization, etc.) and certainly ionic specific, it follows that the cation type effect is stronger at pH 4.0 than at pH 3.0.

The effect of anion type on the aggregation kinetics is shown in Fig. 3a and b for pH 3.0 and 4.0, respectively. In all cases the corresponding cation was sodium. The rate of monomer conversion in both Fig. 3a and b follows the ascending order:

$$H_2PO_4^- < Cl^- < SO_4^{2-} < NO_3^- < ClO_4^-$$

This agrees well with the Hofmeister series, with the only important exception of sulfate, which behaves similarly to nitrate at pH 3.0 and to chloride at pH 4.0.

To better understand the effect of anion type and pH on the extent of oligomerization, we show in Fig. 4 the monomer conversion at a fixed incubation time (6 h) for different anions as a function of pH. It is seen that for chloride, nitrate and phosphate, the protein is more prone to aggregate at pH 4.0 than at pH 3.0 or 4.5, indicating the presence of a maximum aggregation propensity in the pH range

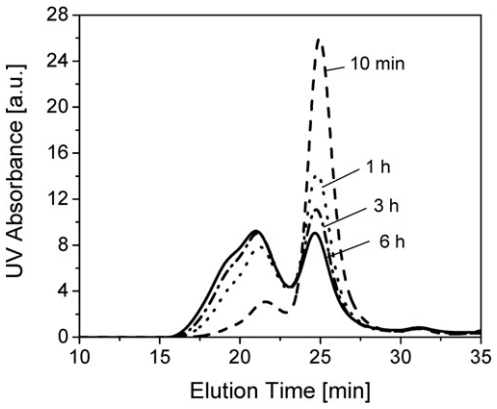

Fig. 1. Typical time evolution of the size exclusion chromatogram during aggregation kinetic experiment. 0.5 g/L antibody solution incubated in 25 mM citric acid buffer and 75 mM Na_2SO_4 at pH 3.0.

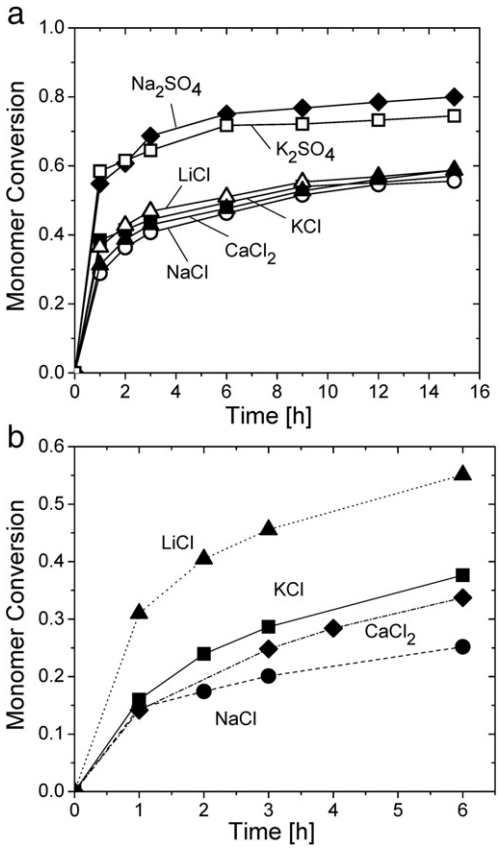

Fig. 2. Cation effect on protein monomer conversion as a function of time: 25 mM citric acid buffer and 150 mM of several Cl and SO_4 salts at pH 3.0 (a) and of several Cl salts at pH 4.0 (b). Protein concentration was 1 g/L in (a) and 0.5 g/L in (b).

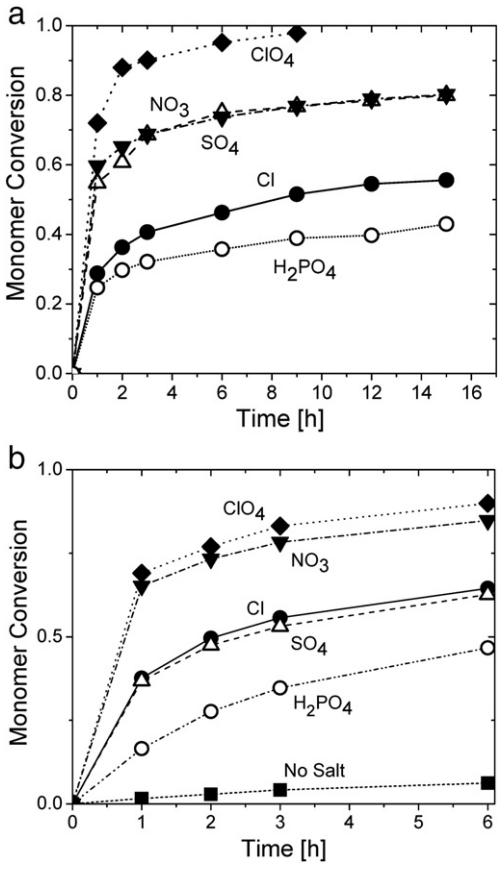

Fig. 3. Anion effect on protein monomer conversion as a function of time: 1 g/L protein solution in 25 mM citric acid buffer and 150 mM of several Na salts at pH 3.0 (a) and pH 4.0 (b).

of 3.0–4.5. On the other hand, for perchlorate and sulfate a monotonic behavior is observed. Moreover, without adding salts, although aggregation does not occur at pH 3.0 and 4.5, it does occur at pH 4.0, as shown in Fig. 4. This indicates the presence of a maximum aggregation propensity as a function of pH also in the absence of salt addition. In general, in the given pH range below the pI, lowering pH leads to an increase in the net charge, and thus in the particle stability, as typically observed for DLVO dispersions. However, what we observed here is that lowering the pH can either increase or decrease the stability, depending on the type of salt. This clearly indicates that the effect of pH on protein aggregation is not purely due to DLVO interactions

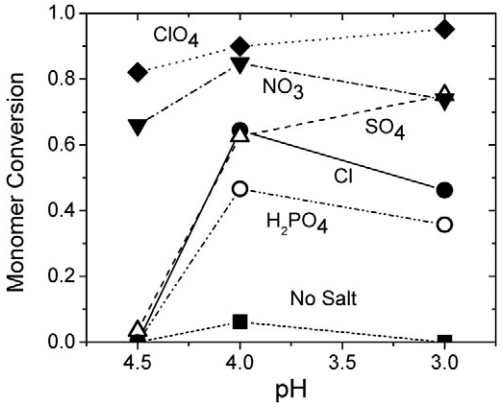

Fig. 4. pH effect on monomer conversion. Monomer conversion values at 6 h of incubation of 0.5 g/L protein solution in 25 mM citric acid buffer with 150 mM of different sodium salts.

and is mostly connected to the pH dependent unfolding of the protein and the formation of an aggregating-prone structure (intermediate).

3.2. Aggregation kinetics: model interpretation

The protein aggregation process discussed above has been investigated in a previous work in the presence of NaCl [23]. The oligomerization process was found to be reversible and quantitatively well described by a modified Lumry–Eyring model including the formation of a reactive intermediate species, *I*:

$$N \underset{}{\overset{K_u}{\rightleftharpoons}} I$$
$$I + I \underset{k_{-1}}{\overset{k_{1,app}}{\rightleftharpoons}} D \qquad (1)$$
$$D + I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} T$$

where *N* denotes the native protein, *I* the intermediate (partially-unfolded) aggregation-prone protein, *D* the dimer and *T* the trimer. Since the overall aggregation is found to be second order with respect to monomer concentration, [23] we can assume that the second step in the above kinetic scheme is the rate limiting step of the process, while the aggregating-prone intermediate, *I*, can be considered in equilibrium with the native state, *N*. Using this model, as described in the Supplementary Material, we can estimate the dimerization equilibrium constant $K_1 = k_{1,\,app}/k_{-1}$ as a function of salt concentration by fitting the SEC experimental data discussed above.

Here we focus on the anion effect, and in particular on two anions: sulfate and chloride, for comparative studies. Sulfate is a peculiar anion involved in the aggregation of proteins in human diseases [26–28] and in the form of ammonium salt is the most common agent used for salting-out of biomacromolecules in industrial purification

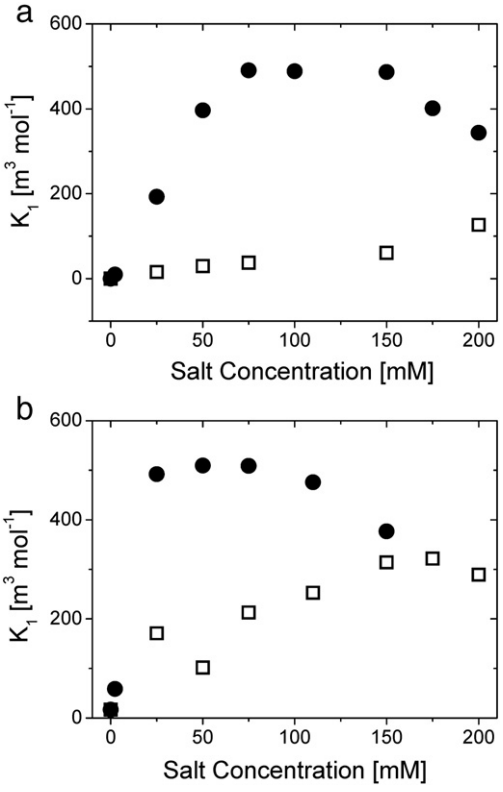

Fig. 5. Dimerization equilibrium constant as a function of salt concentration: sodium chloride (□) and sodium sulfate (●) at pH 3.0 (a) and pH 4.0 (b).

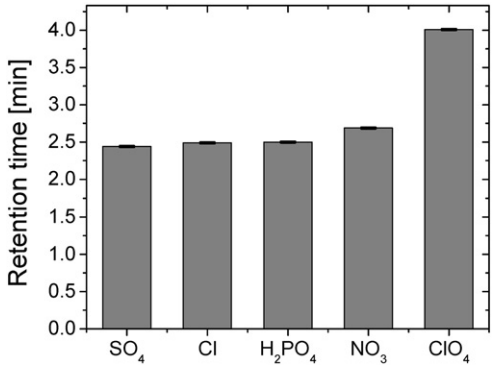

Fig. 6. Retention time of different anions in a reverse phase column. The largest the retention time the largest the affinity of the anion to the hydrophobic surface. Error bars are ±0.01 min.

processes [29]. Fig. 5a and b shows the dimerization equilibrium constant values, K_1, as a function of salt concentration, which have been obtained by fitting the SEC data, at pH 3.0 and 4.0, respectively. The obtained values are in the range 100–500 $m^3\ mol^{-1}$, corresponding to a free energy of dimerization of about 2.7–4 kJ mol^{-1}.

Let us first consider the case of sulfate. In both Fig. 5a and b in the range of low salt concentrations, the K_1 value increases as the sulfate concentration increases, till reaching a maximum value of about 500 m^3/mol and then decreases with the sulfate concentration, thus indicating some kind of re-stabilization effect. The main difference between pH 3.0 and pH 4.0 is that the entire curve moves towards lower sulfate concentrations at pH 4.0. In other words, at low sulfate concentrations, the K_1 value or the monomer conversion is larger at pH 4.0 than at pH 3.0, while at large sulfate concentrations the opposite holds true.

In the case of chloride in Fig. 5, the K_1 value is always larger at pH 4.0 than at pH 3.0 in the given range of salt concentrations. Moreover, at pH 4.0, as the chloride concentration increases, the K_1 value also increases to reach a maximum of around 300 m^3/mol and then decreases. At pH 3.0, however, the K_1 value increases almost linearly with the chloride concentration without reaching a maximum.

When comparing the behavior of the two anions it is seen that in general, the K_1 value is substantially larger for sulfate than for chloride, except for salt concentrations larger than 150 mM at pH 4.0, where it is comparable for the two anions. The electrostatic contribution in DLVO interactions [30] could be used to explain these results at low salt concentrations, i.e., the sulfate anion with its divalent charge is more efficient than the monovalent chloride in screening the electrostatic repulsion between two positively charged protein monomers, therefore promoting faster aggregation. However, at larger salt concentrations other explanations are obviously needed.

Thus summarizing, from the experimental data shown in Figs. 3, 4 and 5, we can draw two main conclusions about the protein aggregation behavior: a) specific effect of salt type and b) a maximum of the aggregation propensity as a function of salt concentration.

Peculiar behaviors of this type in salt–protein systems have been often reported in the literature [31] and their origin is currently still under debate. Ions specific effects are traditionally related to the effect of the ion on the bulk water structure [32]. However, such hypothesis was recently disproved by experiments, showing that the structuring effect of ions is restricted only to the first solvation shell and therefore cannot influence the interaction of the ion with proteins [8,33,34]. More recent studies explain the ion specific effect considering the different polarizability of the different ions, and therefore different ionic dispersion forces acting between each type of ions and the protein [5,35,36]. According to this interpretation, the specific ion effects are related to direct ion-macromolecule interactions more than to bulk properties [8,37]. Specific interactions at the protein interface are also considered to be the reason for the stabilizing effect of co-solutes commonly added in protein formulations, such as arginine [38].

An important aspect involved in ion-protein interactions is the ion binding to the protein charged groups. Different ions have different propensity to bind to the surface charged groups according to the electroselectivity series: [39,40]

$$H_2PO_4^- < Cl^- < NO_3^- < ClO_4^- < SO_4^{2-}$$

Such a series is opposite to the Hofmeister series, with the only exception of the sulfate anion, which due to its divalent charge has the largest tendency to interact with charged components. The sulfate anion has therefore two opposite limiting positions in the Hofmeister and in electroselectivity series and, consequently, two very pronounced and conflicting properties: the largest salting-out activity, which promotes aggregation, and the strongest counterion binding, which increases protein surface dipoles and generates hydration forces with stabilizing effect [12,37]. The competition between these two opposite effects is the source of the peculiar behavior of such anion observed in this work as well as in other systems [41,42]. For divalent ions, the re-stabilizing effect has been explained also by considering charge inversion induced by their binding [43]. However, in this work re-stabilization at large salt concentration has been observed also for chloride anion at pH 4.0 (Fig. 5b), which is obviously not related to charge inversion. Moreover, at large ionic strength, where re-stabilization effects are usually observed, electrostatic repulsion forces would be almost completely screened.

Another aspect to be accounted for is that anions can bind not only to charged groups but also to polar and apolar groups, as well as to the peptide bonds. The less hydrated chaotropic ions will interact more favorably with low solvated and apolar regions of the protein according to the reversed Hofmeister series. We tested the hydrophobicity affinity of the anions by injecting pulses of anions in a reverse phase column (Kromasil® 100-10-C18, Eka Chemicals Bohus, Sweden) and measuring the retention time with a conductivity detector (CDD-10AVP, Shimadzu, Reinach, Switzerland) using a solution of 10 mM HCl with 5.6% w/w acetonitrile at pH = 2.0 as running buffer. As it can be seen from the data shown in Fig. 6, the reversed Hofmeister series was confirmed, with the retention time increasing according to the order:

$$HSO_4^-/SO_4^{2-} \sim HPO_4^{2-} \sim Cl^- < NO_3^- < ClO_4^-$$

The global effect arising from the superimposition of the specific interactions of ions with charged groups, peptide bonds, polar and apolar patches of the protein surface can be described by a single specific (preferential) interaction parameter, introduced by Arakawa and Timasheff to explain the specific salt effect in protein salting-out [29,44] and the stabilizing effects of arginine [45] and sugars [46]. The preferential interaction parameter for a system composed of protein (p), salt (s) and water (w) is defined as $(\partial m_S/\partial m_P)_{T,\ P,\ \mu_W,\ \mu S}$, where m_i and μ_i are the molality and the chemical potential of component i, respectively [44]. This parameter is related to the change in the protein free energy induced by the addition of salt (or a generic cosolute): $(\partial \mu_P/\partial m_S)_{T,\ P,\ m_P} = -(\partial m_S/\partial m_P)_{T,\ P,\ \mu_W,\ \mu S} \cdot (\partial \mu_S/\partial m_S)_{T,\ P,\ m_P}$ [1,29], and represents the type of interaction between the salt and the protein: a negative preferential binding parameter indicates depletion of salt in the proximity of the protein (preferential exclusion) while a positive preferential binding parameter corresponds to preferential binding of the salt. When the ion is preferentially excluded, the protein surface energy increases, and therefore the protein solubility decreases [39]. An increase in the protein surface energy would favor more compact native structure of the protein, but at the same time it would promote also aggregation, since the surface of a dimer is smaller than the total surface of two monomers. These considerations explain the non-intuitive observation that the ions that are the best at stabilizing the protein secondary structure are also the ones that most promote aggregation.

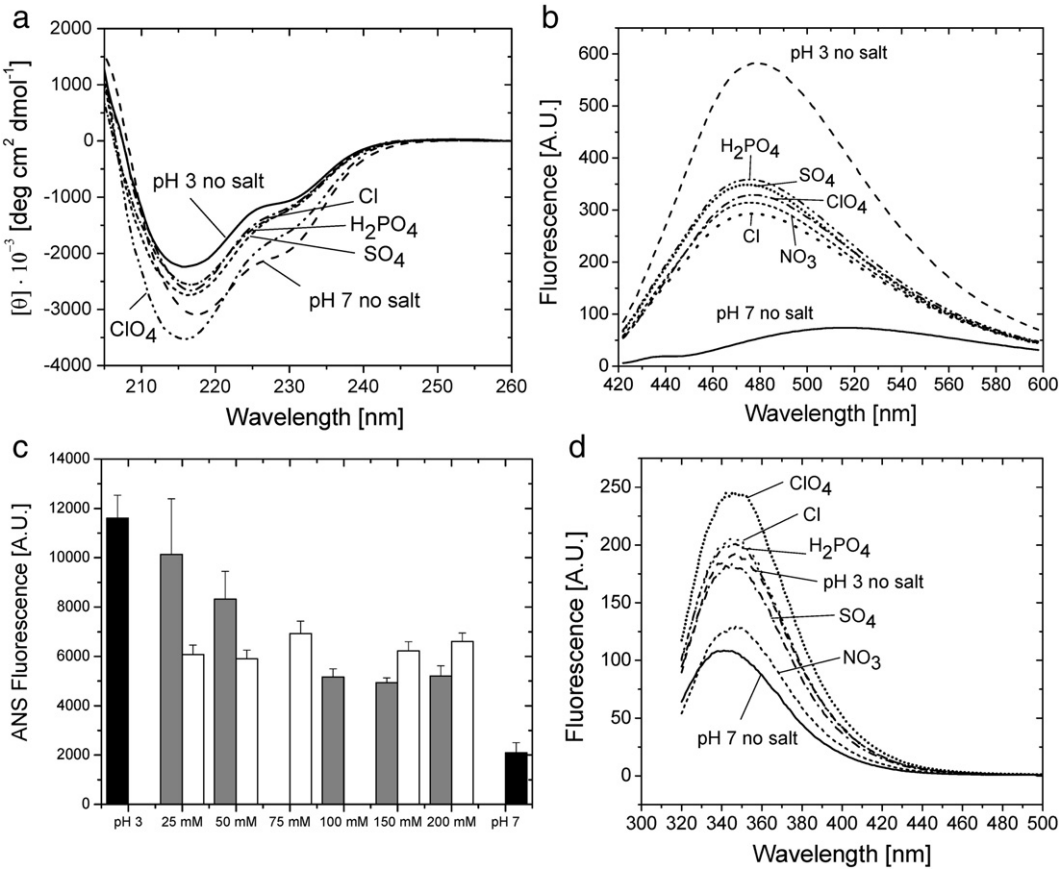

Fig. 7. Salt effect on antibody structure. Circular dichroism spectra (a), ANS fluorescence spectra (b) and intrinsic tryptophan fluorescence spectra (d) of 0.3 g/L antibody solution in 25 citric acid buffer at pH 7.0 and pH 3.0 without salt and at pH 3.0 with 150 mM of different salts. c) The same as in b) but with different concentrations of sodium sulfate (white bars) and sodium chloride (grey bars); pH = 3.

It is worth noticing that the mentioned preferential interactions affect and are also affected by the protein conformational structure and surface charge. Aggregation of globular proteins is commonly triggered by a change in the native protein structure. Since several structural conformations of a protein may be present at the same time [47], the added ions would favor one or another conformation, depending on the specific system, thus promoting or not aggregation. Moreover, protein surface is notoriously inhomogeneous and several binding mechanisms may occur at localized sites. The same ion can preferentially bind or be excluded in different regions of the same protein.

Considering all the above mentioned aspects, it is not surprising that the protein aggregation stability exhibits a rather complex dependence on pH, salt type and salt concentration. Although the various phenomena are strongly interconnected and therefore their independent study is challenging, in the following we investigate separately the salt effect on protein conformational stability and on surface energy.

3.3. Protein structure and surface energy

The effect of salt on protein structure was assessed by a series of spectroscopic techniques at low protein concentration (0.3 g/L) to reduce aggregation during the measurements. Circular dichroism (CD) gives information about the total amount of secondary structures present in a protein. In Fig. 7a the CD spectra of several protein solutions are shown. In physiological conditions (25 mM phosphate buffer solution at pH 7.0), the spectrum shows the characteristic minimum at about 216 nm, representative of the ordered β-sheet structure of immunoglobulins [48]. When pH is reduced to 3.0 (25 mM citric acid buffer without addition of salt), the shape of the CD spectrum remains similar to that in physiological conditions but the intensity is significantly reduced, indicating a loss in the ordered β-sheet content. Such a decrease is due to the intra-molecular repulsion forces generated by the positive charges induced by reducing pH. The addition of 150 mM of different salts at pH 3.0 leads to the CD spectra intermediate between physiological conditions and pH 3.0 without salt. This indicates that the presence of the salts re-generates partially the original β-sheet structure. The re-stabilization of the protein structure with addition of anions has already been observed in the literature and explained by anion-binding, which reduces the intramolecular charge repulsion [49]. Moreover, as stated above, the salt-induced increase of protein surface energy would favor a more compact, stable structure. A peculiar CD spectrum is observed for the very chaotropic perchlorate anion (Fig. 7a), indicating a significant change in the antibody secondary structure. The effect of nitrate anion could not be analyzed due to interference of the anion with CD measurements.

The CD spectra are consistent with the fluorescence spectra of the 1-anilino-8-naphtalene sulfonate (ANS) binding studies shown in Fig. 7b. ANS is a hydrophobic dye whose fluorescence increases upon binding to protein. Thus, ANS binding is considered representative of the global hydrophobicity of protein surfaces [50]. As expected, under physiological conditions the fluorescence value is very low, because folding of globular proteins normally minimizes the exposure of the hydrophobic patches and exposes the more hydrophilic parts to water. Reducing pH to 3.0 leads to a loss in the β-sheet content, as indicated by the CD spectra, and to an increase in hydrophobicity due to exposure of buried patches, in agreement with the increase of the ANS fluorescence signal shown in Fig. 7b. The restoration of

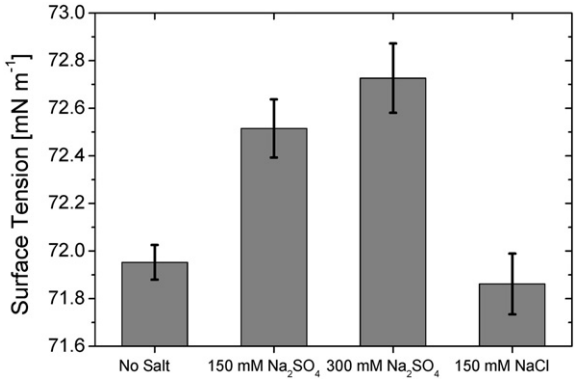

Fig. 8. Salt effect on surface tension. Surface tension of 0.1 g/L lysozyme solutions in 25 mM citric acid buffer at pH 3.0 in the absence or presence of sulfate and chloride anions.

the ordered structure by introduction of the anions corresponds to a reduction in hydrophobicity. Thus, the fluorescence spectra in the presence of anions shown in Fig. 7b are again intermediate between physiological conditions and pH 3.0 without salt. In order to quantify the effect of the anion on the protein hydrophobicity, we have plotted the peak of the ANS fluorescence spectra as a function of the sulfate and chloride concentration in Fig. 7c, which clearly shows reduction in hydrophobicity as the anion concentration increases.

Protein structure changes were analyzed also by intrinsic tryptophan fluorescence measurements. Tryptophan fluorescence depends on the polarity of the environment around the residue: a change of the tertiary structure leading, for example, to more solvent-exposure is reflected by a change in the fluorescence spectrum. As it can be seen in Fig. 7d, the tryptophan fluorescence in physiological condition is very low, indicating burying of the residue. The largest value of fluorescence is observed at pH 3.0 in the presence of perchlorate, thus confirming the denaturation effect of such anion observed by CD analysis (Fig. 7a). At pH 3.0 without salt and with 150 mM of sulfate, chloride, phosphate the spectra are similar, indicating no dramatic change in the tertiary structure of the protein, although the secondary structure content and the general hydrophobicity of the protein is different with and without salt, as discussed earlier based on the CD and ANS results. It is worth noting that the spectrum in the presence of 150 mM of nitrate is significantly different with respect to those of chloride, phosphate and sulfate, indicating that this chaotropic anion, in analogy with perchlorate, affects the antibody structure.

By connecting the results of the structure analyses above to the aggregation kinetics data discussed in the previous section we can conclude that aggregation correlates not only to the hydrophobicity content of the protein but rather to a suitable aggregation-prone intermediate state. A clear evidence of this is that the antibody structure at pH 3.0 without salt has the largest hydrophobicity but its solution is stable, mostly due to its disordered structure and increased number of surface charges. Adding salt should screen progressively the surface charges and trigger aggregation. On the other hand, salt addition promotes the formation of a less hydrophobic and more compact structure, less prone to aggregation. The increase of surface dipoles due to counterion binding also contributes to the reduction of hydrophobicity. The competition among structure re-organization, hydrophobicity reduction and charge screening induced by salt explain the maximum of the aggregation propensity as a function of salt concentration, as observed in Fig. 5.

The behavior of aggregation propensity as a function of pH without salt addition (Fig. 4) is most likely related to the competition among the same effects. In particular, the antibody at pH 4.5 retains ordered structures with low surface hydrophobicity. The opposite situation occurs at pH 3.0, where the hydrophobicity is the highest, but the antibody structure is more disordered and charged. In both situations, no aggregation is observed. Then, the observed aggregation at pH 4.0 is most probably promoted by two factors: reduced charges (with respect to pH 3.0) and less ordered structures (with respect to pH 4.5).

It is worth noticing that only for the chaotropic perchlorate and nitrate anions the increase in aggregation can be connected with changes of the protein structure. For all the other ions, at least in the resolution limit of the applied techniques, no significant effect of salt type on protein structure was detected. This suggests that, for a given salt concentration, ion specific effects are not connected to changes in protein structure properties, but most likely to salt-mediated protein–protein interactions, in particular solvation forces. These include hydrophobic forces due to salt exclusion, hydration forces related to counterion binding and dispersion forces between ion and macromolecule [5].

Hydrophobic forces lead to an increase of the protein surface energy due to the salt exclusion from the apolar portions of the protein surface. This increment is similar to the increase of water surface tension upon addition of salts due to ion exclusion from the air-water interface. Indeed, the increase in the protein surface energy in the presence of a cosolute can be directly linked to the bulk surface tension [1,51]. We checked the capability of the different anions to affect the protein surface energy under the conditions of this work by measuring the surface tension of 0.1 g/L protein solution in the same buffer composition of the aggregation experiments in the presence of sulfate and chloride at different concentrations. Due to the large amount of protein required for the measurements, a globular model protein, lysozyme, was used instead of the antibody and the results are shown in Fig. 8. It is seen that the sulfate anion can increase significantly the surface tension while chloride has a negligible impact. These results are consistent with the larger values of surface tension increment of aqueous solutions reported in the literature for sulfate with respect to chloride [6,39,52] and confirm the impact of the type of salt on protein surface energy and, consequently, on protein aggregation stability.

The experimental evidences discussed above indicate the complexity of these systems and the multiple ways through which salts can affect protein stability. Probably, a comprehensive quantitative description of the salt–protein and salt-mediated protein–protein interactions can be achieved only by molecular dynamic models at the atomic resolution [53–56]. On the other hand, models based on average potential of mean force considering the preferential interaction parameter and the corresponding solvation forces were able to qualitatively explain the observed experimental trends [57–60].

4. Conclusions

The experimental investigation of the salt effect on the aggregation/stability of a model antibody showed the following peculiar features. a) Reducing pH below a critical value of 4.5 induces aggregation to an extent which depends on the salt and the pH value. b) The salt effect is strongly ion specific: particularly, at pH 4.0 both cation and anion type affect aggregation, while at pH 3.0 the cation type does not play any role. The ranking of the anion effect follows the Hofmeister series, with the only exception of the sulfate, while the cation effect does not. c) A maximum of the aggregation propensity (i.e. presence of re-stabilization) as a function of salt concentration is observed.

By correlating the aggregation data to experimental information on protein structure and surface energy, it is found that changes in pH and addition of salt induce aggregation not only by altering electrostatic interactions but also by promoting the formation of an intermediate structure characterized by a certain degree of hydrophobicity and a partially ordered secondary structure. The effect of salt on the intermolecular interactions between two protein monomers is multifold. Apart from the typical charge screening effect, preferential ion exclusion can induce

hydrophobic forces which increase protein surface energy and promote aggregation. On the other hand, counterion binding generates the hydration forces, stabilizing the protein. The complex interactions between the protein structure properties and the various solvation forces explain the presence of the maximum of dimerization extent as a function of salt concentration, as well as the ion specificity and the peculiar behavior of sulfate anion with respect to the protein stability.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bpc.2012.05.004.

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