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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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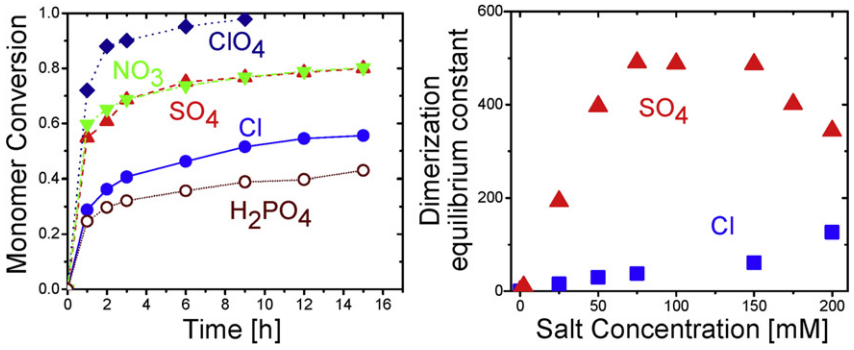
HIGHLIGHTS

- 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,