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THERMAL STABILITY OF BOVINE SERUM ALBUMIN DSC study

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Abstract

A calorimetric study of thermal denaturation of bovine serum albumin in aqueous solutions has shown essential differences in stability of fatty acid containing and defatted albumin. The first one shows a single endotherm peak in DSC curve near 69°C with enthalpy change about 1000 kJ mol^{-1}. Defated albumin melts in two different temperature ranges: near 56 and 69°C with enthalpy changes about 300 and 200 kJ mol^{-1} respectively. Deconvolution analysis shows that the single endotherm is well approximated as the sum of three independent two-state transitions. Two transitions of bimodal DSC curve for defatted albumin are not of a two-state type. This molecule melts probably as two structurally independent parts.

Keywords: albumin domains, bovine serum albumin, DSC, thermal stability, unfolding

Introduction

The thermal stability of albumin, the most abundant protein in mammalian systems, has been widely investigated including differential scanning calorimetry (DSC) technique [1–16]. Most experiments have been made on human serum albumin (HSA), e.g. [1–7] and relatively few investigations were undertaken for bovine serum albumin (BSA) [7–13]. The considerable interspecies differences exist in the denaturation behaviour of human, bovine, dog, rabbit, equine and rat serum albumins [14–16] despite of the similarity (the sequence homologies are greater than 70%) among these mammalian albumins [17]. Various thermal profiles of samples of albumin were obtained depending on the experimental conditions, e.g. the kind of solvent, pH, ionic strength, protein concentration, fatty acid content, and other factors. Single peaks were observed for fatty acid free (FAF) human, porcine, canine albumins and fatty acid containing (FAC) BSA in aqueous solutions. All FAC species, except BSA, exhibited twin peaked endotherms [16].

Albumin contains about 580 amino acid residues (BSA is composed of 582 amino acids) in a single chain with known sequence. The three-dimensional configuration of se-

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rum albumin is composed of three homologous domains (I, II, III). Each domain in turn is the product of two subdomains (IA, IB, etc.), which are predominantly helical, and extensively cross-linked by several disulfide bridges [17, 18]. The three-dimensional structure has been determined crystallographically with resolution 2.8 Å for HSA [19], but from a structural point of view BSA and HSA are probably similar. It is likely that depending on the content of fatty acids, pH, and ionic forces these three domains denature regardless of each other or can unite. It finds expression in the shape of the DSC curve, which may show one or two peaks or a peak having a shoulder.

Many aspects of BSA thermal denaturation have been already examined. The effect of ionic strength [6, 9, 12], pH [7, 8, 12] and sodium dodecyl sulfate (SDS) concentration [11, 12] was studied. In most experiments FAF BSA was used. The studies on the thermal stability of BSA with different amounts of fatty acids indicated that fatty acid-poor and -rich albumin complexes respond differently to pH and ionic strength effects [6]. However some aspects of the unfolding process of the albumin molecule remain still unexplained.

The current study was undertaken in order to elucidate the differences in thermal unfolding of defatted and undefatted BSA in aqueous solutions.

Materials and methods

BSA as a crystallised and lyophilised powder (purity minimum 99%), essentially globulin free (BSG) (lot 79H7614) and essentially globulin and fatty acid free (BSFG) (lot 89H7604) were obtained from Sigma. The BSA commercial products, Fraction V, 96% purity, can contain 4% of other protein bands, seen upon electrophoresis as α_1- and α_2- globulins. These products contain some albumin polymer or complex of albumin with other protein [17] and can be separated into subfractions [13]. Therefore in the present studies essentially globulin free, almost pure products of BSA were used. Distilled, degassed water was used throughout.

DSC was carried out on a VP DSC ultrasensitive microcalorimeter (MicroCal Inc., Northampton, MA) with cell volumes 0.5 mL at heating rates 0.7–1.5 K min^{-1}. DSC scans were obtained in the temperature range 20–100°C. For all the measurements the protein concentrations were in the range of 0.029–0.075 mM L^{-1}, pH was 5.5±0.3.

Degassing during the calorimetric experiments was prevented by additional constant pressure of 1.7 atm over the liquids in the cells. At first, the water was placed in both the sample and reference compartments. A DSC curve corresponding to water *vs*. water run was used as the instrumental baseline. The calorimetric data were corrected for the calorimetric baseline (by subtracting water – water scan). Next, the data were converted to molar excess heat capacity by using the protein concentration (verified spectrophotometrically at 278.5 nm) and cell volume (0.51371 mL) and then corrected for the difference in heat capacity between the initial and the final state by using a sigmoidal baseline in the case of BSG and linear baseline in the case of BSFG.

The calorimetric reversibility of the thermally induced transition was checked by reheating the protein solution in the calorimetric cell after the cooling from the first run.

DSC curves were analysed with MicroCal Origin software. The procedure of correction for instrumental time response (Origin software offers such post-run capability) was applied to albumin calorimetric traces obtained at the faster scan rates (60 and 90°C h^{-1}). The corrected and non-corrected traces were virtually identical at 40°C h^{-1} scan rate.

Statistical analysis of the results was done with Statistica 5.1 using one-way Anova.

Results

Preliminary results of DSC measurements

Figure 1 shows an original DSC recordings for the thermal denaturation of free from globulin bovine albumins: containing endogenous fatty acids (BSG) and fatty acid free (BSFG). DSC protein profiles show broad endothermic transitions, with a positive value for the change in heat capacity ΔC_p, connected with denaturation process (the melting of BSA). As seen in Fig. 1, the DSC curve for BSFG is bimodal while BSG exhibits only one heat absorption peak with a melting temperature region very much resembling the second transition in BSFG.

The results of preliminary analysis based on integration of data in selected temperature range (area below the peak, ΔH, which represents the heat absorbed during the thermal unfolding transition, the thermal midpoint, T_m and width of curve at half height, $T_{1/2}$) are listed in Tables 1 and 2 for BSG and BSFG respectively. Reported values are the means of 3–7 independent replicates. The average standard deviations of T_m, $T_{1/2}$ and ΔH were 0.5, 0.8°C, 47 kJ mol^{-1} in Tables 1 and 0.9, 0.8°C, 17 kJ mol^{-1} in Table 2 respectively. One can see that T_m and ΔH increases slightly with increasing BSG concentration (Table 1). It evidences an increase of albumin stability in aqueous solutions with increasing protein concentration in studied range. This conclusion is not in accordance with finding that stability of HSA decreases with increasing protein concentration [3]. However the experiment for HSA was performed in different conditions (150 mM NaCl, pH 7.0) and in wide concentration range. Besides the thermal behaviour of HSA and BSA may not be identical. The effect of concentration on the BSG transition temperature T_m at two different scan rates: 40 and 90°C h^{-1} illustrates

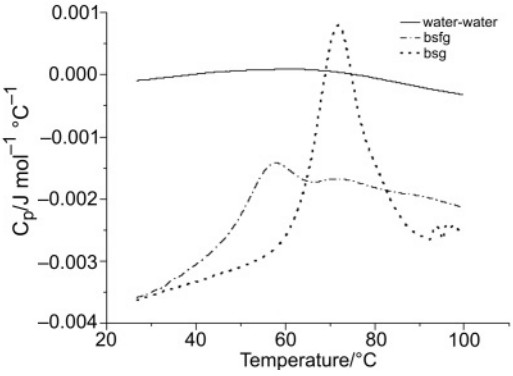

Fig. 1 The raw heat capacity data for BSG (----) and BSFG (–·–·–) aqueous solutions (protein concentration– 0.0745 mM mL^{-1}, scan rate 90°C h^{-1}

Fig. 2. For BSFG only ΔH shows similar tendency, while T_m values are lower for higher concentration (Table 2).

Table 1 The transition parameters for BSG from DSC curves at different concentrations and scan rates

Concentration/ mg mL^{-1}	Scan rate/°C h^{-1}	T_m/°C	$T_{1/2}$/°C	ΔH/kJ mol^{-1}
2	90	68.0	12.9	947
	40	66.9	11.1	708
3	90	69.0	11.1	985
	60	68.9	10.9	1039
	40	68.8	10.0	1014
5	90	70.9	10.0	1098
	40	70.5	8.8	1071

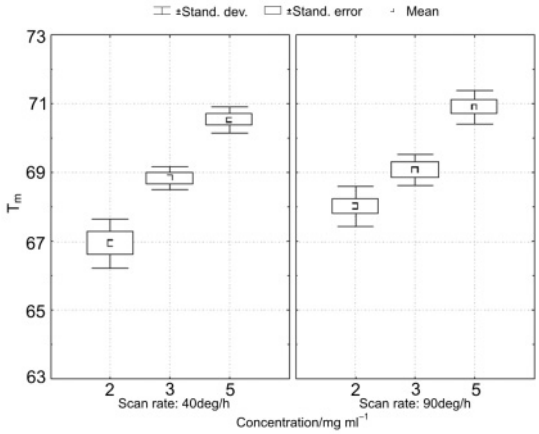

Fig. 2 Effect of concentration on the transition temperature T_m of BSG at two different scan rates

Table 2 The transition parameters for BSFG from DSC curves at different concentrations and scan rates

Concentration/ mg mL^{-1}	Scan rate/°C h^{-1}	T_m/°C	$T_{1/2}$/°C	ΔH/kJ mol^{-1}
2	90	57.3	17.5	440
	40	56.8	16.5	453
5	90	56.2	16.8	550
	40	55.9	17.6	541

The data show that there is a slight increase in T_m as the scan rate increases, however as follows from statistical analysis, the differences are not statistically essential

(p>0.05). Thus, the denaturation process does not rather occur as a kinetically controlled process [20].

Reversibility

The equilibrium criterion usually applied is the reproducibility of the trace in a second heating of the sample, the so-called calorimetric reversibility. It should be noted that the thermal transition of albumin molecule is not reproducible on reheating a sample. The second albumins DSC curves showed only about 10% reversibility. The reversibility of the transitions was checked in detail by the preliminary heat of both kinds of albumin up to 60, 70, 80, 100°C and their cool up to 20°C with subsequent scanning. Figure 3 illustrates the results of this experiment for BSG and BSFG. Percentage reversibility of denaturation process after preliminary heating to different temperatures is shown in Table 3. The estimated uncertainties in reversibility are ± 3%. It catches attention that the differences in reversibility between fatty acid free and fatty acid contain albumins appear only at lower temperatures preheating – 60 and 70°C.

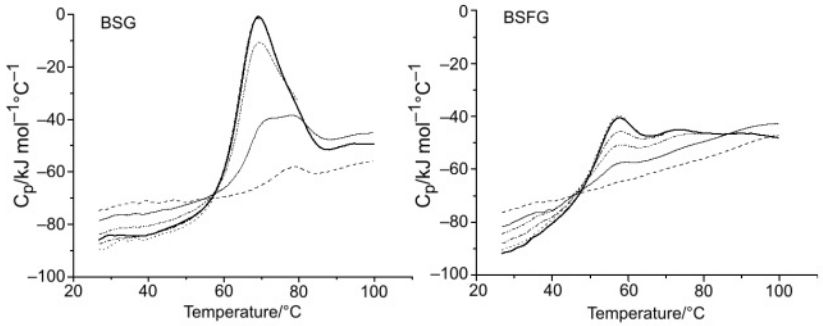

Fig. 3 DSC curves for BSG and BSFG obtained by successively scanning the protein up to different final temperatures in comparison with single (individual) scanning to 100°C (—). The final temperatures at which the protein was heated were: 60°C (----), 70°C (–·–·–), 80°C (–··–··–), 100°C the first time (—), 100°C the second time (----) (BSG and BSFG concentrations – 0.0298 and 0.0745 mM L^{-1} respectively)

Table 3 Reversibility of BSG and BSFG denaturation process after preliminary heating to different temperatures T

T/°C	Reversibility/%	
	BSG	BSFG
60	100	90
70	85	67
80	33	32
100	8	7

Deconvolution analysis

In spite of the calorimetric irreversibility, in some cases it can be considered that the system is in thermodynamic equilibrium during the unfolding process. It takes place when an irreversible alteration of the unfolded state occurs at temperatures higher than the calorimetric transition, and with little heat effect. In such cases, the lack of calorimetric reversibility does not necessarily preclude the derivation of thermodynamic information from the calorimetric trace. This problem was discussed by some authors [21], and similar approach has been applied in the study of the thermal unfolding of HSA and BSA [4, 12].

To obtain detailed information about thermodynamic properties of BSA, a deconvolution of DSC traces were performed. DSC profiles were analysed within the framework of 2-State and Non-2-State models (both models use the Levenberg–Marquardt non-linear least-square method). 2-State model allows the determination of the enthalpy change ΔH_i and the temperature of the maximum, T_{mi}, associated with the unfolding process for each component transition while the Non-2-State model additionally lets to determine the van't Hoff heat change ΔH_{vHi}.

BSG shows a single endotherm peak (Fig. 1) at all concentrations, however an attempt to fit one transition in 2-State model gave very poor result (the fitting not shown). Thus, the unfolding of BSG is not the two-state transition. The best fitting at assumption of one peak was obtained in Non-2-State model. The results are shown in Table 4. The average T_m=70.1±0.8°C and ΔH_{cal}=983±49 kJ mol^{-1} are higher than that reported by Bleustein *et al*. [16] (T_m=65.3±0.5°C and ΔH_{cal}=354±2.3 kJ mol^{-1}) and Nishimura [22] (T_m about 67°C, ΔH_{cal} not determined). This discrepancies may be due to the difference in concentrations, which were about 100 times bigger in works mentioned above (10 and 50% mass/mass respectively) than in this work. The other difference was in albumin samples. In both pre-citated studies [16, 22] samples were the standard fractions V BSA sold by Sigma (only 96% purity, without additional purification).

Table 4 The thermodynamic parameters (±*SEM**) of the one component transition for the thermal unfolding process of BSG in aqueous solutions, obtained from Non-2-State model

Concentration/ mg mL^{-1}	T_m/°C	ΔH_{cal}/kJ mol^{-1}	ΔH_{vH}/kJ mol^{-1}	$\Delta H_{cal}/\Delta H_{vH}$
2	69.4±9	934±71	263±10	3.55
3	70.1±0.5	989±134	303±24	3.26
5	70.9±0.7	1022±42	339±16	3.01

**SEM* – standard error of the mean

The effective van't Hoff enthalpy of the denaturation process was found to be about threefold smaller than calorimetric (Table 4). The ratio $\Delta H_{cal}/\Delta H_{vH}$ suggests three cooperative units per mole of BSG. Denaturation of BSG includes probably three two-state transitions (Fig. 4), but the enthalpies and temperatures of these transitions are not identical – the second transition has a significantly larger enthalpy value (Table 5).

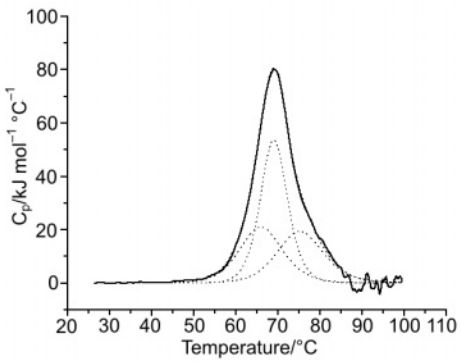

Fig. 4 The curve fitting of DSC profile for aqueous BSG solution in 2-State model

Table 5 The thermodynamic parameters (±*SEM*) of the three-component transition for the thermal unfolding process of BSG in aqueous solutions, obtained from 2-State model

Concentration/ mg mL^{-1}	T_1/ °C	ΔH_1/ kJ mol^{-1}	T_2/ °C	ΔH_2/ kJ mol^{-1}	T_3/ °C	ΔH_3/ kJ mol^{-1}
2	65.7±1.2	249±14	68.2±0.3	396±39	75.0±0.5	313±13
3	66.1±0.5	283±24	69.5±0.7	455±30	75.9±1.6	300±20
5	66.4±0.9	307±36	70.8±0.7	469±28	76.7±1.1	328±14

Using of models based on sequential transitions give worse results.

As we see in Fig. 1, removing of fatty acids from albumin results in a split of the heat absorption peak into two components. The peak with the larger enthalpy is now at lower temperatures. The results of DSC curve fitting in 2-State and Non-2-State models are shown in Figs 5, 6 and Tables 6, 7.

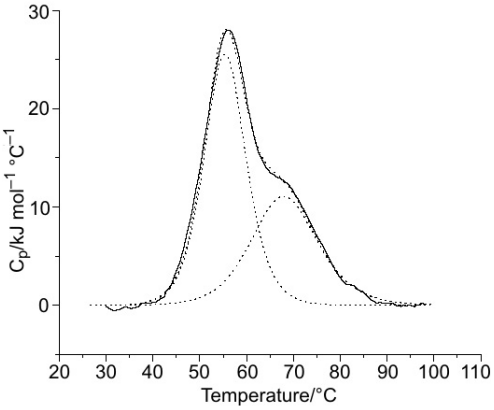

Fig. 5 The curve fitting of DSC profile for aqueous BSFG solution in 2-State model

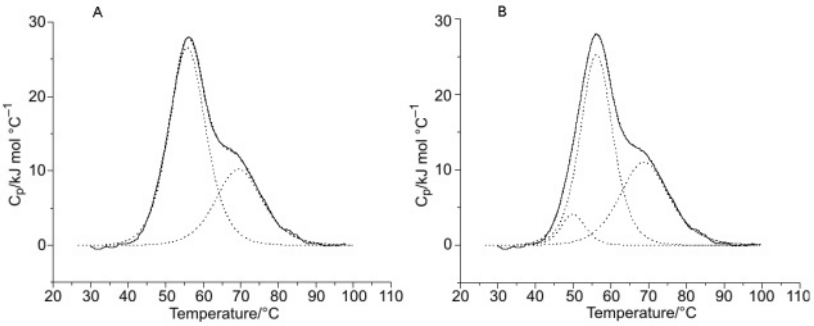

Fig. 6 The curve fitting of DSC profile for aqueous BSFG solution in Non-2-State model: A – with assumption of 2-component transition, B – with assumption of 3-component transitions

Table 6 The thermodynamic parameters (±*SEM*) of the two-component transition for the thermal unfolding process of BSFG in aqueous solutions, obtained from 2-State model

Concentration/mg mL^{-1}	T_1/°C	ΔH_1/kJ mol^{-1}	T_2/°C	ΔH_2/kJ mol^{-1}
2	56.9±0.8	261±11	72.9±1.5	184±15
5	55.5±0.9	311±28	68.5±1.2	228±40

Table 7 The thermodynamic parameters (±*SEM*) of the two-component transition for the thermal unfolding process of BSFG in aqueous solutions (concentration 5 mg mL^{-1}) obtained from the fitting process in Non-2-State model

T_1/°C	$\Delta H_{cal,1}$/ kJ mol^{-1}	$\Delta H_{vH,1}$/ kJ mol^{-1}	$\frac{\Delta H_{cal,1}}{\Delta H_{vH,1}}$	T_2/°C	$\Delta H_{cal,2}$/ kJ mol^{-1}	$\Delta H_{vH,2}$/ kJ mol^{-1}	$\frac{\Delta H_{cal,2}}{\Delta H_{vH,2}}$
55.8±0.5	346±18	277±16	1.25	69.6±0.6	176±18	236±20	0.75

Deconvolution analysis of the heat capacity function shows that the best fitting (the lowest χ^2) gives Non-2-State model with three independent transitions – Fig. 6B. The two main peaks: at about 56 and 69°C are very similar to those obtained at assumption of two transitions (Figs 6A,B). The third, energetically much smaller ($\Delta H_{cal,3}$≈38 kJ mol^{-1}) transition occurs at lower temperature from the range of 49–54°C. The ratios $\Delta H_{cal,1}/\Delta H_{vH,1}$ and $\Delta H_{cal,2}/\Delta H_{vH,2}$ deviate about 9 and 25% from 1.00 in models with three- and two-component transitions respectively. The denaturation mechanism is thus different than a simple two-state process. The smaller than 1.00 ratio $\Delta H_{cal,3}/\Delta H_{vH,3}$≈0.09 suggests some kind of irreversible process [23]. The formation of a crevice in defatted albumin molecule suggested earlier by Yamasaki *et al.* [9] is possible. In the pH range 4.2–7.3 and at an adequate ionic strength a crevice is formed and two peaks appear in the DSC curve. The current pH (about 5.5) and ionic strength (<0.01) set up such conditions.

The comparison of thermodynamic parameters presented in Tables 2, 6, 7 for the thermal unfolding of BSFG with those related in the literature is difficult not only because of different experimental conditions but on account of different deconvolution procedures. At assumption of a double two-state transition in 0.01 M phosphate; pH 7.0 and zero ionic strength, T_1=40.5°C, ΔH_1=65 kJ mol^{-1} and T_2=56.5°C, ΔH_2 =500 kJ mol^{-1} were obtained for FAF bovine albumin [12]. Three-component transition in the same buffer, pH and ionic strength was described by: T_1=59, T_2=68, T_3 =79°C and similar ΔH for all three-transition, each about 300 kJ mol^{-1} [7]. The thermal denaturation of FAF BSA in aqueous solution was characterised by transition with T_m=62°C, ΔH=335 kJ mol^{-1} [16], thus by higher temperature and lower enthalpy change than reported in current paper (T_m≈56.5°C, ΔH≈500 kJ mol^{-1}). Other available in literature results, obtained in more unlike conditions gave: T_m=68°C, ΔH≈535–600 kJ mol^{-1} (in 0.9% NaCl, pH 6.8) [6]; T_m=61.5(56.8)°C, ΔH=568 kJ mol^{-1} (in 0.067 M phosphate buffer, pH 6.0) [14, 15]; T_m=63.9°C, ΔH=785 kJ mol^{-1} (in 0.1 M NaCl, pH 5.6) [9].

Discussion

The denaturation of small compact globular proteins gives usually a single DSC peak, which is approximated well by the two-state transition; i.e., in these proteins the probability of all the intermediate states between the native and the denatured ones is very low and they appear as a single cooperative system. However, under certain solvent conditions, many globular proteins exhibit a significant population of intermediates [24].

For BSG aqueous solution a single peak in the DSC curve is observed, while BSFG DSC curve is bimodal. Defatted BSA was proven to undergo a biphasic denaturation in a particular range of pH and ionic strength [9] or in the presence of a subsaturating level of SDS [11, 12]. The bimodality observed in the DSC curve for undefatted human albumin monomer is explained to origin rather from the denaturation of different kinds of molecules, long-chain fatty acid-poor and -rich species [1–3] than from sequential denaturation of domains within the same molecule. However such explanation may not be valid in the case of FAF albumin.

The thermodynamic parameters indicate the lower stability of defatted bovine albumin in comparison with that containing fatty acids.

This work similarly to [4, 12] shows that the denaturation process of albumin is irreversible, probably due to aggregation of unfolded protein molecules at high temperatures. The aggregation proceeds much more slowly than the unfolding of the native structure.

In the case of BSG the calorimetric curves were deconvoluted as the sum of three independent two-state transitions. These transitions were correlated to the domain structure of BSA. Percentage share in heat effect associated with unfolding of each domain melting in turn with increasing temperature was established as: 27, 42, 31±2%.

Defatted bovine albumin (BSFG) was found to undergo biphasic denaturation in aqueous solutions. It is likely that observed two peaks correspond to the melting of structurally independent parts of the molecule, which are created after the formation of crevice

in albumin molecule. The values $\Delta H_{cal,1}$ and $\Delta H_{cal,2}$ (Table 7) are 66 and 34% of total heat absorption effect respectively. Thus, the subunits into which BSFG is separated near 50°C are not thermodynamically identical. Yamasaki *et al*. [9] suggest that a heat-induced crevice is formed in the vicinity of Trp 212. If we assume that one subunit contains 370 and the other 212 amino acids, it is 64 and 36% of total number of amino acids (582) in BSA molecule. This percentage coincides with percentage contributions of each transition to total ΔH, estimated above from experimental results.

It leads to hypothesis that carboxyl-terminal fragment, which consists of domain III and the bigger part of domain II melts at lower temperatures. The second amino-terminal fragment composed of domain I and a small part of domain II, unfolds at higher temperature.

This conclusion differs from the one proposed by Giancola *et al*. [12], that low temperature transition corresponds to the collapse of the N-terminal BSA domain. They hypothesised that low temperature transition is associated with the denaturation process of one BSA domain, while the high temperature transition – with the cooperative unit composed of the other two BSA domains. However they studied FAF BSA under different experimental conditions.

The crystallographic structure of HSA shows that subdomains IA, IB, and IIA form a particularly compact region as do subdomains IIB, IIIA, and IIIB [18, 19]. Domain II is consequently a relatively open domain, consistent with its susceptibility to cleavage by pepsin to produce PA and PB units. Domain III is unstable in the absence of fatty acids and is responsible for the formation of denaturation intermediate [25] while domain I is not probably involved in the formation of an intermediate during urea denaturation of BSA [15].

In order to determine the role of bound fatty acids in BSA denaturation Ahmad and Qasim have investigated urea-induced denaturation of defatted and fatted BSA by the techniques of UV difference spectroscopy and fluorescence spectroscopy [25]. Their results suggest that binding of fatty acids to BSA prevents formation of denaturation intermediate in this protein. They ascertained the occurrence of denaturation intermediate only in defatted BSA. It is in accordance with results presented in this paper.

Under normal physiological conditions, between 0.1 and 2 moles of fatty acid are complexed with albumin. The two strong fatty-acid-binding sites there are in domain III of BSA molecule [26]. Presumably, binding of fatty acids to the albumin molecule stabilizes this domain. Such conclusion agrees with the results of present study, which suggests lower stability of containing domain III fragment of FAF BSA molecule. This C-terminal fragment unfolds at lower temperatures, before unfolding of smaller fragment containing domain I.

Conclusions

BSFG shows lower thermal stability than BSG. In aqueous solutions BSG melts as a rather compact structure, while in BSFG molecule the crevice is formed before thermal unfolding process. Two peaks observed in the DSC curve of defatted albumin correspond to the unfolding of structurally independent parts of molecule. C-terminal

fragment containing domain III and the greater part of domain II melts probably at lower temperatures. The rest of molecule melts in similar temperature range as nondefatted albumin, however with smaller enthalpy change.

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