Conformational Study of Human Serum Albumin in Pre-denaturation Temperatures by Differential Scanning Calorimetry, Circular Dichroism and UV Spectroscopy

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Thermal conformational changes of human serum albumin (HSA) in phosphate buffer, 10 mM at pH = 7 are investigated using differential scanning calorimetric (DSC), circular dichroism (CD) and UV spectroscopic methods. The results indicate that temperature increment from 25°C to 55°C induces reversible conformational changes in the structure of HSA. Conformational change of HSA are shown to be a three-step process. Interestingly, melting temperature of the last domain is equal to the maximum value of fever in pathological conditions, i.e. 42°C. These conformational alterations are accompanied by a mild alteration of secondary structures. Study of HSA-SDS (sodium dodecyl sulphate) interaction at 45°C and 35°C reveals that SDS affects the HSA structure at least in three steps: the first two steps result in more stabilization and compactness of HSA structure, while the last one induces the unfolding of HSA. Since HSA has a more affinity for SDS at 45°C compared to 35°C, It is suggested that the net negative charge of HSA is decreased in fever, which results in the decrease of HSA-associated cations and plasma osmolarity, and consequently, heat removal via the increase in urine volume.

Keywords: Circular dichroism, Conformational changes, Differential scanning microcalorimetry, HSA, Sodium dodecyl sulphate

Introduction
The mechanism by which proteins fold from a structure-free denatured state to their unique biologically active state is an intricate process. This process is even more complex in multidomain proteins where each domain may be able to refold independently and interdomain interactions may affect the overall folding process (Wetlaufer, 1981; Privalov, 1982; Ptitsyn, 1994). The presence of stable intermediate conformations has helped in understanding of protein folding mechanisms (Redfield et al., 1994; Privalov, 1996). Elucidation of the mechanism of protein denaturation is important for understanding of protein stability (Ptitsyn, 1995). Proteins can be denatured in two ways, namely, chemical and thermal (Santoro and Bolen, 1988). Human serum albumin (HSA) has been used as a model protein for protein folding and ligand-binding studies over many decades (Kracht-Hansen, 1981; Dill et al., 1989). HSA is a single chain protein with 585 amino acids, with a molecular weight of ~67,000 Da. Serum albumin homologs with very similar properties are found in other mammals. The structure of this protein has been determined by X-ray crystallography of high resolution (Sugio et al., 1999); it includes three homologous domains (I-III) that resemble a heart-shaped molecule. Each domain is formed by two subdomains that possess common structural motifs (Tanford, 1968; Carter and Ho, 1994). HSA has one cysteine residue at position 34 (in domain I) with a free sulfhydryl group (Farruggia and Picó, 1999; Sugio et al., 1999).

HSA plays a special role in transporting metabolites and drugs throughout the vascular system and also in maintaining the pH and osmotic pressure of plasma (Ikeguchi et al., 1992;
The basis of two-state model, similar to what is described by Brown and Carter, 1992; Carter and Ho, 1994. Interestingly, HSA structure and dynamics are known to be influenced by a number of factors, like pH, temperature, and binding of different ligands (Brown, 1977).

The thermal denaturation process using differential scanning calorimetric (DSC) method can be described by Eyring and Lumry model (Galisteo et al., 1991):

\[
\text{Native} \xrightarrow{\sim} \text{Unfolded reversibly} \rightarrow \text{Unfolded irreversibly} \\
\text{(scheme 1)}
\]

The first stage includes reversible structural alteration on the basis of two-state model, similar to what is described by Pace (1990).

\[
\text{Native (N)} \xrightarrow{\sim} \text{Denature (D)} \\
\text{(scheme 2)}
\]

while the second stage includes irreversible structural alterations. Irreversible structural changes of HSA occur in temperatures above 74°C (Kragh-Hansen, 1981). Studies from unfolding of acrylodan-labeled HSA probed by steady-state and time-resolved fluorescence methods have shown that increasing temperature to about 50°C is results in irreversible separation of domain I and II. Denaturation by heating to <70°C resulted in irreversible unfolding of domain II, while increasing the temperature to 70°C or higher resulted in irreversible unfolding of domain I (Flora et al., 1998). The denaturation experiments indicate that unfolding of HSA proceeds by the following scheme:

\[
\text{N} \xrightarrow{\sim} \text{E} \rightarrow \text{I} \rightarrow \text{U} \\
\text{(scheme 3)}
\]

where N is the native form of the protein, E is the expanded form, I is an intermediate in which domain II is unfolded but domain I is intact, and finally, U is the unfolded protein. This model is consistent with the previous reports that suggest there is a multi-step unfolding pathway for HSA (Wetzel et al., 1980; Picó, 1997). Scheme 3 shows that unfolding of domain I occurs only when the unfolding of domain II is complete. The U state is not a fully unfolded random coil, but is rather a partially unfolded state (Shaklai et al., 1984). This is likely the result of unfolding of the pocket containing the free -SH group of Cys34, which enables disulfide bonds to be formed within various domains, and/or the presence of some intramolecular cooperation in the thermal denaturation that act to maintain some degree of structure (Flora et al., 1998).

Studies of unfolding and refolding behavior of HSA using circular dichroism (CD) measurements have indicated that the α-helical content of the protein decreases upon thermal denaturation to 75°C and not fully recover upon subsequent cooling. In addition, temperature increase of to 75°C or above promotes HSA aggregation (Wallevick, 1973).

In the present work, using CD, DSC, and UV spectroscopic methods, we studied the thermal behavior of HSA in a temperature range which corresponds to the reversible denaturation of HSA.

### Materials and Methods

**Materials.** Human serum albumin was purchased from Sigma and used without defatting. Sodium dodecyl sulphate was also obtained from Sigma. The other substances were of reagent grade and were purchased from Merck. Phosphate buffer (10 mM, pH = 7.0) was used throughout the study.

**Methods.** DSC experiment was carried out on a Scal-1 microcalorimeter (Russia); the heating rate was fixed at 1 K/min. An extra pressure of 1.5 atm was maintained during all DSC runs to prevent possible degassing of the solutions on heating. The baseline preparation was done by buffer in both sample and reference cells. The concentration of the protein solution was 1 mg/ml for DSC experiments.

The deconvolution analyses and the fittings were done based on the theory of Privalkov and Potekhin (1986), which was integrated in Scal-2 software package (supplied by Scal, Russia). This package enables the user to determine the native and denatured lines based on excess C_p (denoted by C_p<sup>excess</sup> or C_p^C). Then, the deconvolution of C_p<sup>excess</sup> to the corresponding sub-peaks can be done so as to minimize the fitting error. Since a C_p<sup>excess</sup> curve may be deconvoluted in different ways, a convincing deconvolution is a one with the minimum number of sub-peaks, which has also a reasonably low fitting error (typically <1%). In this work, the fitting error was as small as 0.38%.

CD spectra were recorded by a Jasco J-715 spectropolarimeter (Japan). Results are expressed as ellipticity, [θ] (degree cm<sup>2</sup> dmol<sup>-1</sup>), based on a mean amino acid residue weight (MRW). This value was assumed to be 113 Da for HSA. The molar ellipticity was determined as

\[
[\theta] = \left( 0 \times 100 \right) \text{MRW/cm}^2\text{dmol}^{-1}
\]

where c is the protein concentration in mM, l is the length of light path in cm and θ is the measured ellipticity in degree at a given wavelength. The data were smoothed using the Jasco J-715 software, which includes a fast Fourier-transform noise reduction routine. All experiments were repeated three times. The concentration of the protein solution was 0.5 mg/ml for CD experiments. Percentage of secondary structures was calculated with the method of Chen et al. (1972). The α-helix content (f<sub>α</sub>) was estimated from the ellipticity value at 222 nm (10 θ<sub>222</sub>):

\[
[\theta]_{222} = \left( 0 \times 100 \right) \text{MRW/cm}^2\text{dmol}^{-1}
\]

Spectrophotometric experiments were performed using a UV 3100 recording spectrophotometer (Shimadzu, Japan). The sample cell contained 0.8 ml of serum albumin solution at a concentration of 0.3 mg/ml with fixed temperature (25-60°C) and different fixed concentration of sodium dodecyl sulphate (SDS) ranging from 0.00 to 3.0 mM. The reference cell contained buffer and similar concentration of SDS as used in the sample cell, and the absorption of sample cell was recorded at the range of 230 nm to 330 nm. The absorbance values at 280 nm for each temperature were illustrated as a function of SDS concentration.
Results and Discussion

Figure 1 represents DSC thermogram of HSA in the range of 15-110°C. DSC profile includes two transitions, A and B as depicted in Fig. 1; transition A corresponds to the temperatures below 65°C and transition B occurs at temperatures above 70°C. Transition A is a minor transition, while transition B is a major one. The second transition corresponds to the thermal unfolding of HSA via an irreversible process (Saboury et al., 2003), while the first transition is due to the reversible conformational changes that occur before unfolding (Picó, 1997). When the unfolding process is reversible, one can deconvolute its $\Delta C_p$ curve to its sub-transition constituents to determine its energetic domains.

Deconvolution of $\Delta C_p$ curve for the first transition is represented in Fig. 2. It was found that, with a <1% fitting error, it is not possible to deconvolute this $\Delta C_p$ curve with the assumption of only two sub-transitions. Thus, this transition was deconvoluted with the assumption of three sub-transitions (with a fitting error of 0.8%). These three sub-transitions (i, ii and iii), might be related to the links between the three structural domains of HSA (Sugio et al., 1999). The thermodynamic parameters of each sub-transition, such as enthalpy changes and melting temperature values ($T_m$) are listed in Table 1. The three sub-transitions of HSA as depicted in Fig. 2 and Table 1, are sequential and separated by about 9°C considering the $T_m$ values. Fascinatingly, $T_m$ value of the last sub-transition is 42°C, which is equal to the maximum degree of fever in body in pathological conditions. The amount of total enthalpy change of the first transition is equal to 841 kJ/mol. This transition indicates a relatively mild conformational change in HSA structure, since it results in a reversible denaturation without leading the protein’s hydrophobic pockets to be exposed, and thus, leading HSA to aggregation.

Here, we observed a $\Delta C_p$ curve with three sub-transitions. However, other groups have reported two sub-transitions for the reversible denaturation of HSA (Farruggia et al., 2001). This difference is probably resulted from the fact that Farruggia et al. (2001) used defatted HSA in their study. Such a difference in the DSC thermograms has been reported in case of bovine serum albumin (BSA): $\Delta C_p$ curve of defatted BSA has two constituents, while undefatted serum albumin is best deconvoluted to three sub-peaks (Michnik, 2003). This phenomenon is presumably a result of asymmetrical distribution of fatty acid binding sites on HSA, (Curry et al., 1998; 1999; Bhattacharya et al., 2000) which results in clearly distinct thermodynamic properties of different structural domains.

It is mentioned that HSA domain separation occurs at temperatures below 50°C. It is reasonable to assume that this domain separation induces some conformational changes in HSA domains structures. Figure 3 represents CD spectra of HSA in the range of 25-55°C. Note that the spectra corresponding to 25°C and 40°C are fairly similar. In addition, the spectra in 45°C and 55°C are very close to each other.
However, CD spectrum at 40°C shows a relatively considerable difference compared to the spectrum at 45°C. Figure 3 indicates that the temperature change from 40°C to 45°C is a critical event in the course of HSA structural changes. Interestingly, $T_m$ of sub-transition iii (42°C, see Table 1), which is obtained from the DSC study, is in the middle of this range. The ratios of HSA $\alpha$-helix structure at 40°C and 45°C are calculated and illustrated in Table 2; these findings indicate that HSA includes more content of $\alpha$-helix secondary structure (about 3%) in 40°C relative to 45°C. The conformational changes of HSA due to the rise of temperature from 40°C to 45°C is only accompanied by a minor conversion of $\alpha$-helix to other structures; therefore, HSA has a relatively stable conformation in the range of 45°C-55°C, as depicted in Fig. 3.

Figure 4 shows CD spectra in temperatures above 55°C; note that all spectra show reduction in the CD parameter at 222 nm (i.e. $[\theta]_{222}$). The value of CD parameter at 222 nm is proportional to the $\alpha$-helix secondary structure; therefore, decrease in this value is an indication of the reduction of the $\alpha$-helix content of the protein. For better resolution, the amount of CD parameter at 222 nm is plotted as a function of temperature and the plot is compared to a smoothed curve (Fig. 5). Clearly, denaturation occurs in temperatures above 60°C. Compared to the smoothed curve, there are considerable fluctuations in the CD parameter at 222 nm in the pre-denaturation temperatures; at 40°C, the proportion of helix structure is greater than the hypothetical smoothed curve, while a reduction in the helix structure occurs as temperature rises to 45°C. From these observations, it can be concluded that HSA conformation in temperatures above 40°C partially loses its $\alpha$-helix structure and thus, HSA is subjected to conformational changes compared to its native physiological structure.

Sodium dodecyl sulphate (SDS) is an anionic surfactant with a hydrophobic tail and a negatively charged head; the dual properties of this surfactant have made it a common element of many denaturation studies (Moosavi-Movahedi, 2005). Interaction of serum albumin with SDS has been studied by a variety of techniques (Yamazaki et al., 1992; Giancola et al., 1997; Gelamo and Tabak, 2000; Nielsen et al., 2000; Kragh-Hansen et al., 2001). These studies have revealed that this interaction is a complex process: in low concentrations, SDS induces conformational changes in HSA via electrostatic interactions, with a net stabilizing effect on the structure; in contrast, in the higher concentrations SDS leads to serum albumin denaturation via hydrophobic effects (Giancola et al., 1997). In the present work, SDS as a potential probe and denaturant is used for phase resolution in the course of HSA conformational changes.

### Table 2. Percentages of HSA $\alpha$-helix structures at 35°C and 45°C

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>$\alpha$-helix, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>67</td>
</tr>
<tr>
<td>45</td>
<td>64</td>
</tr>
</tbody>
</table>

Fig. 3. Far-UV CD spectra of HSA in 25-55°C.

Fig. 4. Far-UV CD spectra of HSA at temperatures above 55°C.

Fig. 5. CD parameter of HSA at 222 nm as a function of temperature (---). The smoothed curve (----) is added to the plot to facilitate the inspection of fluctuations.
Figure 6 shows absorbance changes of HSA at 280 nm as a function of SDS concentration at 35°C (---), and 45°C (----).

Figure 6. Absorbance changes at 280 nm for HSA, including steps I, II and III, as a function of SDS concentration at 35°C (---), and 45°C (----).

For better understanding this phenomenon, the sigmoidal transitions I, II and III (for 35°C and 45°C temperatures) were analyzed by the “two-state model” of Pace (1990). The determination of free energy ($\Delta G^0$) as a criterion of conformational stability of a globular “module” (e.g. protein, domain, etc), is based on the assumption that the native module (N) can be directly converted to the denatured state (D) via a reversible process (see scheme 2). Assuming a two-state mechanism for each transition, one can analyze the process by monitoring the changes in the absorbance, and calculate the denatured fraction of protein ($F_d$) as well as the equilibrium constant ($K$) of the process.

$$F_d = \frac{(Y_N - Y_{obs})(Y_N - Y_D)}{Y_{obs} - (Y_N - Y_{obs})}$$

$$K = \frac{F_d}{1 - F_d} = \frac{(Y_N - Y_{obs})(Y_{obs} - Y_D)}{Y_{obs} - (Y_N - Y_{obs})}$$

where $Y_{obs}$ is the observed parameter $Y$ (which is absorbance here); $Y_N$ and $Y_D$ are the values of the $Y$ characteristic of native and fully denatured conformations, respectively. Change in the free energy ($\Delta G^0$) can be calculated as:

$$\Delta G^0 = -RT \ln K$$

Note that transition III is roughly similar in the two temperatures, while the other transitions are totally different.

Fig. 7. $\Delta G^0$ curve as a function of [SDS] for HSA transitions I at 35°C (---), and 45°C (----).

Fig. 8. $\Delta G^0$ curve as a function of [SDS] for HSA transitions II at 35°C (---), and 45°C (----).
calculated that at 35°C, as represented in Fig. 8 and Table 3. It can be estimated that the value of $\Delta G^0$ at zero concentration of denaturant ([SDS] = 0) is called $\Delta G^0_{120}$, and can be interpreted as the $\Delta G^0$ of native-to-denature transformation in the absence of denaturant. This parameter is routinely used as a measure of protein stability (Moosavi-Movahedi et al., 1994; Moosavi-Movahedi et al., 1997; Rezaei-Tavirani et al., 2002; Bougie et al., 2004). One can estimate the value of $\Delta G^0_{120}$ by extrapolating $\Delta G^0$ vs. [SDS] line to zero concentration of SDS. Figures 7–9 show the values of $\Delta G^0$ as a function of [SDS]. For all transitions, $\Delta G^0_{120}$ and [SDS]$_{1/2}$ values are listed in Table 3. [SDS]$_{1/2}$ is the concentration of SDS that causes 50% phase transition, i.e. where $F_d = 1/2$, and thus, $K = 1$ and $\Delta G^0 = 0$.

From Fig. 7 and Table 3, it can be read that at 45°C, module I is 3.1 kJ/mol more stable than this module at 35°C. At 45°C, the second module is less stable than its 35°C counterpart by 6.0 kJ/mol, as represented in Fig. 8 and Table 3. It can be calculated that at 35°C the first two modules together are more stable than these modules at 45°C by ~3 kJ/mol (6.0-3.1 = 2.9 kJ/mol). The values of [SDS]$_{1/2}$ in both modules I and II at 45°C are lower than these values at 35°C, indicating that HSA shows a higher tendency for SDS at 45°C. The third modules at 45°C and 35°C are practically the same (see Fig. 9 and Table 3).

Overall, it can be deduced that at 45°C HSA structure becomes unstable to some extent. Increase in the SDS affinity of HSA at 45°C relative to 35°C suggests that the net negative charge density of HSA surface is somehow suppressed during fever. This might be the result of conformational changes that sequester negatively charged residues and/or expose positively charged residues. It is known that HSA provides 80% of the osmotic pressure of plasma (Carter and Ho, 1994) from which ~30% is due to the presence of HSA itself and ~50% is due to the cations (like Na$^+$ and K$^+$) associated with the HSA negative surface. Therefore, temperature increase reduces the concentration of the cations around HSA, which can lead to the reduction of blood osmotic pressure. Reduction of blood osmotic pressure under fever conditions can lead to the increment of urine volume, which is a usual condition in fever. The important biological property of water is its high heat capacity, and therefore, excessive exertion of urine accompanies with the removal of heat from body.

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**References**


![Fig. 9. $\Delta G^0$ curve as a function of [SDS] for HSA transitions III at 35°C (––) and 45°C (-----).](image)

**Table 3.** The values of $\Delta G^0_{120}$ and [SDS]$_{1/2}$ for transitions I-III of HSA at 35°C and 45°C.

<table>
<thead>
<tr>
<th>Transition</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G^0_{120}$ kJ/mol</td>
<td>35°C</td>
<td>19.7</td>
<td>33.0</td>
</tr>
<tr>
<td></td>
<td>45°C</td>
<td>22.8</td>
<td>28.0</td>
</tr>
<tr>
<td>[SDS]$_{1/2}$ mM</td>
<td>35°C</td>
<td>0.48</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>45°C</td>
<td>0.30</td>
<td>0.81</td>
</tr>
</tbody>
</table>


