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

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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 \( \varphi_v \) and compressibility \( \varphi_k \) 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 \( \varphi_v \) and \( \varphi_k \) 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. Generally, structural changes of proteins in water relate to the change in the hydration state. Protein hydration has previously been studied by various methods, such as calorimetry, infrared spectroscopy, osmotic pressure, NMR techniques, dielectric spectroscopy, time-resolved fluorescence studies, 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. The apparent specific volume \( \varphi_v \) and the apparent specific adiabatic compressibility \( \varphi_k \) 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. Thus, any unfolding or refolding process in protein must have impressed 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. Thus, such ultrasonic measurements have recently been used for the characterization of protein conformational states. 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. 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

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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, 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:

\[
d_H = \frac{k_B T}{3\pi\eta D}
\]  

(1)

where \(k_B\) is the Boltzmann constant, \(\eta\) 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 mM 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 RCF (\(E(t')\)) with a sum of exponentials:

\[
R(t) = A + \sum_{i=1}^{N} B_i e^{-t/t_i}
\]

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

\[
a_n = \frac{B_n}{\sum_{i=1}^{N} B_i}
\]

The quality of the curve fitting is evaluated by reduced \(\chi^2\) 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:

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

\(G\), the grating factor is determined following longtime tail-matching technique.

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 \(5 \times 10^{-6}\) g cm\(^{-3}\) and 0.5 ms\(^{-1}\) in density and sound velocity measurements, respectively. Adiabatic compressibility (\(\beta_p\)) of the protein solution can be determined by measuring the protein solution density (\(\rho_p\)) and the sound velocity (\(u_p\)) and applying Laplace’s equation,

\[
\beta_p = \frac{1}{\rho_p u_p^2}
\]

(2)

The apparent specific volume of protein \(\varphi_v\) is given by

\[
\varphi_v = \frac{1}{\rho_\text{w}} + \frac{\rho_\text{w} - \rho_\text{p}}{c_p \rho_\text{w}}
\]

(3)

where \(c_p\) is the concentration of the protein solution and \(\rho_\text{w}\) and \(\rho_\text{p}\) are the densities of the solvent and protein solutions, respectively.

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

\[
\varphi_v = \beta_p \left(2\varphi_v - 2[\varphi_v - 1]\right)
\]

(4)

where \([\varphi_v]\) is the relative specific sound velocity increment given by

\[
[\varphi_v] = \frac{u_\text{p} - u_\text{w}}{u_\text{w} c_p}
\]

(5)

where \(u_\text{w}\) and \(u_\text{p}\) 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 on taking 6 Å to be the thickness of the hydration layer. Such a high value of \(d_H\) could be argued as being due to an aggregation of the protein upon thermal unfolding.

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, d₀ 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 d₀ (~10 nm) throughout the temperature range. However, as the temperature goes beyond 60 °C, appreciable increase occurs in the hydrodynamic 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 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. When the temperature is increased, the content of α-helix decreases to reach 38% at 70 °C (Figure 2b). Earlier Mariyama et al. reported 44% α-helix of HSA at 65 °C. Our finding of 46% α-helicity at 40 °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. reported 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. 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 fluorophore. Flora et al. 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 temperature 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

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The anisotropy of Trp214 residue in HSA at 20 °C whereas the 50 ns component is due to the global tumbling motion of the Trp214 residue within its immediate microenvironment, at different temperatures are fitted biexponentially (Table 2) with fluorescence anisotropy profiles (inset of Figure 3) 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 probe, Trp214, resulting in an increased contribution of the longer

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

<table>
<thead>
<tr>
<th>temperature (°C)</th>
<th>τ1 (ns)</th>
<th>τ2 (ns)</th>
<th>τ3 (ns)</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.13 (56%)</td>
<td>2.11 (20%)</td>
<td>6.63 (24%)</td>
<td>2.06 (1.05)</td>
</tr>
<tr>
<td>20°</td>
<td>0.14 (50%)</td>
<td>2.17 (22%)</td>
<td>6.66 (28%)</td>
<td>2.42 (1.09)</td>
</tr>
<tr>
<td>40</td>
<td>0.05 (93%)</td>
<td>1.57 (4%)</td>
<td>4.98 (3%)</td>
<td>0.27 (1.02)</td>
</tr>
<tr>
<td>40°</td>
<td>0.19 (45%)</td>
<td>2.05 (30%)</td>
<td>5.88 (25%)</td>
<td>2.16 (0.99)</td>
</tr>
<tr>
<td>40°</td>
<td>0.15 (52%)</td>
<td>1.92 (24%)</td>
<td>5.68 (24%)</td>
<td>1.90 (0.94)</td>
</tr>
<tr>
<td>55</td>
<td>0.05 (92%)</td>
<td>1.59 (5%)</td>
<td>4.83 (3%)</td>
<td>0.26 (1.07)</td>
</tr>
<tr>
<td>55°</td>
<td>0.16 (48%)</td>
<td>1.56 (29%)</td>
<td>4.62 (23%)</td>
<td>1.59 (1.05)</td>
</tr>
<tr>
<td>55°</td>
<td>0.05 (93%)</td>
<td>1.35 (4%)</td>
<td>3.99 (3%)</td>
<td>0.21 (1.10)</td>
</tr>
<tr>
<td>60</td>
<td>0.20 (49%)</td>
<td>1.56 (32%)</td>
<td>4.33 (19%)</td>
<td>1.42 (1.02)</td>
</tr>
<tr>
<td>60°</td>
<td>0.05 (93%)</td>
<td>1.36 (4%)</td>
<td>3.99 (3%)</td>
<td>0.22 (1.06)</td>
</tr>
<tr>
<td>70</td>
<td>0.08 (81%)</td>
<td>1.31 (12%)</td>
<td>3.74 (7%)</td>
<td>0.47 (1.07)</td>
</tr>
</tbody>
</table>

* Recovered from 55 °C. ° Recovered from 70 °C.

The apparent specific volume (v) and partial specific apparent adiabatic compressibility (χ_v) values calculated from eqs 3 and 4 are presented in Table 3 and are plotted against temperature in Figure 4. The χ_v value obtained at 20 °C and pH 7.0 in the present study is comparable to the value of 6.6 × 10^−14 m² kg⁻¹ Pa⁻¹ reported by El Kadi et al. and Chalikian et al. for BSA. As evidenced from Figure 4 and Table 3, χ_v increases with increasing temperature and reaches a maximum value at 55 °C. Earlier, Chalikian et al. reported a similar 2-fold increase in χ_v upon increasing the temperature from 18 to 55 °C for a similar transport protein, BSA. When the temperature is increased further, χ_v 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. The χ_v variation during the thermal unfolding process is larger than the corresponding v variation, which is in accord with previous reports. The sharp decrease in χ_v 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 χ_v with temperature can be explained in the following manner:


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

<table>
<thead>
<tr>
<th>temperature (°C)</th>
<th>τ0</th>
<th>τ1 (ns)</th>
<th>τ2 (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.32</td>
<td>0.28 (48%)</td>
<td>50.0 (52%)</td>
</tr>
<tr>
<td>20°</td>
<td>0.25</td>
<td>0.35 (43%)</td>
<td>50.0 (57%)</td>
</tr>
<tr>
<td>20°</td>
<td>0.37</td>
<td>0.37 (64%)</td>
<td>50.0 (36%)</td>
</tr>
<tr>
<td>40</td>
<td>0.30</td>
<td>0.36 (42%)</td>
<td>50.0 (58%)</td>
</tr>
<tr>
<td>40°</td>
<td>0.26</td>
<td>0.35 (49%)</td>
<td>50.0 (51%)</td>
</tr>
<tr>
<td>40°</td>
<td>0.37</td>
<td>0.38 (68%)</td>
<td>50.0 (32%)</td>
</tr>
<tr>
<td>55</td>
<td>0.26</td>
<td>0.26 (55%)</td>
<td>50.0 (45%)</td>
</tr>
<tr>
<td>55°</td>
<td>0.25</td>
<td>0.42 (69%)</td>
<td>50.0 (31%)</td>
</tr>
<tr>
<td>60</td>
<td>0.26</td>
<td>0.24 (58%)</td>
<td>50.0 (42%)</td>
</tr>
<tr>
<td>60°</td>
<td>0.32</td>
<td>0.35 (69%)</td>
<td>50.0 (31%)</td>
</tr>
<tr>
<td>70</td>
<td>0.37</td>
<td>0.34 (69%)</td>
<td>50.0 (31%)</td>
</tr>
</tbody>
</table>

* Recovered from 55 °C. ° Recovered from 70 °C.
The contribution \( \varphi \) is the compressibility and \( \beta \) is the solvent-accessible surface area (SASA) of the protein. In general, the change of SASA and consequently decreases \( \varphi \). Also, the imperfect packing of the amino acid residues due to partial unfolding inside the protein core increases \( \beta \). Although this process is associated with a decrease in \( V_M \), the other two processes overwhelm this decrease, and as a consequence \( \varphi \) 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 \( \varphi \). The increase in \( \beta \) due to the unfolding of compact protein might overwhelm the increase in \( V_M \), and as a consequence \( \varphi \) decreases as observed in the present study. When the temperature is decreased from 70 °C, \( \varphi \) acquires a constant value of \( \sim 4 \times 10^{-14} \) 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 \( \varphi \). When the unfolded state is recovered from 55 °C, the \( \varphi \) value traces back its forward path, and the native state is almost recovered.

We observe a decrease in \( \alpha \)-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; thus, the unfolding and consequent decrease in \( \alpha \)-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 \( \alpha \)-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 \( (\varphi_k) \) 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.

![Image](image3.png)

**Figure 4.** Partial specific apparent adiabatic compressibility \( (\varphi_k) \) 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.

For globular proteins, the value of \( \varphi_k \) is a sum of two contributions:

\[
\varphi_k = \varphi_{km} + \varphi_{kh}
\]

where \( \varphi_{km} \) is the contribution due to the imperfect packing of the polypeptide chains within the solvent-inaccessible protein core and is given by

\[
\varphi_{km} = \beta V_M
\]

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

\[
\varphi_{kh} = M^{-1} \sum_i S_{Ai} K_{Si}
\]

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

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 \( \varphi_k \). Also, it exhibits the largest water-inaccessible core volume with low \( \beta \) (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 \( \varphi_k \). 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 \( \varphi_{kh} \). Also, the imperfect

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

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>( \rho_0 ) (kg m(^{-3}))</th>
<th>( u_k ) (m s(^{-1}))</th>
<th>( \varphi_k \times 10^4 ) (m(^2) kg(^{-1}) Pa(^{-1}))</th>
<th>( \varphi_k \times 10^4 ) (m(^2) kg(^{-1}) Pa(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1000.068</td>
<td>1485.0</td>
<td>7.759</td>
<td>5.929</td>
</tr>
<tr>
<td>25</td>
<td>998.850</td>
<td>1499.5</td>
<td>7.781</td>
<td>7.111</td>
</tr>
<tr>
<td>30</td>
<td>997.485</td>
<td>1511.5</td>
<td>7.807</td>
<td>8.960</td>
</tr>
<tr>
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<td>995.860</td>
<td>1522.5</td>
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<td>10.28</td>
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<tr>
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<td>1531.5</td>
<td>7.853</td>
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<tr>
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<td>1545.0</td>
<td>7.840</td>
<td>11.58</td>
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<td>987.491</td>
<td>1540.0</td>
<td>7.708</td>
<td>13.38</td>
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<td>6.576</td>
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</tr>
<tr>
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<td>1555.5</td>
<td>5.715</td>
<td>2.102</td>
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<td>0.855</td>
</tr>
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<td>1559.0</td>
<td>2.781</td>
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<td>80</td>
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<td>969.300</td>
<td>1564.0</td>
<td>0.413</td>
<td>0.072</td>
</tr>
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<td>0.159</td>
<td>0.032</td>
</tr>
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<td>958.160</td>
<td>1569.0</td>
<td>0.059</td>
<td>0.012</td>
</tr>
<tr>
<td>100</td>
<td>950.068</td>
<td>1571.5</td>
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</tbody>
</table>

temperature, \( \phi_k \) 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 \( \alpha \)-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.