Hydration in Protein Folding: Thermal Unfolding/Refolding of Human Serum Albumin

Rajib Kumar Mitra, Sudarson Sekhar Sinha, and Samir Kumar Pal*

Unit for Nano Science and Technology, Department of Chemical, Biological and Macromolecular Sciences, S.N. Bose National Center for Basic Sciences, Block JD, Sector III, Salt Lake, Kolkata 700098, India

Human serum albumin (HSA) is known to undergo both reversible and irreversible thermal unfolding and refolding, depending upon the experimental conditions (end temperature) at neutral pH. In this report we have used high precision densimetric and ultrasonic measurements to determine the apparent specific volume (φ_v) and compressibility (φ_k) of HSA at different unfolded and refolded states at two different end temperatures, 55 °C and 70 °C. The unfolded and refolded states were characterized using dynamic light scattering (DLS), circular dichroism (CD), picosecond-resolved fluorescence decay, and anisotropy of the single-tryptophan residue in HSA (Trp214). Both the unfolded states were allowed to refold by cooling wherein the former and latter processes were found to be reversible and irreversible, respectively, in nature. The results obtained from the densimetric and ultrasonic measurements reveal that the apparent specific volume and compressibility of the protein in the reversible protein unfolding process is preserved upon restoration of HSA to ambient temperature. However, a significant change in φ_v and φ_k occurs in the process of irreversible protein refolding (from 70 to 20 °C). The experimental observation is rationalized in terms of the exposure of domain IIA to an aqueous environment, resulting in the swelling of the protein to a higher hydrodynamic diameter. Our studies attempt to explore the extent of hydration associated with the structural integrity of the popular protein HSA.

Introduction

Water is essential for the structure and function of proteins.[1] Generally, structural changes of proteins in water relate to the change in the hydration state.[2] Protein hydration has previously been studied by various methods, such as calorimetry,[3] infrared spectroscopy,[4] osmotic pressure,[5] NMR techniques,[6−8] dielectric spectroscopy,[9,10] time-resolved fluorescence studies,[11] etc. The elastic properties of proteins in solution yield information about the amplitude of their structural fluctuations, which are sensitive to interactions with solvent and also to the packing of the structure.[12−14] The apparent specific volume (φ_v) and the apparent specific adiabatic compressibility (φ_k) of a protein are macroscopic observables, which are particularly sensitive to the hydration properties of solvent-exposed atomic groups, as well as to the structure, dynamics, and compressibility of the solvent-inaccessible protein interior.[13−15] Thus, any unfolding or refolding process in protein must have impression on the corresponding

changes in the compressibility and hydration of the protein. Recent advancement in acoustic techniques have made possible high-precision ultrasound velocimetry measurements, leading to the estimation of protein volume and compressibility.[16] Thus, such ultrasonic measurements have recently been used for the characterization of protein conformational states.[13,14,17−24]

The unfolding pathways (both chemical and thermal) of human serum albumin (HSA), one of the most well-characterized proteins, have been examined in previous studies.[25−30] However, to date no crystal structures of HSA in such unfolded states are available, and the conformational changes induced at high temperatures are not known at atomic-level resolution. Also, the knowledge about the essential hydration of this protein molecule at different temperature-induced unfolded and refolded states is lacking in the present literature. Very recently El Kadi et al.[31]

* Corresponding author. E-mail: skpal@bose.res.in.

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have reported the changes of volume and compressibility of bovine serum albumin (BSA) by densimetry and adiabatic compressibility during its conformational transition from pH 7 to pH 2, using ultrasound measurements. The compressibility variation observed corroborates the conformational changes during the transition. In the present study, we report the temperature-induced unfolding and refolding process of HSA with the primary focus to explore the extent to which the transition involves changes in compressibility and hydration and how this observation can be interpreted in terms of the molecular structure. In accordance with the previous reports on the protein HSA,[27,29,30] we adopt two unfolding pathways, (i) increasing the temperature from 20 to 55 °C and then cooling back to 20 °C, (ii) increasing the temperature from 20 to 70 °C and then cooling back to 20 °C. The first process is known to be a reversible one, whereas the latter one is an irreversible one.[27] We characterize the unfolded and refolded states by determining the hydrodynamic diameter (d_h) using dynamic light scattering (DLS) and the secondary structure of the protein using circular dichroism (CD). We also measure the picosecond-resolved fluorescence decay and rotational anisotropy of Trp214 residue at intermediate steps of the unfolding and refolding processes and correlate the results with those obtained from volume and compressibility measurements.

Materials and Methods

Human serum albumin (HSA), and phosphate buffer are obtained from Sigma. The sample solutions were prepared in 50 mM phosphate buffer (pH = 7.0) using water from Millipore system. DLS measurements are done with Nano S Malvern instrument employing a 4 mW He−Ne laser ($\lambda = 632.8$ nm) equipped with a thermostated sample chamber. All the scattered photons are collected at 173° scattering angle. The scattering intensity data are processed using the instrumental software to obtain the hydrodynamic diameter (d_H) and the size distribution of the scatterer in each sample. The instrument measures the time-dependent fluctuation in the intensity of light scattered from the particles in solution at a fixed scattering angle. Hydrodynamic diameter (d_H) of the proteins is estimated from the intensity autocorrelation function of the time-dependent fluctuation in intensity. d_H is defined as:

$$d_H = \frac{k_b T}{3\pi\eta D} \qquad (1)$$

where k_b is the Boltzmann constant, η is the viscosity, and D is the translational diffusion coefficient. In a typical size distribution graph from the DLS measurement, the *X*-axis shows a distribution of size classes in nm, while the *Y*-axis shows the relative intensity of the scattered light. The circular dichroism (CD) studies are done in a JASCO 815 spectrometer with an attachment for the temperature-dependent measurements (Peltier). CD studies are done in 1.5 μM protein solution in 1.0 cm path length cell. The secondary structural data of the CD spectra are analyzed using CDNN software (http// bioinformatik.biochemtech.uni-halle.de/cdnn).

Fluorescence transients have been measured and fitted by using a commercially available spectrophotometer (LifeSpec-ps) from Edinburgh Instrument, U.K. (excitation wavelength 299 nm, ~460 ps instrument response function (IRF)) with an attachment for temperature-dependent studies (Julabo, model F32). The observed fluorescence transients are fitted by using a nonlinear least-square fitting procedure to a function:

$$X(t) = \int_0^t E(t')\, R(t - t')\, dt'$$

comprising convolution of the IRF ($E(t)$) with a sum of exponentials:

$$R(t) = A + \sum_{i=1}^{N} B_i\, e^{-t/\tau_i}$$

with pre-exponential factors (B_i), characteristic lifetimes (τ_i), and a background (A). Relative concentration in a multiexponential decay is finally expressed as:

$$a_n = \frac{B_n}{\displaystyle\sum_{i=1}^{N} B_i}$$

The quality of the curve fitting is evaluated by reduced χ^2 and residual data. For anisotropy ($r(t)$) measurements, emission polarization is adjusted to be parallel or perpendicular to that of the excitation, and anisotropy is defined as:

$$r(t) = \frac{[I_{para} - G \times I_{perp}]}{[I_{para} + 2 \times G \times I_{perp}]}$$

G, the grating factor is determined following longtime tail-matching technique.[32]

Volume and compressibility of HSA have been calculated using the density and sound velocity values measured by a density meter (model DSA5000) from Anton Parr (Austria) with an accuracy of 5×10^{-6} g cc^{-1} and 0.5 ms^{-1} in density and sound velocity measurements, respectively. Adiabatic compressibility (β_p) of the protein solution can be determined by measuring the protein solution density (ρ_p) and the sound velocity (u_p) and applying Laplace's equation,

$$\beta_p = \frac{1}{\rho_p u_p^2} \qquad (2)$$

The apparent specific volume of protein φ_v is given by

$$\varphi_v = \frac{1}{\rho_w} + \frac{\rho_w - \rho_p}{c_p \rho_w} \qquad (3)$$

where c_p is the concentration of the protein solution and ρ_w and ρ_p are the densities of the solvent and protein solutions, respectively.

The partial apparent adiabatic compressibility (φ_k) of the protein is obtained from the following relation:

$$\varphi_k = \beta_w \left(2\varphi_v - 2[u] - \frac{1}{\rho_w} \right) \qquad (4)$$

where $[u]$ is the relative specific sound velocity increment given by

$$[u] = \frac{u_p - u_w}{u_w c_p} \qquad (5)$$

where u_w and u_p are the sound velocities in solvent and protein solutions, respectively.

Results and Discussion

Figure 1 depicts the results obtained from DLS measurement of HSA at different temperatures. The average hydrodynamic diameter (d_H) of HSA at room temperature is ~10 nm (inset of Figure 1), which does not change appreciably when temperature is increased up to ~60 °C, beyond which d_H increases rapidly, and at 75 °C it reaches a value of ~40 nm (inset of Figure 1). d_H of the native protein is consistent with that of the X-ray structure[33] on taking 6 Å to be the thickness of the hydration layer.[34] Such a high value of d_H could be argued as being due to an aggregation of the protein upon thermal unfolding.[27,29,35]

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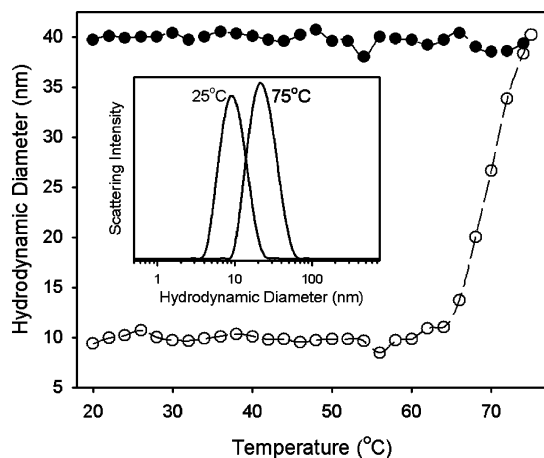
Figure 1. Effect of temperature on the size of HSA in 50 mM phosphate buffer. Open symbols represent forward (unfolding) process, and closed symbols represent backward (refolding) process. DLS signals of HSA at 25 °C and 75 °C have been presented in the inset.

However, our fluorescence anisotropy data (see below) are consistent with the swelling of the protein and *not* with the multimer formation of HSA at higher temperature. When the system is cooled from 75 to 20 °C d_h does not recover its original size (Figure 1). In another case we increase the temperature up to 55 °C and then cool it down to 20 °C. No appreciable change in the hydrodynamic diameter of HSA is observed during the heating or cooling process. These observations reveal that an increase in temperature of HSA up to 55 °C produces no appreciable change in the globular tertiary structure of the native protein as evidenced from the constant value of d_H (∼10 nm) throughout the temperature range. However, as the temperature goes beyond 60 °C, appreciable increase occurs in the hydro-dynamic diameter of the protein, indicating a change in its native tertiary structure. This change is found to be irreversible, as cooling of the unfolded state does not refold back to its native state as evidenced from the DLS measurement. This observation is consistent with previous reports[27,29] wherein the thermal unfolding process of HSA has been reported to be reversible up to 60 °C and beyond this temperature the process is irreversible. The present DLS measurement confirms that the tertiary structure of the native protein could not be revived after thermal unfolding beyond 60 °C.

The effect of temperature on the secondary structure of the unfolded and refolded states of HSA is examined by the temperature-dependent CD measurements (Figure 2a). The CD spectra taken at different temperatures are analyzed to determine the α-helix content, and the results are plotted in Figure 2b. At 20 °C, we found 65% of α-helix, 15% random coil, which is in close agreement with previously reported values.[27,35] When the temperature is increased, the content of α-helix decreases to reach 38% at 70 °C (Figure 2b). Earlier Mariyama et al.[36] reported 44% α-helix of HSA at 65 °C. Our finding of 46% α-helicity at 60 °C is in good agreement with this study. It can be seen from the figure that the change in α-helical content against temperature is quite sharp beyond 55 °C, indicating a rapid loss of α-helicity at high temperature. To check the reversibility of the unfolding process we cooled the protein from 70 to 20 °C and found that the protein can only recover 51% of its α-helicity, indicating the irreversibility of the unfolding process. Mariyama et al.[36] reported
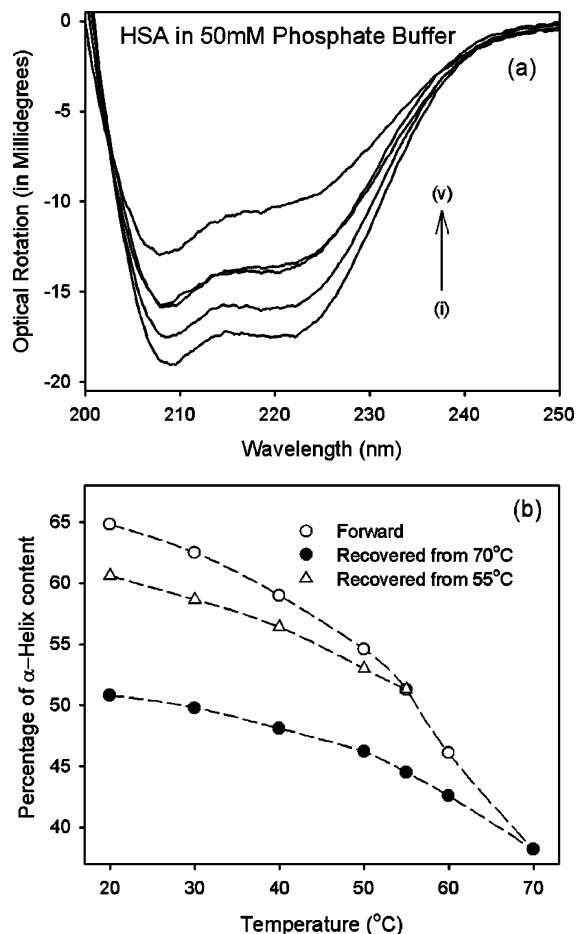
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Figure 2. (a) CD spectra of 1.5 μM HSA in 50 mM phosphate buffer in far UV, (i) 20 °C forward, (ii) 20 °C recovered from 55 °C, (iii) 20 °C recovered from 70 °C, (iv) 55 °C forward, (v) 70 °C forward. (b) Percentage of α-helix content in HSA at different temperatures.

53% recovery of α-helicity when cooled from 65 to 25 °C. On the other hand, heating of HSA up to 55 °C reduces the α-helicity up to 51%, and when the unfolded protein is cooled, it recovers most of its α-helicity (61%). Mariyama et al.[36] reported full recovery of α-helix when cooled from 45 °C. In the present study a possible partial irreversibility of the refolding process perhaps hinders the full recovery from 55 °C.

The presence of a single Trp214 residue in domain IIA turns this protein into an excellent model for following its denaturation process by measuring the fluorescence emission of the fluoro-phore. Flora et al.[27] earlier studied the temperature dependency of the emission spectrum of Trp214 in HSA and found that fluorescence intensity gradually decreased with increasing temperature with a distinct change in slope at 60 °C. In the present study, we also obtained a similar result with a 4−5 nm red-shift of the emission maximum upon increasing the tem-perature from 25 to 70 °C. The decreased intensity associated with a red-shift indicates the exposure of the Trp214 residue to a polar environment at elevated temperature. The intensity does not increase when the system cools from 70 to 20 °C, revealing the irreversible nature of the transition. We also study the picosecond-resolved fluorescence decay transient of the Trp214 residue of the protein to understand the unfolding and refolding processes involved in domain IIA (Figure 3). The transients at 335 nm at different temperatures are well-fitted triexponentially, and the corresponding fitting parameters are presented in Table 1. It can be observed from the table that at 20 °C, the time constants are 130 ps and 2.11 and 6.63 ns with an average time
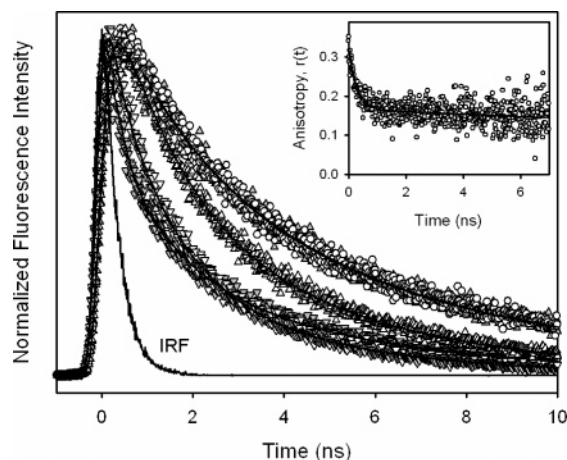
Figure 3. Fluorescence transients of Trp214 residue in HSA at 335 nm (excited at 299 nm) at different temperatures, 20 °C (○), 55 °C (△), 70 °C (▽). The filled symbols represent decay transients at 20 °C recovered from 55 °C (▲) and 70 °C (▼). The fluorescence anisotropy of Trp214 residue in HSA at 20 °C is presented in the inset.

Table 1. Fitted Parameters of the Fluorescence Transients of the Trp214 Residue in HSA at 335 nm at Different Temperatures

temperature (°C)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	$\langle\tau\rangle$ (ns)	χ^2
20	0.13 (56%)	2.11 (20%)	6.63 (24%)	2.06	1.05
20[a]	0.14 (50%)	2.17 (22%)	6.66 (28%)	2.42	1.09
20[b]	0.05 (93%)	1.57 (4%)	4.98 (3%)	0.27	1.02
40	0.19 (45%)	2.05 (30%)	5.88 (25%)	2.16	0.99
40[a]	0.15 (52%)	1.92 (24%)	5.68 (24%)	1.90	0.94
40[b]	0.05 (92%)	1.59 (5%)	4.83 (3%)	0.26	1.07
55	0.16 (48%)	1.56 (29%)	4.62 (23%)	1.59	1.05
55[b]	0.05 (93%)	1.35 (4%)	3.99 (3%)	0.21	1.10
60	0.20 (49%)	1.56 (32%)	4.33 (19%)	1.42	1.02
60[b]	0.05 (93%)	1.36 (4%)	3.99 (3%)	0.22	1.06
70	0.08 (81%)	1.31 (12%)	3.74 (7%)	0.47	1.07

[a] Recovered from 55 °C. [b] Recovered from 70 °C.

constant of 2.06 ns. With the increase in temperature the transient becomes faster. Beyond 60 °C, the transient becomes very fast with an average time constant of 0.47 ns. When the temperature is decreased from 70 °C, the transient remains fast, indicating that the refolding process is irreversible in nature, which is in accord with DLS and CD measurements. The native protein structure is not fully recovered upon refolding, at least in the domain IIA containing Trp214. We check the reversibility of the process when the temperature is raised to 55 °C and then cooled to 20 °C (Table 1). It is found that the time constants are recovered in all the cases, revealing the reversibility of the process. Thus, the domain containing the Trp214 residue perhaps does not unfold, or unfolding is insignificant up to 55 °C. The time-resolved fluorescence anisotropy profiles (inset of Figure 3) of the Trp214 at different temperatures are fitted biexponentially (Table 2) with time constants of several hundreds of picoseconds and 50 ns (fixed). The fast component represents the rotational motion of the Trp214 residue within its immediate microenvironment, whereas the 50 ns component is due to the global tumbling motion of the protein.[37] It can be observed that at the temperature below 60 °C the contribution of the fast component is comparable to that of the 50 ns component, indicating the location of the probe in a restricted environment buried inside the protein. At 70 °C the fast component becomes the major one, indicating the exposure

Table 2. Time-resolved Fluorescence Anisotropy Decay Parameters of the Trp214 Residue in HSA at 335 nm at Different Temperatures

temperature °C	r_0	τ_1 (ns)	τ_2 (ns)
20	0.32	0.28 (48%)	50.0 (52%)
20[a]	0.25	0.35 (43%)	50.0 (57%)
20[b]	0.37	0.37 (64%)	50.0 (36%)
40	0.30	0.36 (42%)	50.0 (58%)
40[a]	0.26	0.35 (49%)	50.0 (51%)
40[b]	0.37	0.38 (68%)	50.0 (32%)
55	0.26	0.26 (55%)	50.0 (45%)
55[b]	0.25	0.42 (69%)	50.0 (31%)
60	0.26	0.24 (58%)	50.0 (42%)
60[b]	0.32	0.35 (69%)	50.0 (31%)
70	0.37	0.34 (69%)	50.0 (31%)

[a] Recovered from 55 °C. [b] Recovered from 70 °C.

of the Trp214 residue in a less restricted environment. When the system is cooled from 70 °C, the fast component remains as the major one, indicating that the mobility of the Trp214 residue is greater in the refolded state than it is in the native state and the protein does not refold to its original form. However, when the system is cooled from 55 °C, it recovers its native time constant values, confirming the reversible nature of the refolding process. It should be noted that the contribution of the slower time constant of 50 ns, indicative of global motion of the host protein, decreases with increase in temperature. It could be recalled that the DLS experiment reveals a ~4 fold increase in the hydrodynamic diameter of the protein upon increasing the temperature beyond 60 °C. A possible aggregation of the protein molecule might have been a reason for this increase, but it should be noted that the peak position of Trp214 emission suffers a red-shift along with an accelerated fluorescence decay at elevated temperatures, which strongly opposes the aggregation phenomenon. The contribution of the longer component (50 ns) in fluorescence anisotropy of Trp214 is found to decrease at higher temperatures (Table 2), indicating labile environments around the probe. The observation also stands against the aggregation of the protein, which might have led to a more rigid environment around the probe, Trp214, resulting in an increased contribution of the longer time constant.

The apparent specific volume (φ_v) and partial specific apparent adiabatic compressibility (φ_k) values calculated from eqs 3 and 4 are presented in Table 3 and are plotted against temperature in Figure 4. The φ_k value obtained at 20 °C and pH 7.0 in the present study is comparable to the value of 6.6×10^{-14} m^3 kg^{-1} Pa^{-1} reported by El Kadi et al.[31] and Chalikian et al.[14] for BSA. As evidenced from Figure 4 and Table 3, φ_k increases with increasing temperature and reaches a maximum value at 55 °C. Earlier, Chalikian et al.[14] reported a similar 2-fold increase in φ_k upon increasing the temperature from 18 to 55 °C for a similar transport protein, BSA. When the temperature is increased further, φ_k value decreases rapidly by an order of magnitude at 70 °C. This unfolding profile is similar to the pH-dependent unfolding pathway of BSA as reported earlier.[31] The φ_k variation during the thermal unfolding process is larger than the corresponding φ_v variation, which is in accord with previous reports.[31,38] The sharp decrease in φ_k beyond 55 °C is comparable to that observed in the CD measurement (Figure 2b) wherein the α-helicity decreases rapidly beyond 55 °C, corresponding to some structural alteration during the unfolding process, which may also involve some nonhelical parts of the molecule. The change in φ_k with temperature can be explained in the following manner:

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Table 3. Result of Compressibility Measurements of HSA at Different Temperatures

temperature (°C)	ρ_p (kg m^{-3})	u_p (m s^{-1})	$\varphi_v \times 10^4$ (m^3 kg^{-1})	$\varphi_k \times 10^{14}$ (m^3 kg^{-1} Pa^{-1})
Heating of 50 μM HSA from 20 to 70 °C			
20	1000.068	1485.0	7.759	5.929
25	998.850	1499.5	7.781	7.111
30	997.485	1511.5	7.807	8.960
35	995.860	1522.5	7.823	10.28
40	994.033	1531.5	7.853	11.63
45	992.023	1539.0	7.868	12.73
50	989.838	1545.0	7.540	11.58
55	987.491	1540.0	7.708	13.38
60	984.986	1553.0	6.576	6.178
65	982.336	1555.5	5.715	2.102
70	979.535	1557.0	4.850	0.855
Cooling of HSA from 70 to 20 °C			
65	982.262	1556.0	5.943	1.376
60	984.907	1553.5	6.819	2.635
55	987.421	1550.5	7.923	4.959
50	989.764	1545.5	7.767	5.190
45	991.962	1539.5	8.054	4.341
40	993.983	1532.0	8.005	3.588
35	995.821	1523.0	7.944	4.258
30	997.460	1512.0	7.883	3.338
25	998.850	1499.5	8.008	4.388
20	1000.064	1485.5	7.772	4.006
Cooling of HSA from 55 to 20 °C			
50	989.835	1545.0	7.549	12.49
45	992.021	1539.0	7.877	12.79
40	994.032	1531.5	7.855	12.08
35	995.857	1522.5	7.832	10.40
30	997.484	1511.5	7.810	8.585
25	998.894	1499.5	7.784	7.274
20	1000.066	1485.0	7.765	5.799

For globular proteins, the value of φ_k is a sum of two contributions[24]

$$\varphi_k = \varphi_{kM} + \varphi_{kh} \qquad (6)$$

where φ_{kM} is the contribution due to the imperfect packing of the polypeptide chains within the solvent-inaccessible protein core and is given by

$$\varphi_{kM} = \frac{\beta V_M}{M} \qquad (7)$$

where, V_M is the volume of water-inaccessible protein interior, β is the compressibility and M is the molecular weight of the protein. The contribution φ_{kh} emanates from the hydration of the surface atomic group of the protein, given by

$$\varphi_{kh} = M^{-1} \sum_i S_{Ai} K_{Si} \qquad (8)$$

where, S_{Ai} is the solvent-accessible surface area (SASA) of the ith residue and K_{Si} is the compressibility contribution of the residue. In general $\sum_i S_{Ai} K_{Si}$ produces negative contribution to φ_k.

In the native state, which is the most compact thermodynamically stable state, the protein exhibits the lowest SASA and consequently the highest hydration contribution to φ_k. Also, it exhibits the largest water-inaccessible core volume (V_M) with low β (due to a tightly packed core). When the temperature is increased to 55 °C, the protein undergoes a reversible change to a compact intermediate state[27] with an overall increase in φ_k. During this change some of the buried residues of the native state become exposed to the solvent. This increases the extent of SASA and consequently decreases φ_{kh}. Also, the imperfect
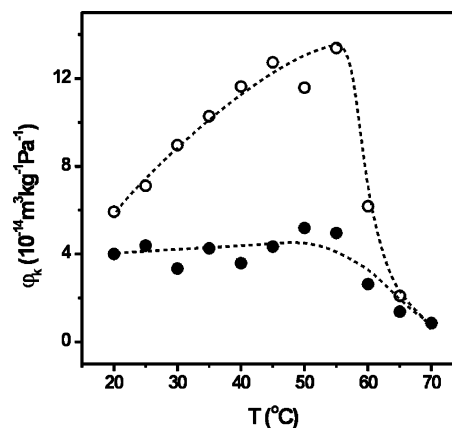

Figure 4. Partial specific apparent adiabatic compressibility (φ_k) of 50 μM HSA in 50 mM phosphate buffer at different temperatures. Open circles represent the forward (unfolding) process, and filled symbols represent the backward (refolding) process. The dotted lines are guides for the eye.

packing of the amino acid residues due to partial unfolding inside the protein core increases β. Although this process is associated with a decrease in V_M, the other two processes overwhelm this decrease, and as a consequence φ_k increases. When the temperature is increased up to 70 °C, the protein transforms into an unfolded state in which SASA increases substantially causing a large increase in φ_{kh}. The increase in β due to the unfolding of compact protein might overwhelm the increase in V_M, and as a consequence φ_k decreases as observed in the present study. When the temperature is decreased from 70 °C, φ_k acquires a constant value of ~4 × 10^{-14} m^3 kg^{-1} Pa^{-1}, indicating the irreversibility of the refolding pathway. The increase in SASA of the unfolded state at 70 °C perhaps could not be recovered due to the imperfect recoiling of the protein in the cooling process, resulting in a low value of φ_k. When the unfolded state is recovered from 55 °C, the φ_k value traces back its forward path, and the native state is almost recovered.

We observe a decrease in α-helicity up to 55 °C, which is recovered upon decreasing the temperature. It could be noted that domain IIIA is prone to refold reversibly because of its relatively fewer interactions with other parts of the molecule;[39] thus, the unfolding and consequent decrease in α-helicity up to 55 °C could be rationalized as a consequence of the unfolding of domain IIIA. When the temperature is increased beyond 55 °C, a part of the broken helical structure might get exposed to the solvent, resulting in an increase in hydration; when temperature is decreased from this state, hydration does not follow its original unfolding pathway. It is observed in the fluorescence anisotropy measurement (Table 2) that at 70 °C the mobility of the Trp214 residue is higher, confirming an increased hydration and a probable solvent exposure of domain IIA. This confirms that beyond 55 °C domain IIA starts to denature irreversibly. As observed from the CD experiment, the α-helicity of HSA suffers an irreversible decrease beyond 55 °C, indicating the exposure of amino acid residues of domain IIA, which are strongly hydrated, and the hydrophobic force is perhaps overcome by the hydration, as they do not recover their native orientations.

Conclusion

Ultrasound and densimetric studies reveal that compressibility (φ_k) of HSA increases as temperature is increased to 55 °C with a corresponding decrease in hydration. With further increase in

(39) Sugio, S.; Kashima, A.; Moshizuki, S.; Noda, M.; Kobayashi, K. *Protein Eng.* **1999**, *12*, 439–446.

temperature, φ_k decreases rapidly with a concomitant increase in hydration. DLS results show an increase in the hydrodynamic diameter of HSA and CD results show rapid loss of α-helicity beyond 55 °C, which exposes the protein residues of domain IIA to the aqueous environment as confirmed by the time-resolved measurements. The time-resolved studies reveal that upon refolding from 70 °C the Trp214-containing domain (IIA) remains exposed to water, revealing the irreversible nature of the refolding process, which has also been confirmed by the hydration study.

Our studies correlate the change in hydration during a protein unfolding/refolding process with the data obtained from DLS, CD, and time-resolved measurements and establish the ultrasound and densimetric techniques to be efficient tools to unravel the essential level of hydration for the structural integrity of proteins.