Interaction of bovine serum albumin with anionic surfactants

Shashank Deep and Jagdish C. Ahluwalia*

Department of Chemistry, Indian Institute of Technology, Hauz Khas, New Delhi-110016, India

Received 2nd July 2001, Accepted 9th August 2001
First published as an Advance Article on the web 19th September 2001

The effect of binding and conformational changes induced by anionic surfactants sodium dodecyl sulfate (SDS) and sodium octyl sulfate (SOS) on bovine serum albumin (BSA) have been studied using differential scanning calorimetry (DSC), circular dichroism (CD), fluorescence and UV spectroscopic methods. The denaturation temperature, van't Hoff enthalpy and calorimetric enthalpy of BSA in the presence of SDS and SOS and urea at pH 7 have been determined. The results indicate that SDS plays two opposite roles in the folding and stability of BSA. It acts as a structure stabiliser at a low molar concentration ratio of SDS/BSA and as a destabilizer at a higher concentration ratio as a result of binding of SDS to denatured BSA. The Brandts and Lin model has been used to simulate the results.

Introduction

The marginal stability of the native globular conformation of proteins, which is a delicate balance of various interactions in the proteins, is affected by the pH, temperature and addition of small molecules such as substrates, coenzymes, inhibitors and activators that bind specifically to the native state. Studies on the interactions of surfactants with globular proteins can contribute towards an understanding of the action of surfactants as denaturants and as solubilizing agents for membranes of proteins and lipids. Extensive studies on the interactions of surfactants with globular proteins have been reported and reviewed.[1,2]

Surfactants can be broadly classified into those which bind and initiate protein unfolding *i.e.* denaturing surfactants and those that only bind leaving the tertiary structure of the protein intact. Commonly used ionic surfactants such as SDS and sodium n-dodecyl sulfonate, generally denature proteins whereas non-ionic surfactants do not. There are however, exceptions to this rule.[3–7]

A number of studies on protein–surfactant interactions, particularly with SDS, indicate that SDS acts as a more potent protein denaturant (effective at much lower concentration) than urea and guanidine hydrochloride, however, at low concentrations, SDS protects against the disorganizing action of extremes of pH or of high concentrations of urea.[8–12] A number of studies have focused on the multifunctional binding properties of BSA[13-17] which binds a wide variety of molecules. The binding function is a means of transporting soluble substances between tissues and organs. Fatty acid transport appears to be albumin's most important function. Binding also functions as a protection against the toxic effects of the bound ligand. Binding studies with BSA find broad and significant applications in the area of rational drug design as many pharmaceuticals are rendered less effective or entirely ineffective by virtue of their interaction with BSA.

With the advent of sensitive differential scanning calorimeters (DSC) studies of the interaction of surfactants with proteins have become very important. A major advantage of the calorimetric method is that it gives values both for the apparent, or van't Hoff enthalpy, and the true, or calorimetric, enthalpy. Thus the measurement affords a direct check on whether the process under study shows a simple two-state behavior, indicated by the equality of these two enthalpies. DSC methods have two other important advantages over equilibrium methods in studies on multidomain proteins, where both a binding domain and regulatory domain contribute to the ligand binding process[18] and in the measurement of very large binding constants.[19]

The action of BSA and HSA (human serum albumin) with SDS has been of considerable research interest. DSC studies on the interaction of SDS with HSA have been reported by Shrake and Ross[20,21] and those of SDS with BSA over a range of concentration ratios have been reported by Yamasaki *et al.*[22] and by Giancola *et al.*[23] Shrake and Ross[20,21] proposed a model that analyses the protein denaturation with attendant equilibrium binding. Yamasaki *et al.*[22] observed a biphasic DSC profile in the case of subsaturation concentrations of the ligand without formulating any model. Giancola *et al.*[23] assumed that the biphasic model is due to the unfolding of two different domains and applied a statistical thermodynamics model that describes the biphasic DSC curves of BSA in the presence of SDS. Though the above DSC studies have provided a greater insight into the interactions of BSA and HSA with SDS complete understanding is lacking and it would be worthwhile to carry out further studies. A systematic investigation using DSC and other techniques involving proteins and surfactants of different chain length should enhance the general understanding of the binding of surfactants by proteins and its consequent effect upon their stability, particularly on the effect of hydrophobic interactions.

The purpose of the present work is to report in detail the effects of binding of the detergents SDS and SOS on the stability of the secondary and tertiary structure of BSA using DSC, CD and fluorescence and UV spectroscopy methods. It also attempts to distinguish between changes brought about by binding and those due to large conformational changes induced by anionic surfactants.

Work reported here includes the determination of denaturation temperature (T_d), van't Hoff enthalpy and calorimetric enthalpy of denaturation of BSA in the presence of SDS and SOS and urea at pH 7. The main technique employed is DSC. CD and fluorescence intensity measurements over a wide range of ligand concentrations were included to complement the DSC data and demonstrate the effects of surfactants on the optical properties of protein aromatic groups and on

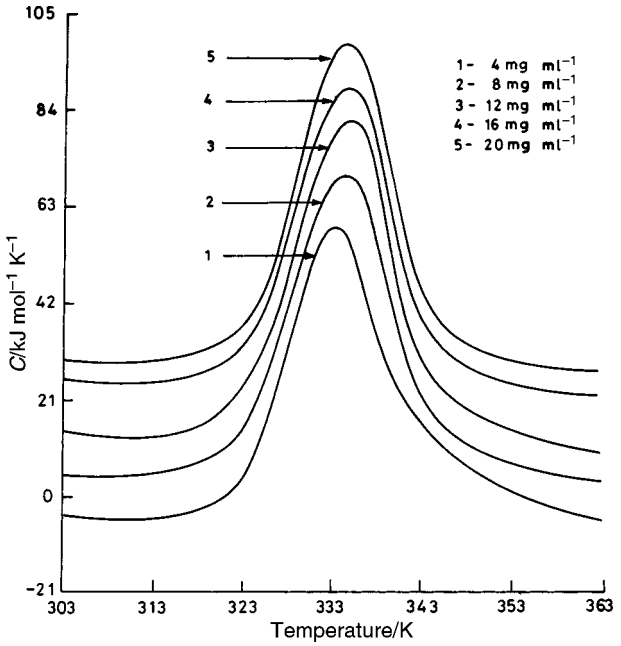

Fig. 1 Thermograms for thermal denaturation of BSA at different concentrations in buffer solution of pH 7.0.

molecular shape. CD and fluorescence studies were also carried out to see the effects of SDS concentration on urea-mediated unfolding of BSA.

Experimental

Chemicals

Crystalline BSA (A-2934, Lot93H0291) was obtained from the Sigma Chemical Company. The SIGMA Fraction V of BSA contains a major component of monomer and two minor components of oligomers.[23] The BSA was deionized by exhaustive dialysis against distilled water at 277 K for over 24 h and was lyophilized before use. SDS, obtained from Koch—Light, England, was recrystallized from 95% ethanol, washed with diethyl ether and dried at 313 K. The water used for buffer preparation was distilled and deionized by passing through a Cole–Parmer mixed bed ion exchange resin column and degassed by boiling before buffer preparation. Urea (U 0631, Lot 86H0778) was also obtained from the Sigma Chemical Company.

Stock solutions of (a) MOPS buffer of pH 7.0, ionic strength 0.01 M (b) 8 M urea solution in MOPS buffer (c) BSA 10 mg ml^{-1} in MOPS buffer (d) 20 mM SDS solution and (e) 20 mM SOS solution were prepared. The solutions of required strength were made by mixing protein and surfactant solution and diluting. The BSA concentration was determined spectrophotometrically on a Perkin Elmer Lambda spectrophotometer. The absorbance was measured at 278 nm and the concentration was determined using an absorbance of 6.58 and molecular mass of 69 kDa.

Calorimetric measurements

DSC measurements were performed with a Setaram microbatch calorimeter. The operation details, calibration and principles of these instruments have been described elsewhere.[24] The calorimeter was operated at a scan rate of 0.5 K min^{-1} from 298 to 378 K with samples of 0.8 to 0.85 g. For each protein concentration, at least three experiments were performed. The amplification applied in the experiments was 25 μV full-scale deflection. Thermal scans were obtained on a voltage *vs.* time scale and converted to excess heat capacity *vs.* temperature scans, following the procedure described by Schwarz and Kirchhoff.[25] Thermodynamic functions of protein denaturation, the transition temperature, T_d, heat capacity, ΔC_{pd} and the enthalpy, ΔH_d of denaturation of BSA in buffer solution and SDS and SOS solutions were determined by least-squares fits of the excess heat capacity data to the two-state model, using EXAM software as developed by Kirchhoff.[26]

Circular dichroism measurements

CD measurements of BSA solutions in MOPS buffer were performed using a Jasco model 720 spectropolarimeter. The spectra of protein solutions were measured in 1 mm cells for far-UV. The solutions were scanned at 50 nm min^{-1} using a 2 s time constant with sensitivity of 20 mdegrees and step resolution of 0.1. The average of five scans was recorded.

Fluorescence meaurements

The fluorescence measurements were carried out using a SPEX fluorimeter with 10 mm square quartz cells. Intrinsic protein fluorescence was determined using excitation wavelengths in the range 275–305 nm. Emission spectra were scanned across the peak, and λ_{max} and the peak height at λ_{max} were determined.

Results and discussion

DSC profile of BSA in presence of SDS

Thermal scans of BSA at different concentrations in a buffer solution of pH 7.0 (0.01 M) are shown in Fig. 1. The reversibility of the unfolding process was checked by reheating the sample of protein after it had been cooled to room temperature in the calorimeter. In the preliminary experiments with BSA it was found that denaturation was irreversible. However the denaturation was found to be essentially reversible, as judged by the area of the DSC curve, provided the first heating was not continued above the temperature at

Table 1 Thermodynamic parameters obtained from DSC scans of BSA at different concentrations at pH 7.0

BSA/mol dm^{-3}	T_d/K	Calorimetric enthalpy/kJ mol^{-1}	van't Hoff enthalpy/kJ mol^{-1}	Cooperativity
0.057	333.4	808	285	2.82
0.114	334.4	941	262	3.60
0.171	334.7	979	267	3.68
0.228	334.7	879	269	3.03
0.285	334.8	920	287	3.21

Table 2 Thermodynamic parameters obtained from DSC scans of BSA in presence of SDS at pH 7.0

SOS/BSA molar ratio	T_d/K		Calorimetric enthalpy/kJ mol^{-1}		van't Hoff enthalpy/kJ mol^{-1}		Cooperativity	
	T_{d1}	T_{d2}	ΔH_{cal1}	ΔH_{cal2}	ΔH_{vH1}	ΔH_{vH2}	η_1	η_2
0.00	334.4							
1	337.6	349.1	819.3	167.2	247.9	409.6	3.30	0.41
2	341.4	352.0	781.7	224.5	239.1	501.6	3.27	0.45
3	344.5	353.6	731.5	316.0	236.2	585.2	3.10	0.54
4	346.4	354.7	693.9	459.8	232.8	627.0	2.98	0.73
5	349.4	355.7	593.6	530.86	273.8	689.7	2.17	0.77
6	351.6	356.3	560.1	560.12	328.6	769.1	1.70	0.73
8	354.2	357.5	522.5	660.44	497.4	999.0	1.05	0.66
10	355.7	358.2	476.5	723.14	631.2	1170.4	0.75	0.62
20	357.7	359.1	459.8	610.28	685.5	1295.8	0.67	0.47
30	355.3	357.9	332.3	451.44	505.8	948.9	0.66	0.47
40	351.6	355.6	204.8	335.23	402.2	668.8	0.51	0.50
50	345.0	350.0						

which denaturation is effectively complete. Sturtevant and co-workers[27–31] and Sanchez-Ruiz[32] and Galistro *et al.*[33] have shown that the usual thermodynamic treatments are applicable in cases where the irreversible steps do not take place during the time the protein spends in the temperature range of the DSC transition, but occur at somewhat higher temperatures. It is thus permissible to apply equilibrium thermodynamics for the evaluation of thermodynamic parameters as functions of temperature and surfactant concentration.

The DSC curve for thermal denaturation of BSA is found to be concentration dependent. The thermal scan of pure BSA exhibits a peak skewed at the higher temperature side at lower concentration (0.057 mM) but symmetric at higher concentration. T_d increases slowly with increasing BSA concentration. This behavior is usually interpreted as being due to a decrease in the extent of oligomerization during the transition.

The values of T_d, ΔH_d as obtained from DSC analysis for different concentrations of BSA are presented in Table 1. Each value represents the average of three to four experiments and the uncertainty represents the standard error of the mean. The T_d values have an experimental error of ± 0.5 K and ΔH_d values have a maximum expected error of $\pm 5\%$ including errors in sample preparation, calibration constant and reproducibility. The van't Hoff enthalpy and cooperativity η, which is the ratio of calorimetric enthalpy to van't Hoff enthalpy for each experiment, are listed in Table 1. The cooperativity of BSA in a buffer solution is within the experimental error of 3.3. Since $\eta > 1$, it may be concluded that one or more states intermediate between native and denatured proteins are significantly populated, which also indicates that thermal

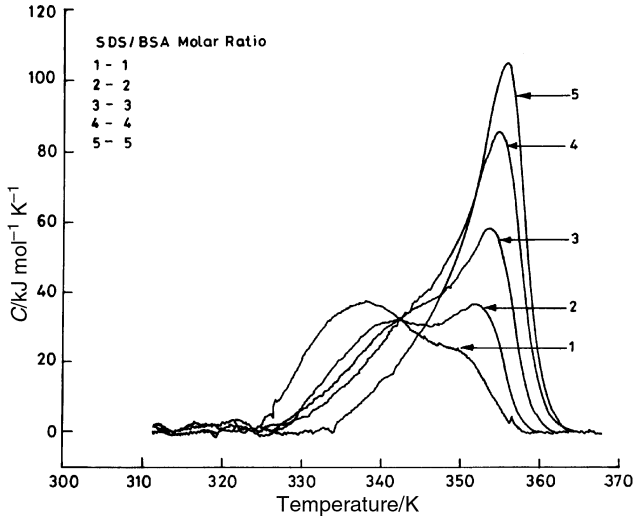

Fig. 2 DSC scans for thermal denaturation of BSA at low molar ratio of SDS/BSA.

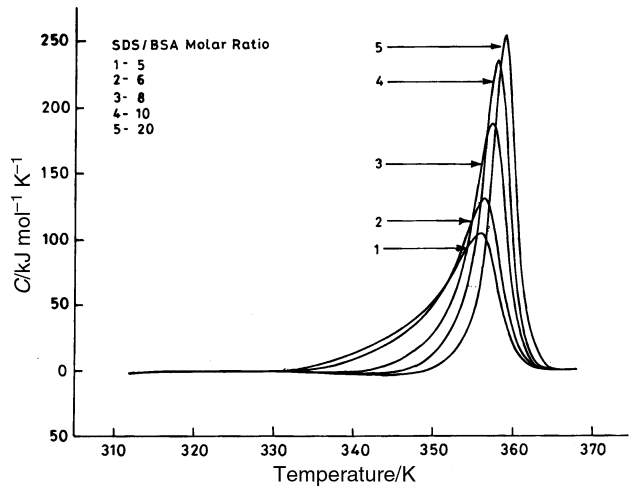

Fig. 3 DSC scans for thermal denaturation of BSA at intermediate molar ratio of SDS/BSA.

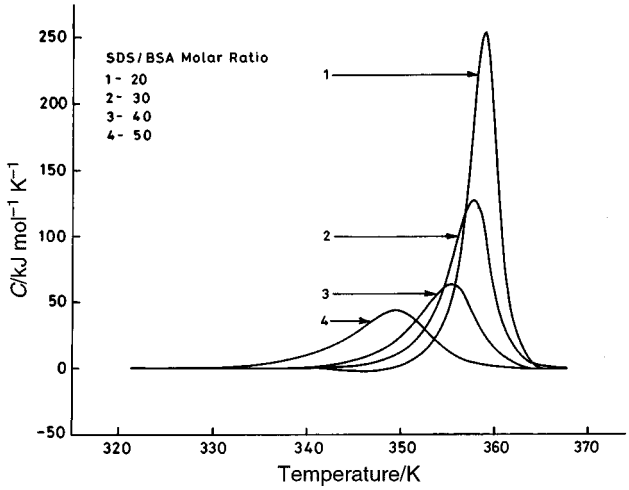

Fig. 4 DSC scans for thermal denaturation of BSA at higher molar ratio of SDS/BSA.

denaturation is not a two-state transition with stoichiometry of more than one.

The values of T_d, ΔH_d as obtained from DSC analysis for denaturation of BSA in the presence of SDS are presented in Table 2. The van't Hoff enthalpy and cooperativity η for each experiment are also listed.

For convenience, the discussion of the interaction of BSA with SDS is divided into two parts:

Lower mixing ratios of SDS/BSA (1–20). Thermograms for the denaturation of BSA in the presence of increasing levels of bound SDS are given in Figs. 2–4. The deconvoluted thermograms are shown in Fig. 5. The thermograms are bimodal with two endotherms; the endotherms at lower and higher temperatures are labelled E_1 and E_2, denaturation temperatures as T_{d1} and T_{d2} and the areas of the two endotherms as H_{cal1} and H_{cal2}. Shrake and Ross[20,21] had also earlier observed in DSC studies of the binding of hydrophobic ligands to HSA that addition of ligand of high affinity can result in the presence of two transitions associated with ligand-poor and ligand-rich molecules of HSA. These effects have been simulated thermodynamically by Shrake and Ross,[21] Robert *et al.*[34] and Brandts and Lin.[19] According to Shrake and Ross,[21] the ligand-induced biphasic protein denaturation described here derives from a perturbation, during the course of thermal denaturation, of the ligand binding equilibrium, which is linked to the equilibrium between the native and denatured forms. The release of bound ligand under subsaturation concentration of ligand by unfolding protein increases the free ligand concentration, which in turn increases the saturation level of the remaining protein; thus increasing T_d and ΔG_{unfold} of HSA. The larger this increase, the greater the tendency for biphasic denaturation; therefore any factor causing a substantial increase in free ligand concentration during denaturation increases the propensity for biphasic protein.

At SDS/BSA = 1, two endotherms E_1 and E_2 appear at 337.6 and 349.8 K with calorimetric enthalpies 820 and 167 kJ mol^{-1}, and $\mu > 1$ for the E_1 and <1 for the E_2. This implies that one or more intermediate states are populated in the denaturation of ligand-poor protein while denaturation of ligand-rich protein involves intermolecular cooperation. E_1 and E_2 shift to higher temperature with increase in SDS concentration, T_{d1} increasing more than T_{d2}. Also, the calorimetric enthalpy of the E_1 decreases while that of E_2 increases. At SDS/BSA = 2, the endotherms shift to higher temperature by as much as 4 K for E_1 and 3 K for E_2. E_1 becomes a shoulder of E_2 at SDS/BSA = 4. When SDS/BSA is increased from 1 to 6, the E_1 shifts by 14 K while E_2 shifts by 7 K. The shoulder E_1 disappears at SDS/BSA = 6. The enthalpies of the two endotherms are *ca.* 560 kJ mol^{-1} at this ratio.

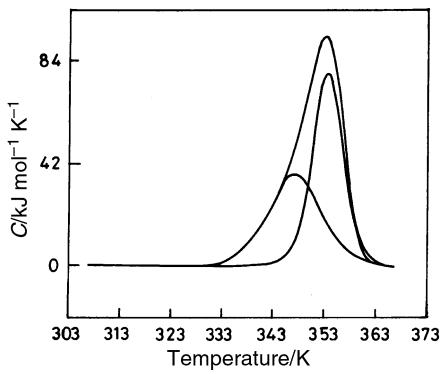

Fig. 5 A typical deconvoluted bimodal thermogram of BSA in the presence of SDS showing two endotherms.

For SDS/BSA between 10 and 20, the two denaturation temperatures are close to each other and approach a limiting value of ~359 K which is ~25 K greater than that of native pure BSA (Table 2). The enthalpy of E_1 decreases while that of the E_2 increases. The cooperativity (η) of both E_1 and E_2 approaches 1 and thermal denaturation of BSA follows a two-state process.

Denaturation temperatures T_{d1} and T_{d2} when plotted as a function of equivalents of SDS per monomer show a sharp increase in both T_{d1} and T_{d2} in this concentration range. With increasing concentration of SDS, T_{d1} increases more than T_{d2}. In addition, the area of the first endotherm ΔH_{cal1} decreases while that of the second endotherm ΔH_{cal2} increases. As a result, the endotherms coalesce to give a single, asymmetric peak, skewed to the lower temperature side.

Calorimetric studies of the effect of BSA concentration on the denaturation of BSA in the presence of subsaturation concentration of SDS (0.5 mM) were carried out. The denaturation temperature decreases with increase in BSA concentration. There is a decrease in calorimetric enthalpy with increase in half height width. The endotherm becomes more asymmetrical. This clearly indicates that there is specific binding of SDS with BSA. Hence the endotherms are affected by the change in SDS/BSA although the SDS concentration is the same.

Binding of SDS to sites with fairly high affinity leads to a large increase in stability. Additional weak interactions and/or an indirect, solvent-mediated effect leads to a continuing but smaller increase in stability at intermediate surfactant concentrations. If we express protein denaturation as a simple equilibrium between the native and denatured states $N \rightleftharpoons D$, on addition of surfactant at lower concentration, the equilibrium shifts towards the native form of the protein because of initial binding of the surfactant with the native protein, whereas it shifts towards the denatured form at a high concentration of SDS due to its binding with the denatured form, as expected according to Le Chatelier's hypothesis.

Heat induced transitions in BSA occur by the electrostatic repulsive forces among the positively charged amino acid residues in a segment Arg 184, Arg 216 containing Trp 212 and the primary binding sites of anions.[35] Small anions at low concentration suppress this transition and thermostabilize BSA by specific and non-specific bindings to these sites.

Since detergent anions are being added to a flexible macroanion, an expansion should ensue, not a contraction. Hence it seems fairly certain that the hydrophobic section of the detergent SDS is not only somehow causing shrinkage to ensue, but makes up for energy and entropy terms: (1) positive free energy terms due to compression of macroanion and charge neutralization of some of the titrable groups at constant pH; (2) negative entropy terms arising from side chain and backbone folding to yield a more compact macromolecule. Presumably the hydrocarbon chain of the detergent also loses several degree of freedom of rotation when it becomes bound, besides its cratic entropy.

The effect of BSA concentration on the denaturation of BSA at a molar mixing ratio of 10 was studied. With increase in concentration, the endotherm shifts to higher temperature with increase in the enthalpy of denaturation, μ also decreases and approaches one. The shifting of the endotherm to higher temperature may be due to a change in the structure of water on addition of SDS. The increase in BSA concentration requires an increase in the concentration of SDS to keep the SDS/BSA ratio constant. The perturbation of the surface free energy of water, *i.e.* the surface tension, is a reflection of the fact that contact between protein and solvent constitutes an interface at which there must be an interfacial tension. According to Gibb's adsorption isotherm, if an agent increases the surface tension of water it will be depleted from the surface layer. SDS, being a surfactant, decreases the surface tension of

water, hence, the concentration in the surface layer is enhanced relative to the bulk solvent. Addition of more SDS will decrease the surface tension further leading to increase in SDS in the surface layer relative to the bulk solvent, which in turn increase the binding of SDS to protein and leads to a shift in the denaturation temperature to higher temperature.

Higher molar mixing ratio of SDS/BSA (20–100). Thermograms for the denaturation of BSA at a higher molar ratio of SDS/BSA (>20) are given in Fig. 4. The results are opposite to those obtained with a low ratio. The T_{d1} and T_{d2} decrease with increase in surfactant concentration. Also, the enthalpies of denaturation for both peaks E_1 and E_2 decrease with increase in half height width. At SDS/BSA = 20, T_{d1} and T_{d2} for E_1 and E_2 are 357.7 and 359.1 K respectively while ΔH_{d1} and ΔH_{d2} are 464 and 610 kJ mol^{-1} respectively. When the ratio is increased from 20 to 30, the T_{d1} decreases to 355.3 and T_{d2} to 357.9 K. With further increase in the level of bound SDS T_{d1} and T_{d2} decrease further, T_{d1} decreasing more rapidly than T_{d2}. As a result, the endotherms become more and more asymmetric, skewed to lower temperature. η decreases with increase in the molar mixing ratios indicating that intermolecular interaction is present during denaturation of the protein–ligand complex. At a ratio of 100, the thermogram shows no endotherm, indicating the denaturation of the proteins.

Thus, SDS plays two opposite roles in the folding and stability of bovine serum albumin. At low concentrations, it acts as a structure-stabilizing additive, increasing the stability toward thermal denaturation. At higher concentration, the binding of SDS to denatured protein is more prominent and unfolding occurs. In other words, the equilibrium N⇌D is shifted towards the right-hand side, making the protein unstable to heat.

Interaction of SOS with BSA

Thermograms for the denaturation of BSA in the presence of aqueous solutions of SOS are shown in Fig. 6. The values of T_d, ΔH_d, the van't Hoff enthalpy and cooperativity η are listed in Table 3. Each value in the table represents the average of three to four experiments and the uncertainty represents the standard error of the mean.

The thermograms consist of a single denaturation peak. With increasing SOS concentration, the T_d increases with concomitant decrease in half height width and increase in C^{ex}_{max} and ΔH_d except at the highest concentration of ligand (40 mM) where ΔH_d decreases slightly. Furthermore, endotherms in the presence of this ligand are essentially symmetric, even at the lowest subsaturation ligand concentrations. At the highest concentrations of added ligand (surfactant/protein ratio of 400), T_d has a maximal value of ~360 K, corresponding to an increase of 27 K in T_d relative to native protein and ΔH_d reaches a limiting value of ~1420 kJ mol^{-1}. The $\Delta H_d/\Delta H_{vH}$

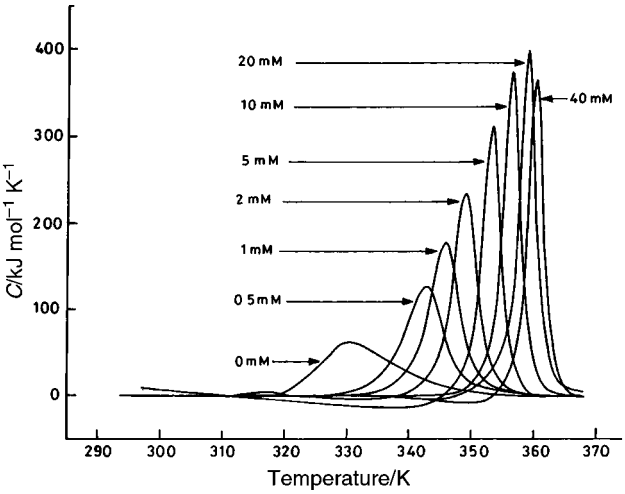

Fig. 6 DSC scans of thermograms of BSA in SOS.

also decreases with increasing SOS concentration. This suggests that as the concentration of bound SOS approaches full saturation the denaturation reaction approach a two-state process.

The effect of increasing concentration of SDS and SOS is compared in Fig. 7. The increase in denaturation temperature is small for SOS in comparison to SDS showing strong binding in the case of SDS. This emphasizes the importance of the hydrophobic part of the surfactant in binding with SDS. Also, SOS does not act as a denaturant at concentrations as high as 40 mM.

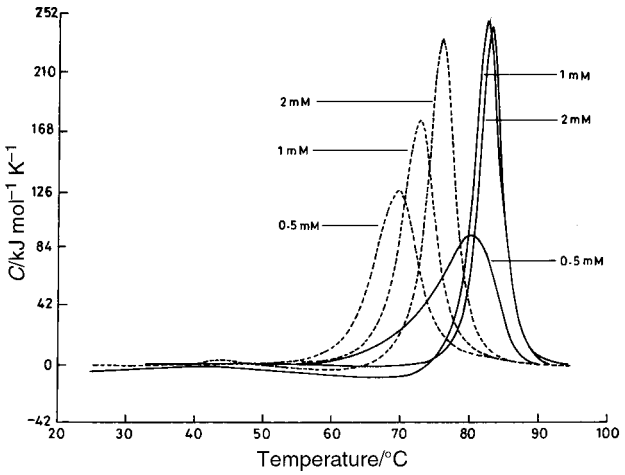

Fig. 7 Comparison of DSC scans for thermal denaturation of BSA in the presence of SDS (——) and SOS (----).

Table 3 Thermodynamic parameters obtained from DSC scans of BSA in the presence of SOS at pH 7.0

SOS/BSA molar ratio	T_d/K	Calorimetric enthalpy/kJ mol^{-1}	van't Hoff enthalpy/kJ mol^{-1}	Cooperativity η
0.00	332.5	941	262	3.60
0.50	342.5	1070	443	2.40
1.00	345.7	1162	581	2.00
2.00	349.1	1221	757	1.60
5.0	353.3	1296	982	1.30
10.00	356.5	1375	1083	1.27
20.00	359.0	1417	1120	1.26
40	360.3	1212	1237	0.98

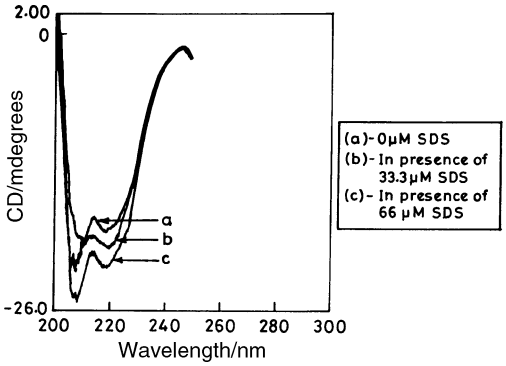

Fig. 8 CD spectra of BSA in the presence of SDS.

In the domain structure of native BSA, three peptide segments are arranged parallel to each other, forming a long hydrophobic groove. This groove forms an especially favorable orientation for the accommodation of several surfactant molecules with multiple hydrophobic bonds. A type of stabilized hydrophobic association could result from the additional binding to cationic sites on the protein surface, specifically to the lysyl, hystidyl and arginyl amino acid side chains.

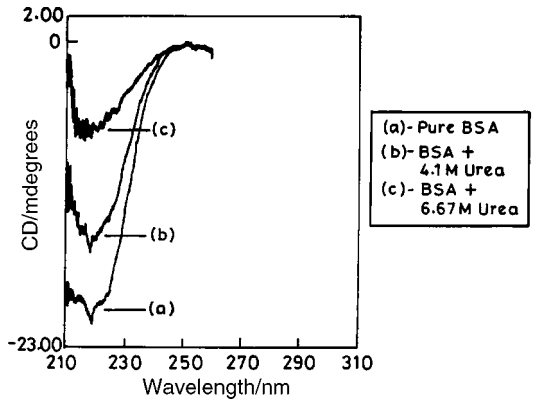

Fig. 9 CD spectra of BSA in the presence of urea.

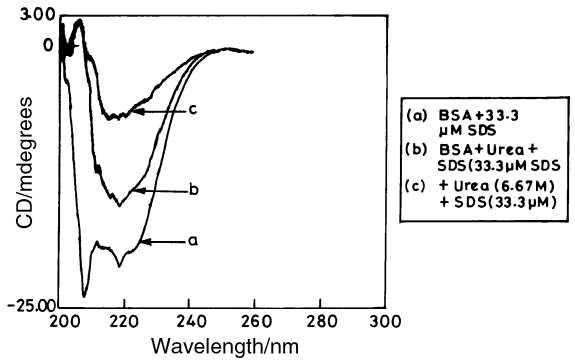

Fig. 10 The effect of 33.3 μM SDS solution on the urea-mediated denaturation of BSA.

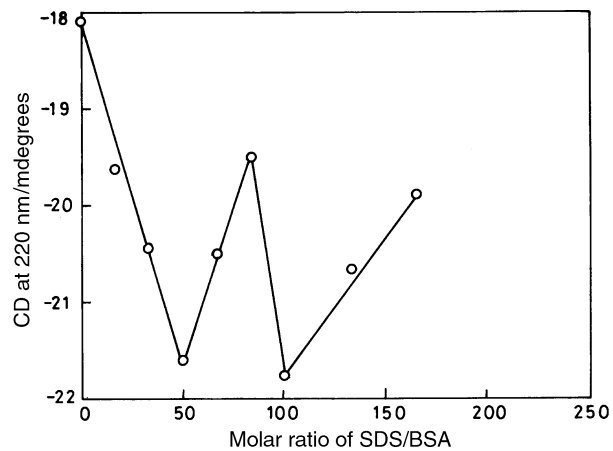

Fig. 11 CD spectra of BSA at different molar ratios of SDS/BSA.

DSC profile of BSA in the presence of urea

Thermograms for the denaturation of BSA in the presence of aqueous solutions of urea consist of a single endotherm. With increasing urea concentration, the ΔH_d decreases together with a decrease in the transition temperature and an increase in half height width. Addition of 5 M urea decreases the denaturation temperature by about 30 K.

There is ample proof that urea acts as a structure breaker.[36–41] The structure-changing propensities of both hydrophobic (structure-making) and hydrophilic (structure-breaking) groups or moieties decrease in a less structured medium.[37,38] Urea may be assumed to have a cosphere containing fewer hydrogen–hydrogen bonds than bulk water, as it destabilizes the water structure.

SDS denaturation of proteins occurs at surfactant concentrations (less than 10 mM) which are far lower than those required for urea (about 5 M). In the case of urea, the denaturation process depends primarily on the effect of the denaturant on the water structure and the weakening of hydrophobic interactions in the tertiary structure of the proteins, while it is the specific binding of the SDS molecule with unfolded protein which causes protein to denature in the presence of SDS.

Circular dichroism spectroscopy

The calorimetric method for studying thermal denaturation has a major disadvantage as compared with optical methods, namely that concentrations higher by an order of magnitude or more are required, which in most cases may lead to increased difficulties due to aggregation, particularly of denatured proteins. CD can provide information about the secondary structure of proteins and nucleic acids and about the binding of ligands to these types of macromolecules.[42,43]

Typical far-UV CD spectra for pure BSA and for BSA (1.99 μM) in the presence of SDS (0–500 μM) are shown in Fig. 8. It is observed that when SDS is added to BSA, the negative band at 220 nm becomes deeper up to 100 μM SDS. Further addition of SDS (100–150 μM) results in collapse of the negative band towards the base line. Adding more SDS, to 200 μM, results in a further deepening of the 220 nm negative band of BSA. The addition of SDS beyond 200 μM results in a collapse of the negative band towards the base line (less negative value). Thus, the curve exhibits two troughs with minima at 100 and 200 μM.

CD spectra for BSA with urea alone and with urea in the presence of SDS are shown in Fig. 9 and 10 respectively. The negative band at 220 nm collapsed toward the baseline (less negative value) with increasing urea concentration. It was observed that the values of CD intensity for BSA in urea solutions are more negative in the presence of SDS (0.01 mM).

The common types of secondary structure adopted by peptides and proteins have distinctive CD spectra in the far-UV. There is considerable variation in the CD spectra with side chains, solvent, and other environmental factors.

The deep trough at 220 nm for BSA is typical of helix-rich protein. The initial binding of SDS to BSA results in deepening of the trough. This is due to increase in the helix content of BSA. The CD rotation of BSA in the presence of SDS is plotted in Fig. 11. The plot exihibits two minima at the molar ratio of SDS/BSA equal to 50 and 100. This indicates the formation of two complexes $BSA(SDS)_x$ and $BSA(SDS)_{2x}$ where $x = 50$. The value of the CD rotation becomes less and less

negative with further increase of SDS concentration. At higher concentration of SDS, the helix–coil transition occurs due to denaturation of protein.

The binding of SDS to BSA revealed that the stoichiometric complex of AS_m (A, albumin; S, surfactant, $m = 1$–12) is formed first by statistical binding. The second and third stages are cooperative binding; the stoichiometric complexes of AS_n and AS_{2n} ($n = 38$–55) are formed by $AS_m \rightarrow AS_n$ and $AS_n \rightarrow AS_{2n}$. When SDS binds to BSA and complex AD_m is formed, BSA is stabilized and becomes resistant to thermal denaturation and urea denaturation.

Fig. 10 represents urea-mediated unfolding curves for BSA. It is clear that in the presence of urea the spectrum collapses toward the base line, suggesting a helix–coil transition. The mid-point urea concentration *i.e.* the concentration of denaturant required for 50% denaturation of protein is close to 5.0 M.

The presence of about 33.3 μM SDS (where binding is supposed to be taking place) in the solution shifted the transition to a higher urea concentration with the midpoint close to a urea concentration of 6.2 M (Fig. 10). This shows the increase in stability of protein in the presence of a low concentration of surfactant which is consistent with the DSC results.

Fluorescence spectra

A typical fluorescence spectrum for BSA showed that the apparent wavelength of maximum emission (λ_{max}) was about 350 nm, regardless of the excitation wavelength (λ_{ex}) in the 275–305 nm range. Fluorescence studies of BSA were carried out at pH 7.0 in SDS solutions over the concentration range (0–55 μM). The fluorescence intensity decreases with increase in SDS concentration. λ_{max} for BSA changes in a simple sigmoidal fashion . Initial binding of SDS has no effect on the λ_{max} of BSA (0 to 20 μM). Addition of SDS over the concentration range 20 to 48 μM SDS resulted in a shift of λ_{max} from 354 to 338 nm. Further addition of SDS (more than 48 μM) had no effect on λ_{max}.

The effects of urea on the fluorescence spectrum of BSA were studied. (Fig. 12). In the presence of urea, excitation at 275 nm had no effect on λ_{max} for BSA but the fluorescence intensity of BSA decreased with urea concentration in a simple sigmoidal fashion. Initial addition of urea (0–3.33 M) had no effect on the fluorescence intensity of BSA. Addition of SDS (20–48 μM) resulted in a shift of λ_{max} from 354 to 338 nm; further addition of SDS had no effect on λ_{max}.

Changes in protein conformation, such as unfolding, very often lead to large changes in the fluorescence emission.[42] In proteins that contain all three aromatic amino acids, fluorescence is usually dominated by the contribution of the tryptophan residues, because both their absorbance at the wavelength of excitation and their quantum yield of emission are considerably greater than the respective values for tyrosine and phenylalanine.

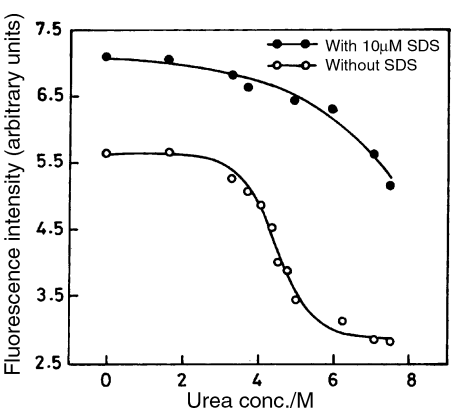

Fig. 12 The effect of urea concentration on the fluorescence intensity of BSA in the absence and presence of SDS.

In proteins that contain tryptophan, both shifts in wavelength and changes in intensity are generally observed upon unfolding. The tryptophan emission of a native protein can be greater or smaller than the emission of free tryptophan in aqueous solution. Consequently, both increase and decrease in fluorescence intensity can occur upon protein unfolding. The emission maximum is usually shifted from shorter wavelengths to about 350 nm upon protein unfolding, which corresponds to the fluorescence maximum of tryptophan in aqueous solution. In a hydrophobic environment, such as the interior of a folded protein, tryptophan emission occurs at shorter wavelength (indole shows an emission maximum of 320 nm in hexane).

The apparent wavelength of maximum emission (λ_{max}) for BSA at about 354 nm (λ_{max} for pure tryptophan in aqueous solution is 354 nm) indicates that the tryptophan residues present in the native protein are on the surface and exposed to an aqueous environment.

Addition of SDS results in a shift of λ_{max} from 354 to 336 nm. Since tryptophan emission occurs at shorter wavelength in a hydrophobic environment, the shift of λ_{max} towards lower wavelength indicates the transfer of protein to a more hydrophobic environment consistent with the binding of SDS near the tryptophan site of BSA. Polet and Steinhardt,[8] on the basis of a UV absorption study of protein, also concluded that tryptophan residues are at, or very near, the binding sites of highest affinity for SDS.

Excitation at 275 nm shows no change in λ_{max} for BSA in the presence of urea. This is because the tryptophan residues in native BSA are already exposed. However, fluorescence intensity changed with urea concentration in a simple sigmoidal fashion (Fig. 12) with a midpoint urea concentration of 5 M. Initial addition of urea (0–4 M) does not have a significant affect on the tryptophan fluorescence intensity but addition of 4–6 M urea (around 5 M) results in a strong decrease in the tryptophan fluorescence due to unfolding of the protein. The distance between the tyrosine and tryptophan residue increases upon unfolding of the protein, and energy transfer from tyrosine to tryptophan become less efficient. In the presence of 0.01 M SDS, this transition midpoint (5 M urea) shifted to a higher urea concentration of about 6 M. The result is consistent with the DSC and CD results that show that a low concentration of SDS stabilizes the protein against thermal or urea-mediated denaturation.

Model of protein–SDS binding

Giancola *et al.*[23] applied a statistical-thermodynamic model that describes the biphasic DSC curves of BSA in the presence of SDS. They assumed that the biphasic transition is due to the unfolding of two different domains. We have applied a different model to simulate the unfolding of BSA in the presence of SDS and analyzed the suitability of this model. It is possible to have a biphasic DSC thermogram with a single domain protein also. The biphasic endotherms are associated with the unfolding of ligand-poor and ligand-enriched protein forms respectively. The basic flaw in the Giancola model is that it assumed that the low temperature calorimetric domain is associated with the denaturation process of one BSA domain and the high temperature calorimetric domain is associated with the cooperative unit composed of the other two domains. If this assumption is true, the enthalpy of denaturation of the second domain should be at least double that of the first domain in all cases. On the contrary, it is seen (Fig. 2) that at a low molar ratio of SDS/BSA = 1, the enthalpy of denaturation of the first domain is found to be about double that of the second domain.

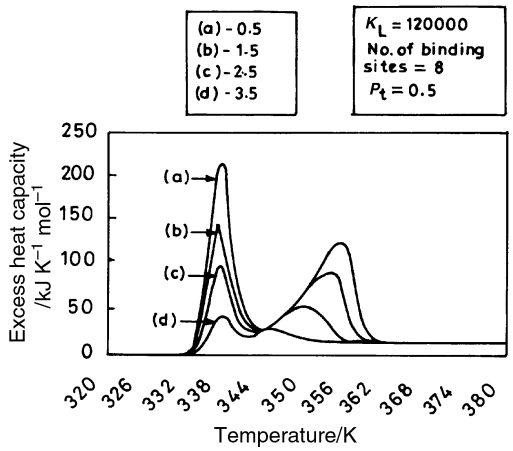

Fig. 13 The simulated curve for the excess heat capacity of BSA with different concentrations of ligand.

A method has also been described in the literature, to simulate the DSC curves to estimate the binding constant and heat of binding. The various equations, as described by Brandts and Lin,[19] used in the simulation are given below:

$$N \rightleftharpoons D$$

$$K = \frac{[D]}{[N]}$$

$$N + L \xrightarrow{K_L} NL$$

$$K_L = \frac{[NL]}{[N][L]}$$

where N is the native globular conformation which binds ligand L, D is the unfolded conformation, NL is the folded-ligand complex, K is the equilibrium constant, K_L is the binding constant of the folded-ligand complex and ΔC_{pL} is the heat capacity of binding of the native state with ligand

Eqn. (1) and (2) for the conservation of mass:

$$[P_t] = [N] + [D] + [NL] = [N] + K[N] + K_L[L][N] \quad (1)$$

$$[L_t] = [L] + [NL] = [L] + K_L[L][N] \quad (2)$$

are solved to obtain [L], [N] and [NL]

Using the values of the input parameters T_0, ΔH_d, ΔC_{pd}, K_L at T_0, ΔC_{pL}, $[P_t]$, $[L_t]$, the concentration of all species are determined at any temperature from eqn. (1) and (2), the equilibrium constants being given by the following equations;

$$K(T) = \exp[\Delta H_d(T_0)/R(1/T - 1/T_0) + \Delta C_{pd}/R(\ln T/T_0) + T_0/T - 1)] \quad (3)$$

$$K_L(T) = K_L(T_0)\exp[-\Delta H_L(T_0)/R(1/T - 1/T_0) + \Delta C_{pL}/R(\ln T/T_0) + T_0/T - 1)] \quad (4)$$

Excess enthalpy is given by

$$H_{xs}(T) = [D]/P_t[\Delta H_d(T_0) + \Delta C_{pd}(T - T_0)] + [NL]/P_t[\Delta H_L(T_0) + \Delta C_{pL}(T - T_0)] \quad (5)$$

and the DSC parameter excess heat capacity is obtained by numerical differentiation over small temperature intervals.

By using the values of T_0, ΔH_d at T_0 in the absence of ligand obtained from DSC, the curves shown in Fig. 13 are simulated. The simulated curve shows the biphasic nature of the thermogram.

Conclusion

SDA plays two opposite roles in the folding and stability of BSA. At low surfactant/protein ratio, it acts as a structure-stabilizing additive. It increases the stability of protein against thermal denaturation. Initially, binding to sites with fairly high affinity leads to a strong increase in stability. Additional weak interactions and/or indirect, solvent-mediated effects lead to a continuing but smaller increase in stability at intermediate surfactant concentrations. At higher concentrations of SDS, the binding of SDS to denatured protein is more prominent and unfolding occurs; this is a consequence of Le Chatelier's principle.

Also, at lower concentrations of SDS, thermograms for the denaturation of BSA are biphasic, as reflected by the two endotherms, while denaturation of pure BSA is monophasic. However, with increasing levels of SDS, the endotherms coalesce to give a single, asymmetric peak, skewed to the lower temperature side. The biphasic endotherms are associated with the unfolding of ligand-poor and ligand-protein forms respectively.

Thermograms for the denaturation of BSA with added SOS consist of a single denaturation peak at lower as well as at higher concentration. With increasing SOS concentration, the denaturation temperature increases with concomitant decrease in the half height width. The increase in the denaturation temperature in SOS is less than in SDS which indicates that SDS binds BSA more strongly than SOS. This emphasizes the importance of the hydrophobic part of a surfactant in binding with BSA. In the domain structure of BSA, three peptide segments are arranged parallel to each other, forming a long hydrophobic groove. This groove forms an especially favorable orientation for the accommodation of several surfactant molecules with multiple hydrophobic interactions.

Addition of SDS at a low SDS/BSA ratio increased the mid-point urea concentration for the CD and intrinsic fluorescence parameter. Increase in the SDS concentration leads to a shift in the wavelength of the maximum of the intrinsic fluorescent emission with quenching of fluorescence. It is consistent with the calorimetric results that show that SDS acts as a stabilizer at low concentration while it destabilizes at higher concentrations.

Acknowledgements

J. C. Ahluwalia is grateful to the Indian National Science Academy for the award of INSA Senior Scientist and to the Indian Institute of Technology, Delhi for providing the necessary facilities for the research work.

References

1 M. N. Jones, in *Biochemical Thermodynamics*, ed. M. N. Jones, Elsevier, Amsterdam, 1st edn., 1985, ch. 5, p. 112.
2 M. N. Jones and A. Brass, in *Food Polymers, Gels and Colloids*, ed. E. Dickinson, Royal Society of Chemistry, 1991, pp. 65–80.
3 M. N. Jones, P. Manley and A. E. Wilkinson, *Biochem. J.*, 1982, **203**, 285.
4 M. N. Jones, P. Manley, P. J. W. Midgley and A. E. Wilkinson, *Biopolymers*, 1982, **21**, 1435.
5 C. A. Nelson, *J. Biol. Chem.*, 1971, **246**, 3895.
6 M. N. Jones, A. Finn, A. Mosavi-Movahedi and B. J. Waller, *Biochim. Biophys. Acta*, 1987, **913**, 395.
7 M. Y. El-Sayert and M. F. Roberts, *Biochim. Biophys. Acta*, 1985, **831**, 133.
8 H. Polet and J. Steinhardt, *Biochemistry*, 1968, **7**, 1348.
9 R. Lovrien, *J. Am. Chem. Soc.*, 1963, **85**, 3677.
10 M. L. Markus, R. L. Love and F. C. Wissler, *J. Biol. Chem.*, 1964, **239**, 3687.

11 M. L. Meyer and W. Kauzmann, *Arch. Biochem. Biophys.*, 1962, **99**, 348.
12 J. D. Teresi and J. M. Luck, *J. Biol. Chem.*, 1952, **194**, 823; S. P. Manly, K. S. Matthews and J. M. Sturtevant, *Biochemistry*, 1985, **24**, 3842.
13 T. Peters, *Adv. Protein Chem.*, 1985, **37**, 161.
14 Kragh-Hansen, *Pharmacol. Rev.*, 1981, **33**, 17.
15 T. Peters, *Albumin: An Overview and Bibliography*, Miles Laboratory, ElKhart, Inc., 1988.
16 J. F. Foster, in *Albumin Structure, Function and Uses*, ed. V. M. Roesnoer, M. Oratz and M. A. Rothschild, Pergamon, Oxford, 1977, pp. 53–84.
17 J. R. Brown and P. Shockley, in *Lipid–Protein Interactions*, ed. P. Jost and O. H. Griffith, Wiley, New York, 1982, vol. 1, p. 25.
18 J. F. Brandts, C. Q. Hu, L.-N. Lin and M. T. Mas, *Biochemistry*, 1989, **28**, 8588.
19 J. F. Brandts and L. N. Lin, *Biochemistry*, 1990, **29**, 6927.
20 Shrake and P. D. Ross, *J. Biol. Chem.*, 1988, **263**, 15392.
21 Shrake and P. D. Ross, *J. Biol. Chem.*, 1990, **265**, 5055.
22 M. Yamasaki, H. Yano and K. Aoki, *Int. J. Biol. Macromol.*, 1992, **14**, 305.
23 Giancola, C. D. Sena, D Fessas, G. Graziano and G. Barone, *Int. J. Biol. Macromol.*, 1997, **20**, 193.
24 S. Gopal and J. C. Ahluwalia, *J. Chem. Soc., Faraday Trans.*, 1993, **89**, 2769.
25 F. P. Schwarz and W. H. Kirchoff, *Thermochim. Acta*, 1988, **128**, 267.
26 W. H. Kirchoff, in *EXAM* (*DSC Analysis Version*), Chemical Thermodynamics Division, NIST, Gaithersburg, USA, 1988; J. M. Sturtevant, *Annu. Rev. Phys. Chem.*, 1987, **38**, 463.
27 J. M. Sturtevant, *Annu. Rev. Phys. Chem.*, 1987, **38**, 463.
28 S. P. Manly, K. S. Matthews and J. M. Sturtevant, *Biochemistry*, 1985, **24**, 3842.
29 V. Edge, N. M. Allewe and J. M. Sturtevant, *Biochemistry*, 1985, **24**, 5899.
30 C. Q. Hu and J. M. Sturtevant, *Biochemistry*, 1987, **26**, 178.
31 V. Edge, N. M. Allewe and J. M. Sturtevant, *Biochemistry*, 1988, **27**, 8081.
32 J. M. Sanchez-Ruiz, *Biophys. J.*, 1992, **61**, 921.
33 M. L. Galistro, F. Conejero-Lara, J. Nunez, J. M. Sanchez-Ruiz and P. L. Mateo, *Thermochim. Acta*, 1992, **199**, 147.
34 C. H. Robert, S. J. Gill and J. Wyman, *Biochemistry*, 1988, **27**, 6829.
35 M. Yamasaki, Y. Hiroshige and K. Aoki, *Int. J. Biol. Macromol.*, 1990, **12**, 263.
36 G. Barone, E. Rizzo and V. Vitagliano, *J. Phys. Chem.*, 1970, **74**, 2230.
37 T. S. Sharma and J. C. Ahluwalia, *J. Phys. Chem.*, 1972, **76**, 1366.
38 K. N. Prasad and J. C. Ahluwalia, *Biopolymers*, 1980, **19**, 273.
39 N. Desrosiers, G. Perron, J. G. Mathieson, B. E. Conway and J. E. Desnoyers, *J. Solution Chem.*, 1974, **3**, 789.
40 M. Y. Schrier, P. J. Turner and E. E. Schrier, *J. Phys. Chem.*, 1975, **79**, 1391.
41 L. L. Bright and J. R. Jezorek, *J. Phys. Chem.*, 1975, **79**, 800.
42 R. W. Woody, *Methods Enzymol.*, 1995, **246**, 34.
43 F. X. Schmid, in *Protein Structure: A Practical Approach*, ed. T. E. Creighton, IRL Press, Oxford, 1989, p. 251.