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GENERAL AND APPLIED PHYSICS

Thermal-Induced Denaturation and Aggregation Behavior of Lysozyme and Bovine Serum Albumin: a Thermodynamic and Structural Study

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Abstract Solution studies permit a direct investigation of the particles on a well-defined environment. Fluorescence, circular dichroism, scattering, and calorimetry provide, individually, very important information among the protein structure, overall shape, and thermodynamic equilibrium. In this work, a combination of these techniques is presented for the study of denaturation induced by temperature of two well-known proteins, Henn Egg lysozyme and bovine serum albumin. A detailed thermodynamic and structural investigation is shown for these proteins, providing interesting information on the thermal-induced changes in the protein structure and aggregation behavior.

Keywords Proteins · Small-angle X-ray scattering · SAXS · Modeling · Protein structure · DSC

1 Introduction

Proteins are macromolecules essential for living systems. They are found in all cells and in all parts of the cells and are responsible for the many intricate and correlated biological processes [1]. From a set of (only) 20 types of amino acid residues, a defined sequence of residues is build (known as primary sequence), and due to hydrophobic effects, this sequence folds forming the secondary and tertiary structures. The tertiary structure defines the shape of the protein in solution and is directly related to the protein function and dynamics [2]. In several cases, the proteins agglomerate on a well-defined manner (quaternary structure) forming functional supramolecular systems like, for example, the ribosome [3]. The knowledge on the structure of the macromolecule can be very useful to understand its function on the system. There are several methods for investigate protein structure, function, and dynamics. When is possible to crystalize the protein, protein crystallography methods can be used to retrieve atomic resolution information for the system. Nuclear magnetic resonance also permits atomic resolution information, but usually the measurements take very long time and an elaborated data analysis. This technique permits the investigation of systems in solution but is limited to low molecular weight proteins (<50 kDa). Electron microscopy techniques (TEM and Cryo-EM) had an impressive advance on the recent years [4] but still requires elaborated sample preparation and are indicated for large complexes (of the order of hundreds of kDa or higher). Scattering techniques, namely, small-angle scattering, either using neutrons or X-rays, permit structural investigations directly in solution, with relative simple sample preparation, providing an experimental tool for real time monitoring the system and the retrieval of overall size, shape, and low-resolution structure [5, 6]. The combination of this technique with other experimental results from complementary biochemical techniques like circular dichroism, calorimetry, fluorescence, and sedimentation provides valuable information for the studied system. In the literature, a large number of articles present the thermal-induced changes on lysozyme and BSA. However, in most of the cases, the results are related to one specific technique and it is not given detailed structural description of the system. As will be presented in this work, the systematic combination and correlation of experimental results from DSC, CD, FE, and SAXS can provide new interesting results even for very well-known proteins like lysozyme and BSA. Using the calorimetric data as guideline, a systematic investigation on overall size, shape, and

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aggregation state of the proteins is performed and correlated with the corresponding changes in secondary and tertiary structures. As a result, a detailed description of the structural changes and thermodynamic behavior is obtained for each case.

2 Materials and Methods

2.1 Sample Preparation and Experimental Conditions

Both proteins were purchased by Sigma-Aldrich as lyophilized powder and dissolved in PBS buffer. The concentrations of the stock solutions were measured using ultraviolet light absorbance at 280 nm and the known extinction coefficients for each protein. In both cases, initial solutions of 50 mg/ml were prepared and diluted according to the experiment.

2.2 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was performed using a MicroCal VP—DSC, placed at the Institute of Chemistry, University of São Paulo. The temperature scans were performed at a speed of 1 °C/min, varying from 20 °C up to 85 °C. After several tests, the optimum sample concentration was 50 mg/ml. The reference panel was filled with MiliQ water. Data treatment was made using the equipment software.

2.3 Fluorescence Spectroscopy

Steady-state fluorescence measurements (FE) were carried out using a Cary Eclipse fluorescence spectrophotometer (Varian Ltd.) placed at the Institute of Physics, University of São Paulo. Sample concentration was 0.2 mg/ml. The excitation was set at 290 nm with the emission range between 300 and 500 nm. Measurements were performed in a 10-mm quartz cuvette (Hellma, Germany) as an average of three accumulations with a scanning speed 200 nm/min. Both excitation and emission slits widths were 5 nm. The temperature scans started at 20 °C up to 90 °C (1 °C/min) and were performed automatically by the acquisition system.

2.4 Circular Dichroism

Far-UV circular dichroism (CD) were recorded in a 1-cm quartz cuvette on a JASCO J-715 spectropolarimeter (Jasco, Japan) placed at the Institute of Chemistry, University of São Paulo. Sample concentration was 0.2 mg/ml. Data at two temperatures were measured, 20 and 90 °C. Wavelength scans were recorded in the range of 195–260 nm (bandwidth 1.0 nm and path 0.1 cm). The scanning speed was 20 nm/min and the final results corresponds to an average of four accumulations. Background contributions from the buffer were subtracted. The results are presented as the mean residue molar ellipticity [θ] (deg cm^2 $dmol^{-1}$) versus wavelength (nm). The secondary structural contents of BLA were analyzed on DICHROWEB using SELCON3 algorithms [7–9].

2.5 Small-Angle X-ray Scattering

Small-angle X-ray scattering (SAXS) data was measured at the Institute of Physics, University of São Paulo, on a laboratory equipment, Bruker-NANOSTAR™, upgraded with microsource X-ray generator Genix3D™ (Cu kα edge, $\lambda = 1.54$ Å). The X-ray beam is focused by a FOX3D™ optics and collimated by two sets of scatterless slits, all provided by the company Xenocs. In this configuration, a typical flux of 10^8 photons/s/mm^2 is obtained. The samples are placed on reusable quartz capillaries of 1.5 mm in diameter, mounted on stainless steel cases. Therefore, the capillaries can be washed a rinsed, permitting the measurements of the sample and buffer in exactly same conditions. The sample to detector distance was 0.67 m, giving a q range of $0.01 < q < 0.35$ $Å^{-1}$, where q is the reciprocal space momentum transfer modulus, defined as $q = 4\pi\sin(\theta)/\lambda$, 2θ being the scattering angle and λ the radiation wavelength. The typical acquisition time was 1800 s, and the temperature was varied from 20 up to 80 °C. Temperature control was performed using a sample holder with Peltier system, operated directly from the acquisition software. Therefore, it was possible to perform automatic temperature scans. The data treatment was performed using the SUPERSAXS package (Oliveira and Pedersen, unpublished) and normalized to absolute scale using water as primary standard. For the SAXS data, the Indirect Fourier Transformations were performed using program package GNOM [10] and the ab initio modeling using program DAMMIN [11]. Both programs are available at the ATSAS package [12].

3 Results and Discussion

The strategy for these experiments is the following: from the DSC, experiment is possible to obtain the temperature interval where the denaturation occurs. Then, systematic measurements on the above mentioned techniques were performed around this peak (below and above), in order to investigate the changes on the sample induced by the temperature.

3.1 Hen Egg Lysozyme

In Fig. 1a, the DSC results for lysozyme in solution are shown. As one can see, there is a peak for the specific heat at around 73 °C, indicating a phase transition. This transition is well known in the literature [13] and is related to the denaturation process. The lysozyme is a very stable protein, due to strong disulfide bridges [14] and it undergoes a thermal denaturation on an almost two-step process: folded → unfolded [13, 15].

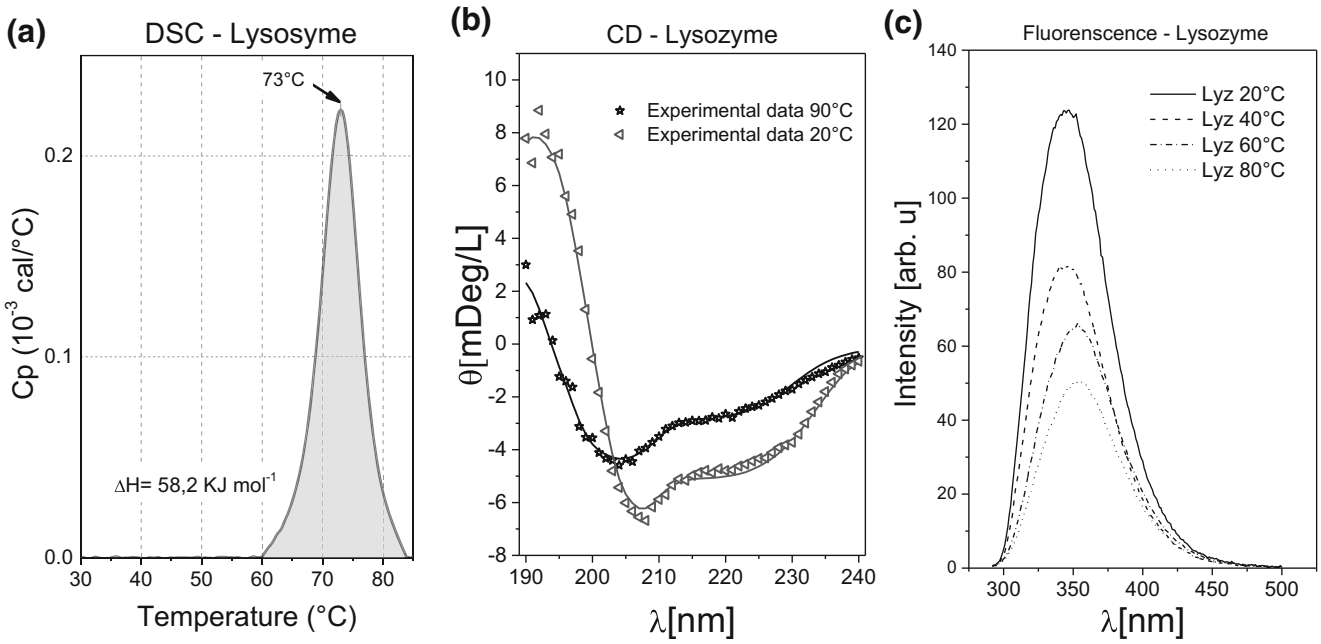

Fig. 1 Calorimetric, fluorescence, and circular dichroism results for hen egg lysozyme. **a** DSC results. The maximum of the specific heat is at 73 °C. From the peak integral, an enthalpy of 57.2 kJ/mol is released. **b** CD results at 20 and 90 °C. The results indicate an important loss of secondary structure. **c** Fluorescence results at 20, 40, 60, and 80 °C. The maximum at the curve shifts to longer wavelengths, indicating an exposure of the tryptophan residues. The decrease of the curve high is due to suppression of the quantum efficiency, due to temperature increase

The transition gave an enthalpy value of 57.2 kJ, which indicates that the lysozyme absorbs heat to unfold. This energy uptake is necessary to break the internal covalent bounds which are responsible for the protein stabilization.

The DSC result is very useful to determine the temperature of unfolding and the change in enthalpy but it does not give any structural information. From the fluorescence result, In Fig. 1c, one can clearly see a maximum around 346 nm which shifts to around 354 nm when the temperature is increased. This shift indicates an exposure of the tryptophan residues to the polar solvent which can be related to the loss of secondary structure. Also, the curve high decreases, which is a consequence of suppression of quantum efficiency of fluorescence. This process is well known when protein systems are subject to temperature variation [13, 14]. Interestingly, in Fig. 1b, where the CD results for 20 and 90 °C are shown, clear differences are seen on the curves for these two temperatures, indicating changes on the secondary structure. The CD spectra were analyzed using program DICHROWEB [8] and as a result, the amount of secondary structure elements is described. A summary of the CD results for lysozyme is shown in Table 1. The experimental values for 20 °C are similar to the ones obtained from the atomic resolution model (2lyz.pdb). Besides some differences on the obtained value for the α-helices and β-sheets, the total amount of folded regions (almost 50% of the whole structure) was correctly indicated by the experimental data. The differences are related to the limited range of the CD spectra, statistical uncertainties and limitations on the fitting procedure. Interestingly, for 90 °C, one sees an increase of the unfolded regions, reaching almost 71%, which indicates the thermal denaturation of the protein, in agreement with DSC and fluorescence results.

The DSC data provided the transition temperature and fluorescence and CD provided indications on the change of the protein internal structure but no information is provided about the overall structure of the protein and aggregation state. In this way, SAXS experiments were performed for the sample in solution in order to retrieve this data. In Fig. 2a, the SAXS curves for the lysozyme sample from 20 up to 80 °C are shown. The frames were obtained on steps of 5° but since there were not important changes between 20° and 50°, some plots were skipped. This is in agreement with the DSC results where we clearly see that some variation on the specific heat only appears after 60°. From the theoretical fit of the SAXS data (solid lines in Fig. 2a), one obtains the pair distance distribution functions p(r) shown in Fig. 2b. Interestingly, the comparison of the p(r) curves indicates that the overall structure of lysozyme remains unchanged up to 50 °C, presenting a small increase of the maximum size for at 55, 60, and 65 °C. From the IFT results, one can calculate the particle radius of gyration and average molecular weight, as shown in Fig. 2c, d. At 70 °C, there is an increase on the radius of gyration but not on the molecular mass, which indicates that the protein changed its conformation (expanded) but remains as monomers. Interestingly, at 75 °C, there is an important increase on size and molecular mass, which indicates agglomeration of the proteins (almost 3 molecules/aggregate) and at 80 °C, there is a systematic increase for the aggregate size and mass.

The ab initio modeling results are shown in Fig. 3. As it can be seen, up to 60 °C, the overall size and shape of the protein in solution

Table 1 Values of α-helices, β-sheets e other structures (turns or random coil) in relative proportion (percentage), for 20 and 90 °C. The theoretical value for lysozyme at 20 °C is based on the crystallographic structure 2lyz.pdb

	Lysozyme—20 °C			Lysozyme—90 °C		
	Secondary structure elements			Secondary structure elements		
	α (%)	β (%)	Other (%)	α (%)	β (%)	Other (%)
Theoretical values	41	10	49	–	–	–
Experimental values	56	0	44	19	10.2	70.8

is very similar to the one in crystallographic structure. At 70 °C, it is possible to see a small increase on the particle size and at 75 and 80 °C, the important increase on the maximum sizes indicates protein aggregation. All these conclusions were obtained from the analysis of p(r), radius of gyration and molecular mass, but the ab initio procedure provides a low resolution 3D model for each step.

3.2 Bovin Serum Albumin

In Fig. 4a, the DSC results for BSA in solution are shown. Interestingly, for BSA, there is a peak around 58 °C, almost 15 °C lower than the one for lysozyme, indicating that the structure of BSA is less stable than lysozyme under

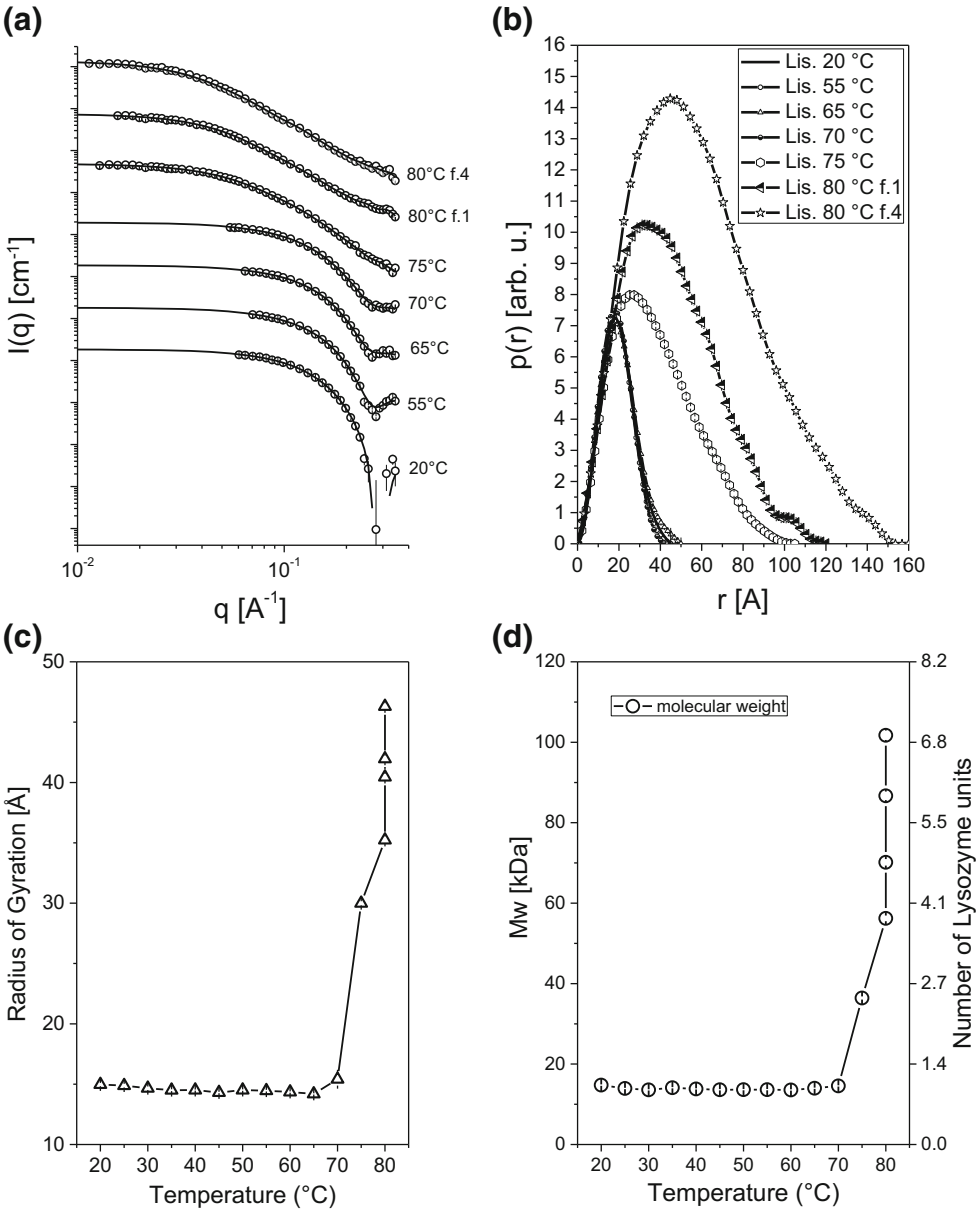

Fig. 2 SAXS results for lysozyme. **a** SAXS data for lysozyme. *Open symbols*: scattering data for several temperatures, as indicated. The curves were shifted for clarity. For some cases, a few points at low angle were deleted due structure factor effects (repulsion). At 80 °C, shorter frames were collected (10 min). In this plot, the first and the forth frame at 80° are shown. *Solid lines*: IFT fits. **b** p(r) curves for lysozyme. For each temperature, a p(r) curve is shown. From the IFT fit the radius of gyration at each temperature (**c**) is shown. From the forward scattering (I(0)), the expected molecular weight (**d**) is calculated. In plots **c** and **d,** the values for all temperatures are shown

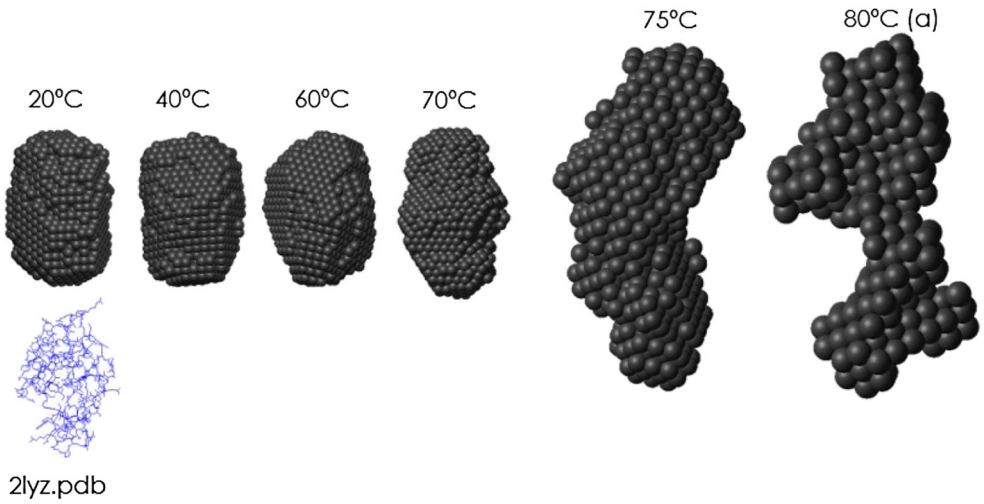

Fig. 3 Ab initio modeling for the SAXS data from lysozyme. *Dark gray spheres*: low resolution models obtained from the ab initio modeling program DAMMIN. All models provided very good fits of the experimental data (data not shown). Ten independent models were generated for each temperature and an average was performed using program DAMAVER [16]. Wireframe model: structure of crystallographic model 2lyz.pdb for the lysozyme

temperature variation. The transition gave an enthalpy value of 58.2 kJ, which indicates that the BSA also absorbs heat to unfold. This transition is well known in the literature [17] and it is related to the denaturation process. BSA is larger than lysozyme, with a larger number of amino acids. Also, the known crystallographic structure of BSA demonstrates the presence of a large cleft on the center of the structure [18] which can trigger structural flexibility and instabilities under stress. The denaturation of BSA can be seen as a multistep process depending on the denaturating agent. For example, SDS induced denaturation gives a multistep process that is related to the binding of SDS molecules on the structure [17]. Thermal-induced denaturation of BSA is well known to induce structure changes, unfolding, and aggregation [17, 19] and a recent article proposed a model for the kinetics of thermal-induced denaturation and aggregation [20], but without information on the shape of the formed aggregates.

From the fluorescence results (Fig. 4c), one can clearly see a maximum around 355 nm which shifts to around 345 nm when the temperature increases. This shift indicates that less tryptophan residues are exposed to the polar solvent. Therefore, even though the temperature increase induces denaturation, the structural rearrangement somewhat promotes a protection of the tryptophan residues. Also, the curve high

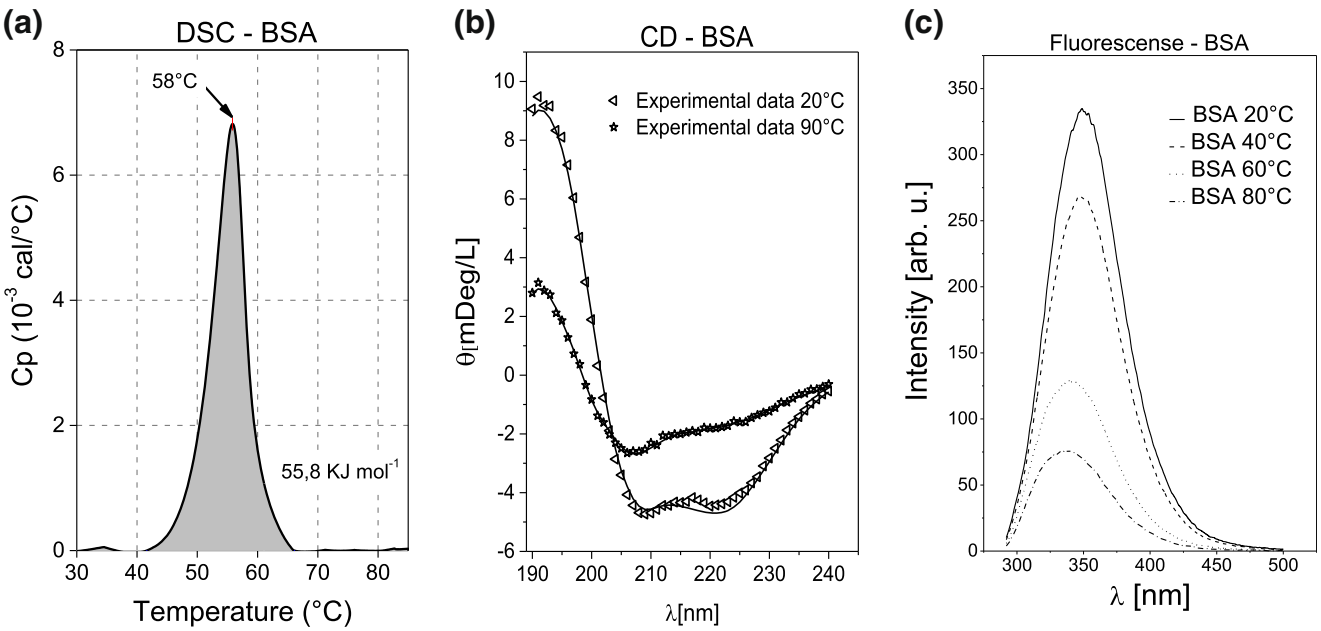

Fig. 4 Calorimetric, fluorescence, and circular dichroism results for BSA. **a** DSC results. The maximum of the specific heat is at 73 °C. From the peak integral, an enthalpy of 55.8 kJ/mol is released. **b** CD results at 20 and 90 °C. The results indicate an important loss of secondary structure. **c** Fluorescence results at 20, 40, 60, and 80 °C. The maximum at the curve shifts to shorter wavelengths, indicating that the tryptophan residues are somewhat less exposed to water at high temperatures. The decrease of the curve high is caused by the suppression of the quantum efficiency, due to temperature increase

Table 2 Values of α-helices, β-sheets e other structures (turns or random coil) in relative proportion (percentage), for 20 and 90 °C. The theoretical value for BSA at 20 °C is based on the crystallographic structure 4f5s.pdb

	BSA—20 °C			BSA—90 °C		
	Secondary structure elements			Secondary structure elements		
	α (%)	β (%)	Other (%)	α (%)	β (%)	Other (%)
Theoretical values	73	–	27	–	–	–
Experimental values	46	12	42	21.4	21.6	57

decreases, which is a consequence of suppression of quantum efficiency of fluorescence, similarly to the lysozyme results.

In Fig. 4b, the CD results for 20 and 90 °C are shown. Clear differences are seen on the curves for these two temperatures, indicating changes on the secondary structure. The CD spectra were analyzed using program DICHROWEB [8] and as a result, the amount of secondary structure elements is described. A summary of the CD results for BSA is shown in Table 2. The experimental values for 20 °C are somewhat different from the ones obtained from the atomic resolution model (4f5s.pdb). These

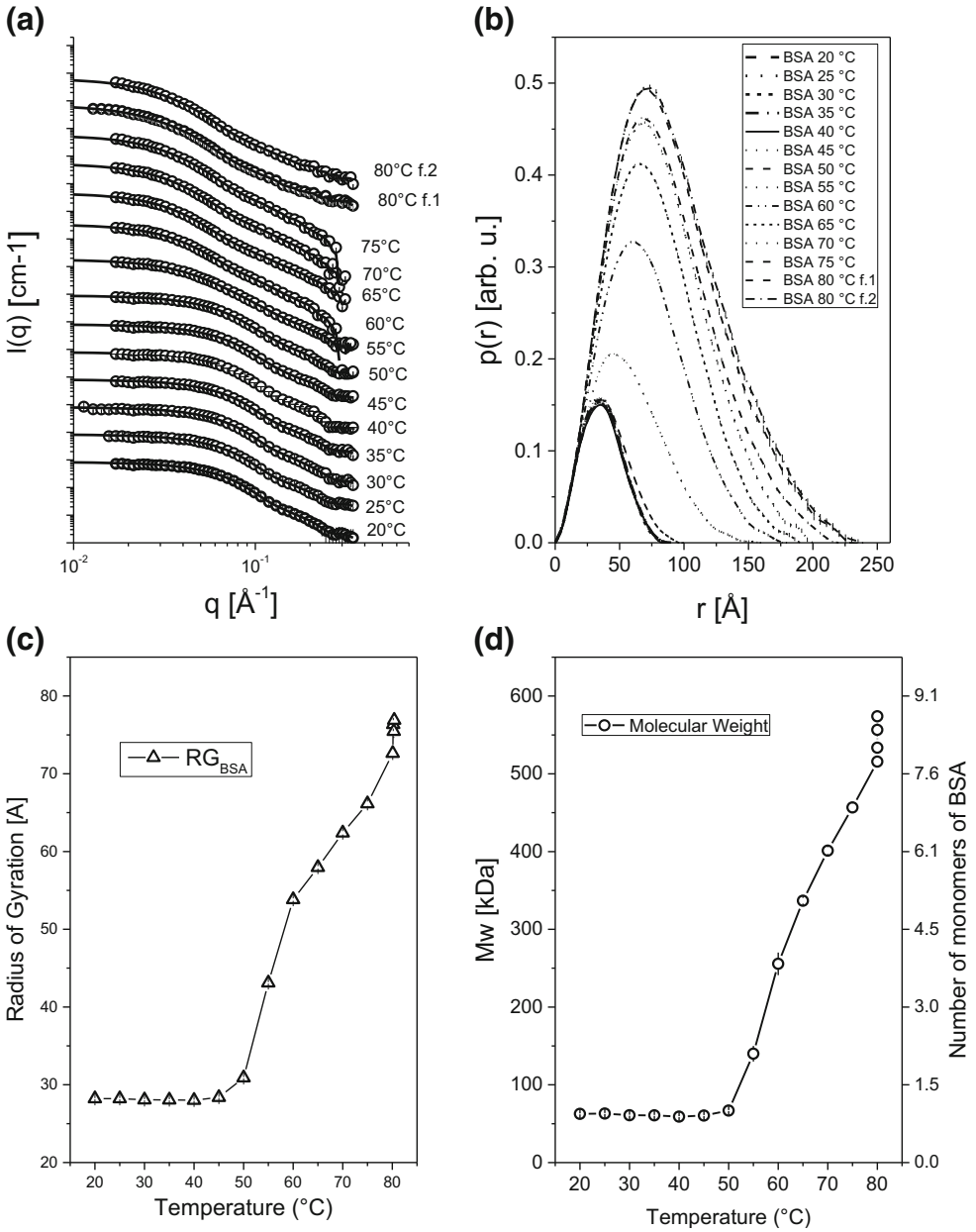

Fig. 5 SAXS results for BSA. **a** SAXS data for BSA. *Open symbols*: scattering data for several temperatures, as indicated. The curves where shifted for clarity. For some cases, a few points at low angle were deleted due structure factor effects (repulsion). At 80 °C, shorter frames were collected (10 min). In this plot, the first and the forth frame at 80° are shown. *Solid lines*: IFT fits. **b** p(r) curves for BSA. For each temperature, a p(r) curve is shown. From the IFT fit, the radius of gyration at each temperature (**c**) is shown. From the forward scattering (I(0)), the expected molecular weight (**d**) is calculated

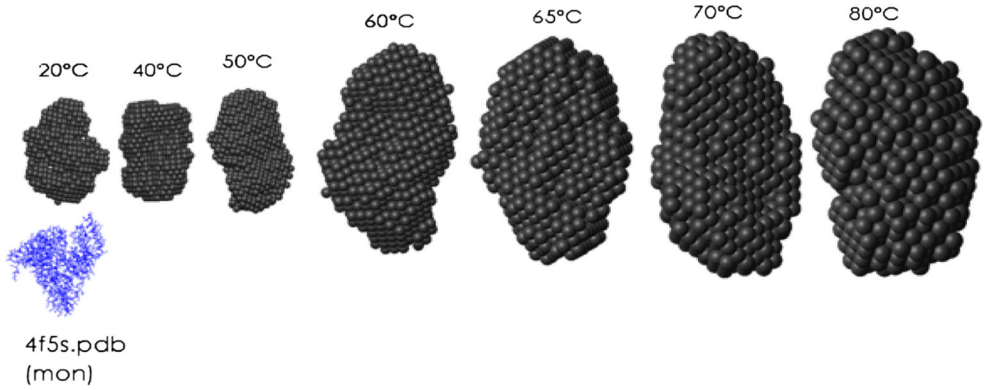

Fig. 6 Ab initio modeling for the SAXS data from BSA. *Dark gray spheres*: low resolution models obtained from the ab initio modeling program DAMMIN. All models provided very good fits of the experimental data (data not shown). Ten independent models were generated for each temperature and an average was performed using program DAMAVER. Wireframe model: structure of crystallographic model 4f5s.pdb for the BSA. In this model, the protein is shown as dimers but in this image only the monomer is consider

differences are probably related to the limited range of the CD spectra, statistical uncertainties and limitations on the fitting procedure, which does not give perfect fits. In any case, the experimental data indicates that the major part of the protein is structured, which agrees with the theoretical data. Interestingly, for 90 °C, there is an important increase of the unfolded regions, up to 57% of the whole structure. The same tendency was previously obtained by Takeda et al. [19]. The CD results together with the DSC and fluorescence results indicate that the increase of temperature induces protein denaturation, with loss of secondary structure but with a lower exposure of tryptophan residues to the solvent.

In order to retrieve information on the overall structure and aggregation state, SAXS experiments were performed. In Fig. 5a, the SAXS curves for BSA are shown, varying from 20 up to 80 °C. In Fig. 5b, the pair distance distribution functions obtained from the IFT analysis are shown. From 20° up to 45 °C, the SAXS curves and p(r) are almost identical indicating that there was no large changes on the protein shape and overall size. The calculated radius of gyration (Fig. 5c) and molecular weight (Fig. 5d) also indicate that the protein remain as monomers in solution. This is in agreement with the DSC results where we clearly see that important variations on the specific heat only appear after 45 °C. At 50 °C, there is an increase on the particle size, as indicated by the p(r) and radius of gyration values. However, there was no increase on the molecular mass, which indicates that the protein is still as monomers but its structure probably adopted a more open conformation. There is a systematic increase of the maximum size, radius of gyration, and molecular mass for 55, 60, 65, 70, 75, and 80 °C. The results indicate that the aggregate is formed by ~2 BSA molecules at 55 °C increasing to ~7 BSA molecules at 75 °C. At 80 °C, a faster aggregation is triggered, as can be seen from the p(r) curves, radius of gyration and molecular weight, reaching ~9 BSA molecules per aggregate.

The ab initio modeling results for BSA SAXS data are shown in Fig. 6. As it can be seen, up to 40 °C, the overall size of the protein in solution is very similar to the one in crystallographic structure. The obtained shape is not too much similar to the crystallographic structure but this is due to the limited amount of information and low resolution of the SAXS data. The BSA protein has a complex shape, with an important cleft on the middle of the structure, which is very difficult to be reproduced by ab initio methods. In any case, the SAXS data indicates that the protein was monomeric in solution. At 50 °C, it is possible to see a small increase on the particle size and after 60 °C, there is the formation of large aggregates. Similar to the lysozyme results, the ab initio procedure provides a low resolution 3D model for each step, which can be correlated with the other experimental results.

4 Conclusions

In this work, a combination of calorimetric and spectroscopic methods was presented for the investigation of thermal denaturation of proteins. As mentioned in the text, the two investigated proteins, lysozyme, and BSA, are widely studied in the literature and many of the results, individually, can be found in several articles. The main goal of this work is to demonstrate that a systematic investigation combining the abovementioned techniques provides thermodynamic and structural information for the system directly in solution. The correlation of the obtained results provides a detailed and comprehensible description of the changes on the protein structure. For lysozyme, the results confirmed the high stability of the protein structure, providing the temperature and of denaturation, decrease on the secondary structure, exposure of the tryptophan residues and detailed information on the overall size, molecular weight and also, low resolution models for the protein structure in each temperature. Interestingly, for BSA, it was shown that the denaturation takes place at lower temperature values than the one for the lysozyme, with a decrease on the secondary structure, less exposure of the tryptophan residues.

A detailed structural description was also obtained, with the generation of low resolution modeling based on the SAXS data. In both cases, the combination of the presented techniques provided self-consistent thermodynamic and structural information for the studied samples. The investigation strategy used in this work can be applied as guideline for the characterization of protein systems in solution.

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