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TOWARDS PROTEIN CRYSTALLIZATION
Some thermodynamic studies

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The work describes the development in calorimetric studies on the aggregation process of simple globular proteins solutions induced by the presence of electrolytes. It also provides information on theoretical models and experimental methods applied to the examination of those processes.

Keywords: *aggregation, crystallization processes, experimental methods, small globular proteins, theoretical models, thermodynamic models*

Introduction

Though the first protein was crystallized over a hundred years ago, crystallization has largely remained an art rather than science. This opinion was formulated by Feher and Kam [1] more than 20 years ago. Although significant progress has been achieved, it is true also for the current situation. Most of the known recipes for protein crystallization were found by means of the trial and error method.

The basis of the specificity of the association processes occurring between proteins and other biomolecules is the complementary shape of the interacting molecules. Therefore, in order to investigate the mechanisms of basic biochemical reactions one needs to study the structures of interacting molecules at the atomic level. Only if three-dimensional structures of proteins and nucleic acids are known, we can describe macromolecular processes at high level of accuracy and learn about their interactions per se and their interactions with ligands (including drugs) which modulate and regulate their biological actions. This need is becoming more and more severe with the rapid advances in biology, biotechnology, molecular pharmacology and molecular medicine that require the understanding of biological processes at the atomic level.

Three-dimensional, atomic resolution structures of protein molecules can be determined by X-ray or neutron crystallography and nucleic magnetic resonance spectroscopy (NMR). Crystallography is the most frequently chosen method and more than 80% of known protein structures have been solved by X-ray crystallography (statistics are on the www.resb.org). Although the calculation of a protein structure from the diffraction patterns is not a trivial task, the major obstacle on the way to study protein structures by means of crystallography is protein crystallization [2]. Indeed crystallization of proteins and crystallization of inorganic crystals obey the same laws, none the less the differences arise from size, low symmetry, large solvent content, and relatively small number of contact points that result in small binding energies per unit volume. The latter causes the protein crystals to be soft and sensitive to small changes in external conditions [3], concentration of precipitant and protein, temperature, pH, pressure, purity, cleanliness, age, buffers, others. There is also a biological reason of difficulties with protein crystallization. In the cell proteins function in crowded environment – the total concentration of molecules is higher than the protein concentration used in crystallization set-up [2, 4].

Due to diversity of proteins, though we know more and more on the conditions of the course of the process of their crystallization, it is hardly probable that a theory will be elaborated in the near future. Therefore, what is left is to continue to learn as much as possible on the basis of crystallization process, including by means of thermodynamic investigations described in this review.

Progress in thermodynamic investigations in the past 10 years

Theoretical models

Prausnitz and Hagar in the paper entitled 'Three Frontiers in the Thermodynamics of Protein Solutions' [5] give three examples, which illustrate the versatility and

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usefulness of biothermodynamics: 1) calculation of a phase diagram for an aqueous target protein in a separation process where addition of a salt causes protein precipitation [6]. This example concerns the calculation of phase diagrams for aqueous lysozyme solutions with addition of sodium salts (NaCl, NaSCN, NaI of concentration 0.2 M, pH=4.3, T=298 K). Calculations were made with a new potential of mean force that takes Hoffmeister effect into account (the Hoffmeister series in the arrangement of ions in the decreasing order in which they salt out or coagulate colloids); 2) effect of addition of acetone to the aqueous solution of insulin, which dramatically increases the rate of crystallization by nearly one order of amplitude [7, 8]; 3) studies in the reactions of two drugs that deactivate the HIV protease realized using titration calorimetry [9]. The progress in thermodynamic studies concerns not only the research directions described by Prausnitz and Hagar.

Kierzek and Zielenkiewicz in the paper entitled: 'Models of protein crystal growth' published in Biophysical Chemistry in 2001 [10] presented the progress in elaboration: 1) theoretical model of association based on Gibbs free energy transfer from gas phase to the solution [11]; 2) microscopic models of crystallization processes [12–14]; 3) model of association based on the Kauzmann concept of hydrophobic effect enriched by the approaches given in the papers of Chotia and Janin [15], Eisenberg and McLachlan [16] and then by Horton and Lewis [17].

Vallone *et al*. [18] calculated the association free Gibbs energy of hemoglobin mutants using Horton and Lewis approach [19]. They obtained a very good agreement between the calculated and the experimental values. According to the author's knowledge, it is presently the only method providing consistent results of theoretical and experimental studies.

Experimental methods

In the last 10 years the progress was also made in experimental investigations. The mechanism of crystallization processes was investigated by several experimental methods, chemical factors and experimental techniques. Unsaturated, dilute protein solutions were often investigated by spectroscopic methods such as: UV, fluorescence, CD-spectroscopy, osmometry, whereas crystal growth was investigated by e.g. light scattering, both static and dynamic, interferometry, X-ray topography, low angle X-ray and neutron scattering, atomic force microscopy and most recently by confocal microscopy [20].

There are several levels of intermolecular rearrangement involved in the nucleation process from monomers to higher oligomers, fractals (amorphous precipitates) and nuclei. In the study of this process determination of the level of protein aggregation have been marked by a particularly intensive use of dynamic light scattering. This method was also used to evaluate Gibbs free energy of association as well as Mc Milan Meyer virial coefficients.

Experimental thermodynamics contributed to the explanation of crystallization processes by e.g. the solubility and phase diagrams determinations as well as determinations of various conditions of crystallization process. However, calorimetric methods were rarely used in the study of crystallization processes [19–22].

Calorimetric investigations

In 1997 we undertook the calorimetric investigations of aggregation process with the use of simple globular proteins: salting by NaCl, LiCl, KCl, K_2SO_4, Li_2SO_4, $(NH_4)_2SO_4$.

Home-made conduction microcalorimeter was applied to the study of kinetics of the aggregation process of three times crystallized lysozyme, dialyzed against water, lyophilized and stored at 4°C. All experiments were performed in a buffer containing 0.1 M sodium acetate, pH 4.5. Monodispersity of the preparations was controlled by dynamic light scattering (DLS) without added electrolyte before each series of experiments. Various lysozyme and NaCl concentrations were used in experiments conducted continuously for a few hours. Pronounced heat-power peaks that can be attributed to nucleation and growth, appeared at finite times which, in turn, depended on the supersaturation level. For example, for NaCl concentration equal 0.639 M and concentrations of lysozyme corresponding to 2.60, 2.78, 2.95, 3.16 mM, respectively, the elapsed time between mixing of the components (lysozyme solution, buffer, NaCl) and the occurrence of the heat-power peak maximum in the thermogram corresponded to 13.17, 6.86, 4.15 and 2.1 h. In line with those studies for the same samples and conditions of measurement (temperature, pH, stirring of solution) DLS experiments were conducted. The qualitative accordance of the calorimetric results with the nucleation behavior deduced from small-angle scattering experiments was obtained [23]. Kinetics of NaCl induced lysozyme aggregation in which the aggregates size distribution was monitored independently by DSL and nuclear magnetic resonance (NMR) was analyzed. DSL experiments showed a systematic increase of the aggregates size, while upon NMR diffusion-ordered spectroscopy (DOSY) experiment in the hour time scale no significant changes of the diffusion coefficient were observed. After ~70 h of NMR experiment with 2.1 mM lysozyme solution in 0.5 M NaCl solution started to crystallize/aggregate, which was indicated by a decrease in both translational

and rotational diffusion coefficients [24]. DSL method was also employed to investigate the behavior of nucleating ovalbumin solutions when varying the concentration of $(NH_4)_2SO_4$ [25].

Changes of the enthalpy *vs.* salts concentration, using ITC MicroCal titration and LKB 10700-2 microcalorimeters, were determined. In the case of experiment with lysozyme solution of constant concentration of 0.1 mM *vs*. varied concentration of NaCl in the range 0.3–1.1 M it was showed that: a) the salting process is connected with negative enthalpy; b) the increase of salt concentration enlarges the negative value of the enthalpy changes; c) in the concentrations of about 0.3 M NaCl and 0.65 M NaCl the inflections in the curve $\Delta H=f(m\text{NaCl})$ were observed. Similarly, densimetric determinations brought us to the conclusion that the changes of the apparent molar values in relation to the buffered lysozyme solution *vs.* NaCl concentration followed a similar pattern as the enthalpy changes.

The obtained values of enthalpy and apparent molar volumes *vs*. NaCl concentration were compared with the solubility data presented in the paper of Retalleau *et al*. entitled: 'No salting-in of lysozyme chloride observed at low ionic strength over a large range pH', [26]. In this paper it was stated that the solubility of lysozyme always decreased as soon as sodium chloride was added and no salting-in occurred. The curves corresponding to the course of solubility changes *vs*. NaCl concentration, presented for various pH and NaCl concentration, showed bimodal variation of lysozyme solubility: 1) at first, a steep decrease down to 0.2–0.3 M NaCl and then a more moderate decrease at higher NaCl concentration; 2) then, the slope of a solubility curve *vs*. NaCl concentration changed also slightly in concentration exceeding 0.6 M NaCl. For these values of lysozyme and NaCl concentrations the maxima on the enthalpy change curves and apparent molar volumes curves were also obtained. The deflections occurring on these curves were most visible for the function $\Delta V_{\phi,3}=f(m\text{NaCl})$ (Fig. 1), with $\Delta V_{\phi,3}=0$ corresponding to the $V_{\phi,3}$ of lysozyme solution when NaCl was not added. The curve show bimodal variation of lysozyme solubility: According to the course of this curve, in the NaCl concentration range 0–0.3 M, the apparent molar volume of lysozyme decreased by $2.98\cdot10^2$ cm^3 mol^{-1}. Starting with the concentration of m_{min}=0.3 M NaCl apparent molar volume begins to rise to achieve a value of $108\cdot10^2$ cm^3 mol^{-1} at concentration m_{max}=0.68 M NaCl, i.e. between minimum and maximum values of the apparent molar volume. If one assumes that the minimum occurring in the $\Delta V_{\phi,3}$ is the starting point of the process, whereas the maximum value (0.68 M NaCl) indicates the completion of

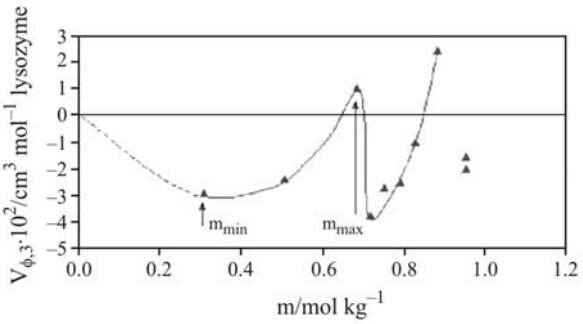

Fig. 1 $V_{\phi,3}=f(m\text{NaCl})$ from paper

the process, then the difference in apparent molar volumes of various electrolytes Li_2SO_4, $MgCl_2$, $(NH_4)_2SO_4$ gives quite similar increment averaging $\Delta\Delta V_{f,3}=3.4\cdot10^2$ cm^3 mol^{-1} for monovalent ions and twofold larger for divalent ions [27].

Such changes occur also in the course of the enthalpy and apparent molar volume changes of other simple proteins studied like bovine albumin (A) and albumin from human serum (B), whereas, depending on the protein and salt used, both ΔH, $\Delta V_{\phi,3}$ and $\Delta m=m_{min}-m_{max}$ differ [28, 29]. However, the course of the functions: function $\delta\Delta H=f(\Delta m)$, (Fig. 2) and function $\delta\Delta V_{\phi,3}=f(\Delta m)$ (Fig. 3) is similar to the linear course [30].

The disturbances occurring on the enthalpy and apparent molar volumes curves at low concentration of salts are caused by the disturbance of solvation shell due to the formation of pairs between protein molecules and ions, whereas at higher salt concentration

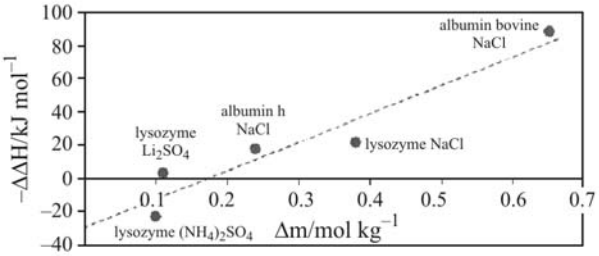

Fig. 2 The changes in the increment of the enthalpy, of salting $\Delta\Delta H$ *vs*. $\Delta m=m_{min}-m_{max}$ [29]

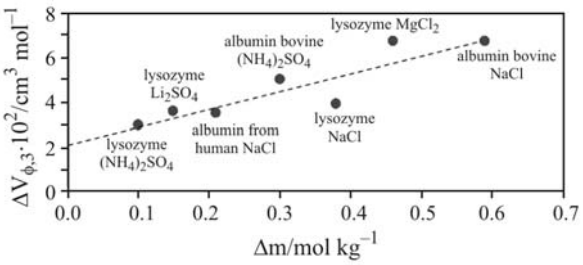

Fig. 3 The changes in the increment, of $\Delta V_{\phi,3}$ *vs.* $\Delta m=m_{min}-m_{max}$ [29]

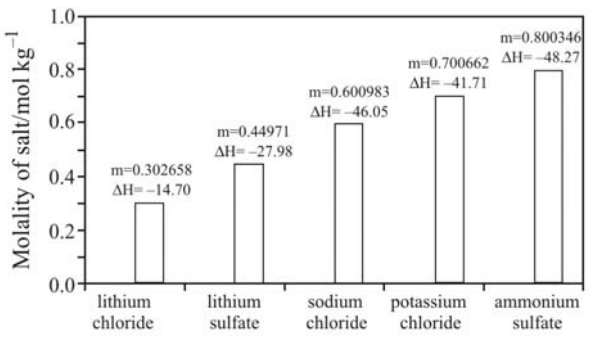

Fig. 4 Local maximum of salting [31]

preferential solvation of salts ions can desolvate proteins causing their aggregation, precipitation or crystallization. This results in the occurrence of the maximum on the curve at concentration m_{max} and intensive aggregation/precipitation process [31]. For example, the ability of various electrolytes to aggregate lysozyme solution at m_{max} concentration and evaluated values of the enthalpy are presented in Fig. 4 [32]. The determination of enthalpic pairwise coefficients evaluated from enthalpy data confirmed previous considerations. It results from them that at low concentrations the protein–electrolyte interactions play the main role, whereas as the concentration increases the protein–protein interactions gain significance [31, 33].

The conducted calorimetric investigations demonstrate usefulness of this technique for studying aggregation processes. Their continuation will be concerned with the study of aggregation and crystallization processes in various pHs, buffers and temperatures. The subject of the study will not only be simple globular proteins but also protein-components of human blood with various ligands (including drugs).

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