UNIVERSITE DE LAUSANNE - FACULTE DES SCIENCES

DIPLOME DE BIOLOGISTE*

Effet de l'Amylose sur l'Ovalbumine en Solution Aqueuse en Mélange avec la Tricaprine et le Caprate de Sodium

Une étude Thermodynamique de Systèmes Ternaires Utilisant la Calorimétrie à Balayage Différentiel (DSC) et la Dispersion Statique de la Lumière d'un Laser

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1. INTRODUCTION

1.1. Foreword

Protein conformation stability has been recognised as the primordial parameter affecting the functional properties of food [1,2,3]. According to thermodynamic laws the native state protein folds into such a conformation that the free-energy used up to maintain it is minimal. However, protein free energy is rarely minimal during and after food processing, for example, in treatments in which heat intervenes. Indeed, in most food products, proteins are partially or totally denatured, i.e. are subjected to conformational modifications. In food processing heat denaturation usually leads to total unfolding of proteins: starting from the native folded stable structure they adopt a completely random coil conformation at the end of the treatment. During the transition between these two extremes heat is absorbed or liberated by the protein, modifying its internal energy, i.e. its enthalpy. As heat is swapped between the protein and the medium, thermodynamic methods may be used to measure it. Among them differential scanning calorimetry (DSC) is of the highest importance. Its main interest lies in the fact that this method quickly provides direct thermodynamic parameters, such as denaturation, enthalpy, heat capacity and temperature, which are closely dependent on protein conformation. This explains why DSC has been intensively used for the last 25 years to study protein stability. During this period most work has been focused on individual proteins and binary interactions between protein and another food component, but little interest has been focused on ternary systems.

The aim of the present work is to associate the three main components of real food: proteins, polysaccharides and lipids. The thermodynamic behaviour of hen ovalbumin in the presence of potato amylose, sodium caprate and tricaprin is characterised.

1.2. Comment on the presentation of the report

It was decided to accompany the calorimetric studies by light-scattering experiments on ovalbumin and amylose. This is a well known method for characterising interactions between molecules. As light-scattering experiments represent a small part of the whole work, each main chapter of the report will end with this subject.
Tricaprin and sodium caprate are both considered as lipids.

1.3. Choice of compounds

By far the most important protein of hen egg white [4] (ca. 2/3 of the total protein [5,6]) ovalbumin is so outstandingly equilibrated in its amino acid composition that it is one of the best nutritional model proteins. This glycoprotein (3.5% of carbohydrate [7]) is very important in food technology since egg white is widely used in food processing due to its whipping [8], gelling [3,9] and foaming [10] properties and glycoproteins are extensively employed as emulsifiers [11]. Moreover, ovalbumin is known to gel under various conditions[12], especially after heat treatment[13] and owing to this property egg white is also used in many food products which require gel structure. Thus, ovalbumin is a good model for studying protein functional properties.

Ovalbumin has been already investigated by DSC since it is a heat-sensitive globular protein of compact native conformation [14] which easily denatures when heated [13]. Moreover its DSC thermogram profile is representative of all the proteins of the egg matrix [15].

Together with amylopectin amylose is the most widely eaten carbohydrate in the world [16]. This linear polysaccharide is widely used in food technology to improve gelling structure. In natural food products starch gelling property mainly comes from amylose. Indeed, during starch heating, amylose exudes from the granule and diffuses into the solution whereas amylopectin molecules still provide starch structure. After cooling amylose molecules recover their helical structure which forms the gel. Moreover, according to this phenomenon, one can say that, when heat is applied, amylose is the first starch component to enter into contact with the surrounding molecules of the medium.

Compared with e.g. wheat starch, potato starch contains almost no lipids [17] and thus is suitable for interaction studies with lipidic molecules.

'Sodium caprate is a salt of capric acid (systematic name: decanoic acid) which is a saturated

fatty acid of 10 carbons. Like fatty acids shorter than 14 carbons, capric acid is seldom a constituent of food triglycerides, apart from coconut (6%) and palm seed (4%) [4] oils. This fatty acid was nevertheless chosen due to its short chain length and, therefore, its greater solubility in aqueous medium than longer chain lipids.

Tricaprin is a triglyceride with three capric acids. Owing to its non polar nature, tricaprin better symbolises food lipids than sodium caprate.

1.4. Thermodynamic theory of protein conformation and heat denaturation

The native form of a protein is Stabilised by different kinds of forces. Apart from specific driving forces such as hydrogen bonds, disulphide linkages, electrostatic and van der Waals interactions, protein folding and Stabilisation is mainly caused by the so-called hydrophobic effect. This effect is linked to the high ability of water to form strong hydrogen bonds.

By definition non polar amino acid residues cannot form hydrogen bonds with water. Consequently, when water molecules surround the hydrophobic elements, they are tidily ordered through hydrogen bonds. This firm structure is associated with low entropy. Since free energy is defined by

$\Delta G = \Delta H - T\Delta S$

(eq.1)

(where ΔG is the change in Gibbs free energy, ΔH is the change in enthalpy, T is the absolute temperature and ΔS is the change in entropy), a small entropy change leads to a high ΔG. This goes against the fundamental thermodynamic law of biopolymers which consists of minimising free energy [8]. In fact, in the native form, protein tends to "hide" its apolar residues from water, i.e. to bury them in the structure [18]. Consequently the effect on AG of apolar residue burying is two-fold: destructuring of surrounding water molecules causes a large …… …… …… …… … …… … …… ……..

increase in entropy; and the dense concentration of residues in the interior of the molecule leads to lower enthalpy. Thus, the free energy of a native protein is minimal. After food processing protein structure is usually different from the native conformation. Most of the time like the protein looks like a random coil whose hydrophobic residues are exposed to the water molecules. Obviously the free energy of such a protein is higher than the minimal value.
This change of conformation is called denaturation. The phenomenon is provoked by physico-chemical changes in the molecular environment. One of them, heat, is the denaturing factor the most relevant to food processing[8,18]. The large number of publications consecrated to thermal denaturation of proteins leads to the conclusion that it is a transitional process of co-operative multistate nature[19]: between the native and unfolded states many intermediate structures of progressive denatured stages follow on from each other. An exception is most small globular proteins whose transition is considered to be a two-state process, i.e. lacking of intermediates[20]. The transition which occurs during thermal denaturation of protein and which starts from a well structured, stable conformation to finally end with the complete unfolding of the molecule is a process in which all the structures are disrupted. Given that the terminal state of a given protein is the same for all processes, the more structures broken during the process, the more stable is the protein in its native conformation. The measure of this disruption may be the unfolding enthalpy since the enthalpy change during scanning is associated with a change owing to the unfolding of the protein[21-22]. The enthalpy change is given by the sum of the breaking of internal hydrogen bonds in protein and in water, and the formation of protein-water bonds and hydrogen bonds between water molecules around the apolar groups[23].

1.5. Principle of Differential Scanning Calorimetry (DSC)[a]

Differential scanning calorimetry is based on the thermal denaturation process. Starting from the native state, the molecule to be denatured is subjected to a linear programmed increase in temperature until it reaches its unfolded form. During the process, the breaking of the linkages maintaining the native structure is accompanied by heat absorption or liberation. This exchange of energy between the molecule and the solvent is the basis of the so-called "null-balance" principle[25] of DSC method described as follows:
DSC instruments, as imagined by their designers (see e.g. reference 24), are equipped with two cells, one containing the molecule to be denatured surrounded by its solvent and the other, the reference cell, holding the solvent alone. By increasing the temperature, heat is supplied to the cells, both of which receive the same amount of energy. The heat content difference between the two cells, i.e. the heat coming from the exchange between the molecule and its solvent, is continuously checked by a thermal captor. When a difference occurs the captor immediately compensates for it through a withdrawal or a supply of heat flow in the reference cell to maintain the two solutions at the same temperature. The compensating heat flow is then recorded for each temperature difference. As the rate of heat flow into a sample is proportional to its instantaneous specific heat, or heat capacity[25], Cp, one can obtain the latter by comparison with a standard under the same conditions. For most DSC instruments the standard is provided through a precise supply of heat of known energy.

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[a] A complete paper should be consulted, reference 24

In practical heat capacity measurement a function of temperature leads to thermogram conception. A typical thermogram picture is obtained from protein thermal denaturation. An example is given in figure 1. Denaturation occurs between the native form, symbolised by A, and the denatured form, expressed by F. The denaturation process starts from point B and ends at point E. Point C symbolises the transition point at which half of the protein is in the native form, the other half being denatured. Extrapolation on the ordinate of the AB segment gives the heat capacity of the native molecule (Cp(N)) whereas EF provides the unfolded protein heat capacity (Cp(D)). Heat capacity change, $\Delta_d Cp$, shown as (G-H) segment, is positive for most proteins[26]. The reason is still unclear but for many authors $\Delta_d Cp$ indicates the degree of exposure, or the solvating, of hydrophobic groups in the denatured state[14,28,29] and seems to be correlated with the amount of hydrophobic contacts[26], i.e. groups close enough to stabilise the structure[21] by the hydrophobic effect presented above. It has been estimated that at least 80% of the $\Delta_d Cp$ values are due to hydrophobic solvating[20].

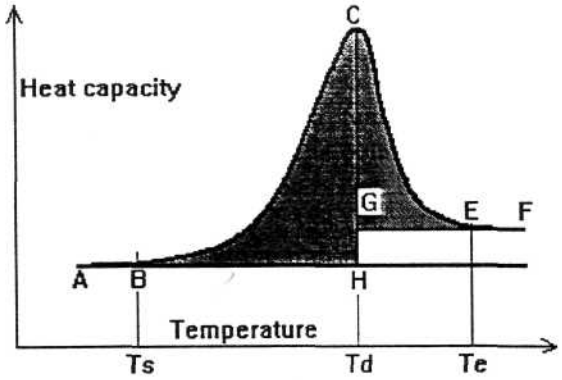

Figure 1. Typical DSC thermogram for protein heat denaturation

Apart from heat capacity parameters the thermogram allows to calculate the calorimetric, or practical, enthalpy of the denaturation reaction, $\Delta Hcal$, and the effective, or theoretical, enthalpy, namely the van't Hoff enthalpy ΔH_{VH}. The first is obtained from the surface of the denaturation curve[20]. Indeed, since the difference in enthalpy between native and denatured state at temperature T, $\Delta H(T)$, is defined by the integration[28]

$$\Delta H(T) = \int C_p dT, \quad \text{(eq.2)}$$

$\Delta H(T)$ represents the area under the peak (symbolised by the points BCEGH on figure 1). The van't Hoff enthalpy applies to a transition process where a molecule transforms in another without intermediates (or with a very low probability of having intermediate forms), called the two-state transition. It can be obtained from the thermogram by one of the two following equations (the first one from Privalov and Potekhin[77] and the second one from Privalov and Khechinashvili[21])

$$\Delta H_{vH} = 4RT_d^2 \times \Delta Cp \times M^{-1} \times \Delta H_{cal}^{-1}$$

$$\Delta H_{vH} = 4RT_d^2 \times \Delta T_{1/2}^{-1} \times M^{-1}$$

where, R is the gas constant, 8.314 J mol^{-1} K^{-1}, T_d is the temperature at the transition point (K), i.e. at the maximum of heat absorption in thermogram (the point C at which half of the protein is denatured[30,32,33]), ΔCp is the height of the curve at the transition temperature (JK^{-1}) M is the molar mass (g mol^{-1}) and $\Delta T_{1/2}$ is the width of the calorimetric transition at half peak height (K).
Comparison of both enthalpies measures the similarity of the theoretical and experimental treatments: as the van't Hoff enthalpy is only available for protein characterised by a two-state transition, the equality of both enthalpies assesses that the protein studied denatures in a two-state manner. As it has already been said these two values are comparable for most globular proteins[20].

The usual parameters taken to provide an estimation of protein stability are ΔHcal and T_d[34], whereas Δ_dCp is assessed to characterise the relation of the molecule to its environment and the ΔHcal/ΔHvH ratio is calculated to verify the two-state transition. According to Privalov et al.[35] $\Delta T_{1/2}$ evaluates the co-operativity of protein unfolding: if denaturation occurs within a narrow range of temperatures, the transition is a very co-operative process. The lower the $\Delta T_{1/2}$> the higher is the co-operativity[36].

1.6. Light-scattering

1.6.1. Principle

According to Kratochvil[37] light-scattering (LS) seems to be the most universal method for the mass determination of macromolecules. Moreover LS can lead to various other indications like e.g. thermodynamic interactions between a molecule and its environment, characterised by the second virial coefficient, the size of the particle, given from the radius of gyration, and polydispersity of a polymer in solution. The fundamental importance of these physical and thermodynamic parameters and the great number of publications in which they have been applied for more than 50 years makes this technique one of the most important methods for physical investigations on molecules.
To outline the situation, the principle of LS for molecules is the following[b,]: When light comes into contact with any molecule, the oscillating electric field component induces dipole moments in the particle, i.e. makes the electrons of the molecule oscillate. The oscillating electrons are in turn a source of new electromagnetic radiation, scattered light. The light scattered is commonly characterised by the so-called Rayleigh ratio, $R\theta$, which expresses the intensity of the light scattered $i'_{u,\theta}$ as a function of the incident light intensity, Io:

[b] The following theory has been formulated essentially according to references 37 (mainly), 38, 39, 40 and chapters 5, 6, 10 and 17 from reference 41

$R_\theta \sim (i'_{u,\theta} / Io)$, (eq.5)

$R_\theta = i'_{u,\theta} r^2 / Io\ (1+ \cos^2\theta)$[c]

where θ is the angle formed between the scattered light and the continuous incident light, r is the distance between the scattering molecule and the detector and the subscript u means that the incident beam is non polarized.

1.6.2. Single polymer solutions

If the particle is small, i.e. at least 20 times smaller than the wavelength of the incident beam (less than 200kDa) so that it can be considered as a single point, assuming that the particle is isolated and that the medium is perfect (close to an ideal gas), the scattered light can be expressed as:

$$i'_{u,\theta} = Io\ (1+ \cos^2\theta)\ (8\ \pi^4\alpha^2/r^2\lambda^4)\ ^{37}, \quad \text{(eq.6)}$$

where a is the polarizability of the scattering molecule and λ is the wavelength of the incident light in the medium.
Since the polarizability of a molecule is difficult to obtain experimentally, it is easier to express it through the refractive indexes by the relation:

$$\alpha = (M/2\pi N_A)\ (dn/dc) \quad \text{(eq.7)}$$

where M is the molar mass (g/mol), N_A is the Avogadro number and dn/dc is the refractive index increment (change of the refractive index, n, of a medium with concentration, c, of the components), c is usually expressed in g/cm^3. Thus:

$$i'_{u,\theta} = Io\ (1+ \cos^2\theta)\ (2\ \pi^2/r^2\lambda^4 N_A^2)\ (dn/dc)^2\ M^2 \quad \text{(eq.8)}$$

In a system containing many molecules it is obvious that the total scattered light will depend on the number N of the molecules per unit volume, V, (density of molecules N/V) in the system which is correlated with the equation:

$$N/V = cN_A/M \quad \text{(eq.9)}$$

and therefore:

$$i'_{u,\theta} = Io\ (1+ \cos^2\theta)\ (2\ \pi^2/r^2\lambda^4 N_A)\ (dn/dc)^2\ M\ c \quad \text{(eq.10)}$$

For a molecule in solution, let us multiply $i'_{u,\theta}$ given for an ideal solution by n_0, the refractive index of the solvent and replace λ by λ_o/n_o, λ_o is the wavelength in a vacuum:

$$i'_{u,\theta} = Io\ (1+ \cos^2\theta)\ (2\ \pi^2 n_o^2/r^2\lambda_o^4 N_A)\ (dn/dc)^2\ M\ c \quad \text{(eq.11)}$$

[c] If light is polarised in a vertical direction, $R\theta = (i'_{u,\theta} / Io) \sin 9$, assuming that r = 1

Considering now that a normal dilute polymer solution is subjected to random density fluctuation of solute molecules, i.e. that all different dV of the solution do not contain the same concentration of solute molecules, it is usual to express this new parameter by the so-called virial expansion formulated by the power series of the dependence of the osmotic pressure π (sometimes also of the chemical potential, μ , of the solvent[37]) of a molecular solution at the molecular concentration[29,42]:

$$\pi / c = \text{const}\ (1/M + 2A_2c + 3A_3c^2 + ...) \qquad \text{(eq.12)}$$

where the A terms are the virial coefficients. The first term of the right hand of the equation describes an infinitely dilute solution, in which no interaction between solute molecules occurs. The other terms are corrections for the interactions which occur between the solute molecules at higher concentrations. The virial coefficients, dependent on the shapes of the molecules and the intermolecular forces[39], are important parameters characterising the thermodynamic interactions among the solute molecules at a given temperature[43]: When $A_{2,3,...} > 0$ it means that the polymer molecules prefer contact with solvent molecules rather than with other polymer molecules, and thus the solvent is a good one for the polymer. For $A_{2,3,...} < 0$, the polymer-interaction is favoured and the fluctuation probability is high. If $A_{2,3,....} = 0$, polymer-polymer and polymer-solvent contacts are energetically equivalent. The integration of the fluctuation component into (eq.11) gives:

$$i'_{u,\theta} = Io\ (1+ \cos^2\theta)\ (2\ \pi^2 n^2/r^2\lambda^4 N_A)\ (dn/dc)^2\ c\ 1/(M^{-1}+2A_2c + 3A_3c^2 + ...) \qquad \text{(eq.13)}$$

with n, the refractive index of the solution $\approx n_0$, in dilute solutions.

For $K_u{}^d = (2\pi^2 n_o{}^2/\lambda_o{}^4 N_A)(dn/dc)^2$ (eq.14)

and with (eq.5), one can express the Rayleigh ratio as:

$$R_\theta = K_u c / M^{-1}+2A_2c + 3A_3c^2 + ... \qquad \text{(eq.15)}$$

to finally lead to the usual equation for light-scattering from solutions of small macromolecules:

$$\mathbf{K_u c/R_\theta = M^{-1}+2A_2c + 3A_3c^2 + ...} \qquad \text{(eq.l 6)}$$

For larger particles, that is of hundreds of nm (hundreds of kDa) the light scattered can interfere with other scattered beams issued from the same particle. Thus, contrary to small particles, the light scattered do not spread all directions with the same intensity since the scattering phenomenon strongly depends on the shape and orientation of the particle. Consequently, characterisation of light-scattering of large particles must be performed from many different angles, whereas for light scattered by small molecules measurement from a single angle is sufficient (see the relevant chapter in the materiel and methods section). It

[d] For vertical polarised light $K = 2K_U$

Thus $K' = (2\pi^2 n_o{}^2) / (\lambda^4 N_A)$

seems obvious that at zero angle there is no intramolecular interference. Thus, for this angle, the fundamental equation given for small particles may be re-written for large particles.

1.6.3. Solutions of polymer mixtures

For multi-component systems, usually binary polymer mixture, the fundamental equations of light-scattering have to be re-written using most of the parameters obtained for single polymer solutions expressed in a different nomenclature: the first component carries the subscript 2 and the second the subscript 4. According to the common convention M_2 and M_4 are respectively the molar masses of polymer components 2 and 4, c_2 and c_4 their concentrations and v_2 and v_4 their specific refractive index increments. A_{22} and A_{44} are their second virial coefficients and characterise the interaction of two identical polymer, whereas the second virial coefficient A_{24} symbolises the interactions of polymer 2 with polymer 4. It is important to add that A_{24} is independent of the molar masses of the polymers[37]. The third coefficients are expressed with three subscripts, e.g. A_{224} which characterises the interaction of 2 molecules of polymer 2 with one molecule of polymer 4.
In a sufficiently dilute solution, all parameters can be obtained from experimental results for single polymer solutions except A_{24}. Concentrations are usually expressed by the mass fractions of the polymers:

$$w_2 = c_2/c_2 + c_4 \text{ and } w_4 = c_4/c_2 + c_4 \qquad \text{(eq.17)}$$

In analogy to eq.16 the basic equation for multi-component system is given by eq.18:

$$K_u^{'e}(c_2 + c_4)/R_e = 1/(V_2^2M_2w_2+V_4^2M_4w_4) + \\ + 2((v_2^2M_2^2w_2^2A_{22}+2v_2v_4M_2M_4w_2w_4A_{24}+v_4^2M_4^2w_4^2A_{44})/(v_2^2M_2w_2+v_4^2M_4w_4)^2)(c_2+c_4). \qquad \text{(eq.18)}$$

The practical use of this complicated equation is explained in the relevant part of the following chapter.

[e] For mixed solutions the square of the refractive index increment is omitted (in ref 37 p.274).

2. MATERIEL & METHODS

2.1. Preparation of solutions

Chemical products. Chicken egg albumin (Grade III A-5378 Lot 43H7010), potato amylose (Type III A0512 Lot 63H3910) and tricaprin ($C_{33}H_{62}O_6$, M = 554.85 g/mol, F7517 Lot 71H8455) were purchased from Sigma, Na-decanoate (or sodium caprate, $C_{10}H_{19}O_2Na$, M = 194.25 g/mol) from the Russian technology. All substances were assumed to be sufficiently pure without further purification.

Buffer. For all solutions twice distilled water was used. A buffer stock 0.1M, pH 7.0 was prepared at 25°C (for 1 litre, 39.0ml of NaH_2PO_4 0.5M + 53.6ml Na_2HPO_4 0.5M). Buffer 0.01M was used for all solutions. As the preparation of amylose solution needs an alkali treatment with NaOH (see further) and, due to neutralisation with HC1, 0.04M of NaCl was prepared. The ionic strength of the amylose solution was 0.05M. For ovalbumin solutions buffer was corrected with 0.04M NaCl.

Single component solutions. Ovalbumin solutions were prepared using a 0.05M NaCl-buffer (0.04 NaCl + 0.01M buffer) by addition of lyophilised protein at a concentration of 0.5% for measurements with lipidic molecules and at a concentration of 1.0% for the mixtures with amylose. Ovalbumin was stocked at 4°C for no longer than 4 days. At the concentrations ovalbumin does not gel[13] and DSC can be performed[21]. Moreover, since interactions between protein residues easily occur when the molecule is unfolded, leading to aggregation, solutions must be dilute enough to avoid interactions.
For amylose, as for most polysaccharides, problems were encountered in obtaining aqueous solutions of more than 5%. Moreover it is known that after lyophilisation solubility is lost. Therefore, solutions of more than 2% can not be obtained by the process described below. Amylose is first added to 2 vol buffer; after 1 minute 1 vol NaOH 2N is slowly added to wet, and thus solubilise, all amylose clusters; the solution stirred gently for 5 min; buffer is then progressively added until the desired concentration is reached and the pH is finally neutralised by titration with ca. 2 vol of HC1. The solution is then heated to 85°C for 1 hour to overcome associations of molecules, i.e. to increase solubilisation, cooled to 30°C and centrifuged during 20 min at 4'000t/min to remove the non soluble particles. The supernatant was stocked **at** 40°C (temperature low enough to prevent evaporation and high enough to limit retrogradation) for no longer than 4 days.
Tricaprin was solubilised in100µl ether and sodium caprate in 100µl of water with a Hamilton syringe at concentration from 0.05 to 6.55mM and from 0.05 to 20.0mM respectively.

Concentration determination. The concentration of protein was determined by UV absorbance (280nm) using extinction coefficient s obtained by dry weight measurements. For amylose the concentration was checked directly after the centrifugation by the "Phenol Sulphuric Acid Colorimetric Method" from Dubois et al.[44] with solutions diluted up to 0.01% (1ml sample + 1ml phenol 5%(w/v) + 5ml conc H_2SO_4, 480nm). The extinction coefficient was obtained by dry weight measurements. For the days following the preparation the concentration was estimated measuring the volume of the stock solution lost by evaporation. For ovalbumin $\varepsilon = 6.0$ and for the polysaccharide $\varepsilon = 78.8$.

Mixture solutions. For protein/lipid solutions tricaprin in ether was added to ovalbumin solution using a 25µl Hamilton syringe and the solution stirred uncovered for 15 minutes at room temperature before thermostated stirring to remove the solvent This step is obviously not necessary for sodium caprate/ovalbumin solution. For protein/polysaccharide solutions two equal volumes of each twice concentrated pure solutions were mixed. For ternary solutions an equal solution of polysaccharide twice concentrated was added to a twice concentrated solution of protein/lipid mixture.

All solutions were shaken for 45 minutes at 30°C before being measured. Transfer to the calorimeter cells and to the filtration process preceding the LS measurements was done as quickly as possible to avoid cooling the solutions. Indeed capric acid has a PF of 31.3[4] and white solid globules have been observed in solution at a temperature of < 27.0°C.

2.2. Differential Scanning Calorimetry

2.2.1. Description of the device

The differential adiabatic scanning microcalorimeter (DASM) designed by Privalov and his co-workers[24] (Institute of Protein Research of the former USSR Academy of Science) in the early 70's is among the most famous calorimeters used for measurement of biopolymer systems[45]. These instruments are called microcalorimeters owing to their great sensitivity to temperature.

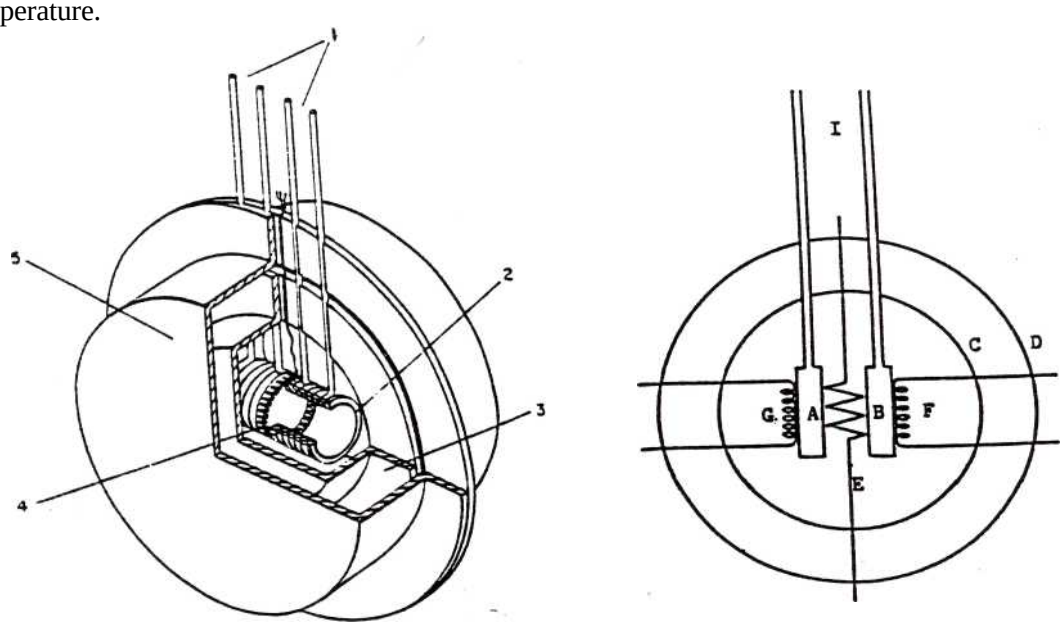

Figure 2 Calorimetric block of the capillary scanning microcalorimeter DASM-4 (from reference 24) on the left (1-capillary inlets, 2-capillary cell heater, 3-intemal shield, 4-thermopile, 5-extemal shield). Schematic picture of the measuring chamber of the DAS Microcalorimeter models (from reference 27) on the right (A and B-cells, C and D-internal and external adiabatic shields, E-thermopile system between the two cells, F and G-main heaters, I-capillary filling tubes)

This sensitivity is necessary since biopolymers have high molecular weights giving very small enthalpy changes per unit mass and, furthermore, most biopolymers are subjected to aggregation in concentrations of more than 1%[46]. They are equipped with adiabatic shields to control pressure when solutions need to be heated at a temperature higher than the boiling point. The DASM-4M used for this work (designed in 1978 by the Special Design Bureau of Biological Instruments, Russian Academy of Sciences, Moscow[24]), presented in figure 2, is equipped with two platinum cells with equal volume of 0.5 cm^3 each. Both cells are in contact with a thermopile system which detects the temperature differences between them. The measuring system is surrounded by two concentric adiabatic shields (Figure 2). For more details one can refer to e.g. the references 24, 32 and 47.

2.2.2. Experiments and measures

The cells are filled with up to ten volumes of solution to avoid bubbles which induce strong irregularities on the curve drawing. An excess pressure of 1 bar is then applied to the surface of both liquid phases to avoid the development of air during the heating process. A constant heating rate of 2°C/min is progressively set to allow both solutions to be well equilibrated at the same starting temperature. The latter is in any case higher than 30°C to overcome risks of precipitating tricaprin and, therefore, of formation of hard micelles which could contribute to enthalpic deviations. The baseline drawing is carried out with solutions lacking in protein, e.g. solution containing buffer plus lipid or/and amylose in suitable concentration. The calibration of heat capacity is performed during the baseline measurement by an impulsion of 50×10^{-6} Watts for a few minutes. The height of the calibration rectangle gives a heat capacity scale of 1.5×10^{-3} J x K^{-1} and the area is the calibration of the enthalpy in Joules. When the temperature has reached 100°C the solution is cooled to 50°C for solutions with lipidic molecules and 70°C for solutions containing amylose. After use the cells are cleaned approximately 30-times with warm twice-distilled water and with a chromic acid solution once every ten days.

Let us remember that two values measure thermal stability of proteins: the denaturation temperature or transition temperature, Td, and the enthalpy of denaturation ΔHcal. Td is the temperature at the heat absorption maximum in the thermogram. ΔHcal is represented by the area under the peak formed by the curve itself and the base-line drawn under the peak. The baseline was not constructed, as done by many workers[48] that is by simply joining the denaturation start and end points because the heat capacity difference between both points differ strongly with the additive. Instead the baseline obtained by heating the appropriate solution was translated and fitted under the peak. Areas were measured with a planimeter. According to Bershtein and Egorov[49] errors on ΔH when measured with a planimeter are ca. 3 to 5%. This is in accordance with the experimental errors obtained on duplicates reproducible within 2% (a variation of 0.2 to 0.4 J g^{-1} for ΔHcal). Note that for the same duplicates the variation in Td is 0.1°C and up to 0.06 J $g^{-1}K^{-1}$ for ΔCp.

To evaluate the co-operativity of protein unfolding the width of the calorimetric transition at half peak height, $\Delta T_{1/2}$, is often measured[35]. If denaturation occurs within a narrow range of temperature (sharp peak), that is if the rupture of intramolecular bonds occur within a narrow temperatures range, the transition is a very co-operative process[50].

To know if the process is of a two-state type, i.e. the protein denatures according to a direct transition between two states (native and unfolded) one can compare the enthalpy change estimated from the sharpness of the transition, given by the difference of Van't Hoff enthalpies

of the extreme states ΔHvH, and the heat effect determined from the peak area ΔHcal. When the ratio of the two enthalpies is close to unity the process is of a two-state type[51]. Conversely if the two enthalpies are not equal the protein denatures through intermediates or intermolecular associations[52].
The values for ΔCp and Δ_dCp are also reported to express the evolution of the protein molecule with its environment, the first being measured from the height of the denaturation peak and the second from the difference of heat capacity between the native and denatured states of the protein[26].

2.3. Light-scattering

2.3.1. Description of the device

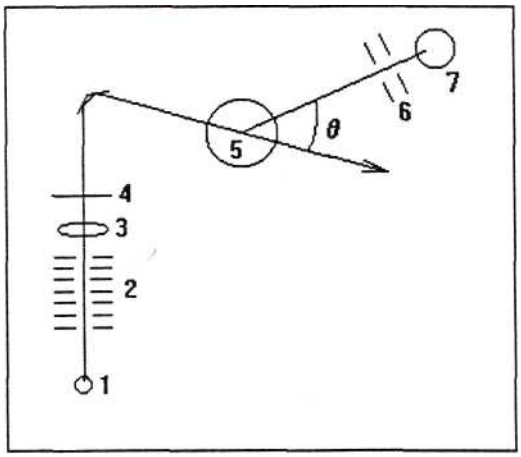

Figure 3. General scheme of a light-scattering photometer. Explanation can be found in the text.

The incident beam is emitted by a laser (1). A monochromatic source is required because of the dependence of the scattered intensity on the reciprocal of the fourth power of the wavelength of the incident light. It passes through a set of slits (2) provided to reduce the incident intensity so as not to destroy the photo multiplier when zero angle is used. A lens system (3) makes the rays parallel (this is not necessary for a laser source since lasers are characterised by parallelism of the beam), a polariser (4) is insert to select a vertical polarised beam. When the cell containing the solution (5) is crossed, the primary beam is absorbed and the scattered light registered by a photo multiplier (7) after being isolated by a system of slits (6). The photo multiplier is fixed onto a rotatable arm, coaxial with the measuring cell. The usual instruments do not allow measurement of angles smaller than 30° because at those angles the intense primary beam, whose intensity is by more than 10^6 higher than scattered beams, will greatly interfere with the scattered light. Moreover at smaller angles the scattering of dust particles is greater than for higher angles; the solutions have to be of a high purity.

2.3.2. Preparation of solutions

This stage is very important since the presence of dust in the solution may completely alter the results. The clarification of the solutions may be by filtration of by centrifugation. For this work, the first method was chosen. The solutions containing amylose were forced through the filter by suction and the temperature was maintained at 40°C so that amylose does not gel in the filters. For ovalbumin solutions no pressure was needed. The passage through the membrane is accompanied by removal of this largest molecules. The intensity of light-scattering, even if the concentration of the polymer is almost unchanged, may change a lot, particularly at small angles. Thus care must be observed during filtration: a slow process gives a better yield. If the solvent is good for the molecule, the clarification is also better. The filtration is problematic for polydisperse molecules. In this case filters with larger pores must be used, which obviously decreases the clarification. Comparison of refractive index increment of solutions before and after filtration gives a loss of up to 15%. No criterion of the efficiency of clarification exists, but since the scattering intensities must be the same at angles θ and (180°- θ), thus symmetrically to the direction of $\theta = 90°$ a maximal asymmetry up to 1.1 is tolerated between the measures of two symmetric angles (see further). Moreover excessively large scattering intensities at a low measurement angle produce a strong curvature in the co-ordinate K_C/R_θ. This curvature is also a good indicator of inefficient clarification. The measuring cells are cleaned by soaking in a chromic acid solution for 1 min, washing in tap water then in double-distilled water and drying with compressed air. The solutions of benzene and protein solutions are filtered on a Sympor (Tchekoslovakia) ultrafilter of pore size 0.23µm without addition of pressure. The solutions containing amylose were clarified under pressure on the ultrafilter with pores of 0.45µm so as not to exclude the larger molecules from the solution.

The intensity at 0° without a solution in the measuring cell was noted 30 min after switching on the device with adequate reduction of intensity through appropriate slits (generally: reduction of 10^6). The value is taken for the rest of the day.

Prior to measurement the solutions are kept for 15 min at 25±0.25°C in the measuring cell to stabilise the temperature and to let the last dust particles sediment. Intensity measurement at 135°C and 45°C is performed and the intensity ratio $i'_{v,45}/i'_{v,135}$ is checked. If the intensity ratio is higher than 1.1 the solution is re-filtered until the desired ratio is obtained. Note: a reciprocal ratio much higher than 1.1 is a good indicator of a dirty cell.

2.3.3. Measurements

The experiments were carried out on a russian laser photometer (LS-01 model from the VA Instruments, Saint-Petersburg) and conducted under the following conditions: pH 7.0, ionic strength of the solvent of 0.05M and temperature controlled at 25 ± 0.25°C by a thermostated system.

The measurements are performed in two phases. The first stage to calculate the constant K_v and the second to obtain the Rayleigh ratio.

According to eq.14,

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