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A spectroscopic approach of pH effect on thermal denaturation of human and bovine serum albumins

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Thermal conformational changes of human serum albumin (HSA) and bovine serum albumin (BSA) in different buffer solutions, in the pH range 3.0 - 9.0, are investigated by fluorescence spectroscopy. Temperature increasing in the range 25 - 90 °C induces partially reversible changes in the 3D structure of the two serum albumins. At pH = 3.0 (albumin form E) and pH = 8.0 (B form) the renaturation degree is greater than that manifested at pH = 4.0 (F form) and pH = 7.0 (N form). Instead, at pH = 5.0 and pH = 6.0, both serum albumins present states, whose thermal denatured conformations cannot be renatured by a stepwise temperature decrease.

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1. Introduction

Albumins are the most abundant serum proteins, both in human (human serum albumin, HSA) and bovine (bovine serum albumin, BSA) organisms. These multifunctional proteins, synthesized in the liver, are secreted in the circulatory system [1; 2] and are present in all tissues and body fluids. There are many functions of albumins in both organisms: from the accomplishment of important antioxidant activities [3], to the binding and the transport of metal ions [2], hormones [4], fatty acids [5; 6], drugs [7; 8], and to the balance of the osmotic pressure and blood pH [9].

Details of conformation change of HSA and BSA, at molecular level, under the influence of various physical and chemical agents, are important keys for all processes in which these serum proteins are involved. It is known that albumins undergo several stabile conformations, at different pH, from the extreme acidic to the extreme basic values. Conformational transitions of both HSA and BSA, with the change of pH, are essential for the physiological roles of these proteins *in vivo*. In the case of HSA, at pH = 2.0, far-UV CD spectra showed largely keeping of the secondary structure, but near-UV CD spectra showed a great loss of the tertiary structure [10]. At pH below 3.0 and pH around 4.0, HSA presents two isomeric forms, the *E* form and the *F* form, respectively [11]. Spectroscopic studies on BSA, at basic pH values, show a *molten globule*-like state, around pH = 11.2. At pH = 13.0, BSA seems to acquire an unfolded conformational state, but it can regain nearly 40 % of its native secondary structure, by reversibility study, the tertiary structure being irreversibly damaged [12]. Another study [13] has investigated albumin conformational changes at pH around 7.0 (the normal form, *N*), at pH around 8.0 (the basic form, *B*) and at pH near 10.0 (the aged form, *A*). One can say that pH is a parameter that has a critical influence on both HSA and BSA conformations.

The sensitivity of the Trp emission to the polarity and viscosity of environment is an important tool property permitting the studies of protein structure and dynamics [14; 15]. In the present work, the fluorescence technique is used to obtain information on thermal denaturation, under acidic and basic pH of both HSA and BSA structures. The fluorescence spectroscopy has been chosen for its advantages: high sensitivity, versatility, and fast data acquisition.

2. Materials and methods

Proteins. Bovine and human serum albumins (purity over 98 %) were purchased form SIGMA. Protein concentrations were measured using absorption spectroscopy, on the basis of standard molar absorption coefficients (ε_{280} = 44,000 M^{-1} cm^{-1} for BSA and ε_{280} = 36,000 M^{-1} cm^{-1} for HSA). The absorption spectra were recorded by a *Perkin Elmer Lambda 2S* spectrophotometer in the ultraviolet (UV) range.

Buffer solutions. Proteins were dissolved in two aqueous buffers: HEPES (50 mM) and Tris-HCl (50 mM), at different pH values, between 3.0 and 8.0, using bi-distilled water. pH of the buffers and protein solutions was adjusted using a *inoLab* 720 pH-meter.

Fluorescence. Fluorescence emission spectra were recorded for each pH, for different temperatures, by a *Perkin Elmer MS 55* spectrofluorometer, in the spectral range 300 - 450 nm, with 500 nm/min speed. Measurements were done in 10 mm × 10 mm quartz cells. The excitation wavelength, for each albumin, was 290 nm and the emission was recorded between 300 and 450 nm.

Protein concentrations were kept constant at 3.5 μM, for HSA and 8.0 μM, for BSA. The thermal denaturation was monitored in the range, 25 - 90 °C.

3. Results and discussions

Structural information on albumins can be obtained by studying selected properties of these molecules, involved in interaction with the environment, one of the most studied being the pH influence [16]. Conformational changes in HSA and BSA could be studied by different spectroscopic methods, but fluorescence emission spectroscopy was chosen, because both proteins contain tryptophan residues (Trp-214 in HSA; Trp-213 and Trp-134 in BSA), which are responsible for their fluorescence, when excited at 290 nm (where tyrosine fluorescence is negligible) [14]. Trp fluorescence allows us to monitor the protein molecule exposure to the solvent.

3.1. Thermal denaturation of BSA

Understanding of bovine serum albumin folding and unfolding is based on information provided by various methods, NMR studies for BSA in solution [17; 18], optical rotary dispersion [19], viscosity [20; 21], Raman spectroscopy [22] and circular dichroism [23 - 25].

Thermal unfolding of proteins is an important process that can affect, partially or totally, the protein functions. Because BSA shows an intrinsic fluorescence, due to its Trp, the emission spectra were recorded by excitation at 290 nm.

In the case of BSA, different forms of the protein appear, when pH is changing. We investigated the unfolding and refolding processes of BSA, as a function of temperature, at pHs between 3.0 and 8.0 (Fig. 1). It is noticed that pH values affect the denaturation in a significant way. At pH = 3.0, 4.0, 7.0 and 8.0 BSA seems to unfold in one step process, while at pH 5.0 the protein unfolding is a two steps process, with an inflection point around 65 °C. Thermal unfolding is more rapid at acidic pH (3.0) than at basic pH (8.0). The renaturation process of BSA (Fig. 1) follows the unfolding process at pH 3.0 (*E* form), pH 4.0 (*F* form), 7.0 (form N) and 8.0 (*B* form). At pH 3.0 the renaturation process is almost complete. The renaturation of BSA does not take place at pH 5.0 and 6.0, putting in evidence the *molten globule* states, in which the protein does not recover any of its space structures, when the temperature is decreased at the initial value of 25 °C.

The effect of pH on the fluorescence properties of BSA structure was analyzed using the 25 ^{0}C data from Table 1. As one can see, Fig. 2 shows BSA emission maximum intensities and the corresponding wavelengths, in the denaturation and renaturation processes, as a function of pH, recorded at 25 °C, when BSA (8 μM) was dissolved in 50 mM HEPES buffer. The change in emission maximum intensity, as a function of pH, is presented in Fig. 2A. One can see on this figure, that at 25 °C, the denaturation and renaturation intensities vary with pH, the denaturation being a more reversible process at pH 3.0 and 7.0.

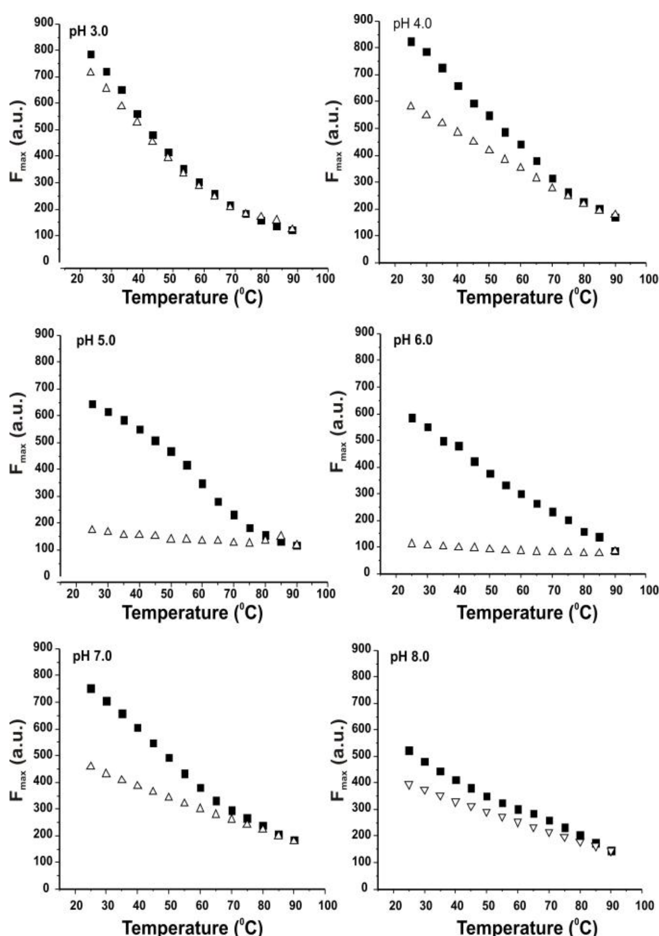

Fig. 1. Thermal induced unfolding (■) and refolding (Δ) of BSA, monitored by fluorescence emission (λex = 290 nm). Protein (8 μM) was dissolved in 50 mM HEPES, at pH between 3.0 and 8.0.

The changes in intensity (Fig. 2A), with the pH variation, occur in parallel with the change in the emission maximum wavelength (Fig. 2B). The Fig. 2B shows a wavelength red shift with pH increase. This tendency could be explained by different degrees of Trp exposure to the hydrophilic environment. A similar result was obtained for human serum albumin denaturation, induced by urea [26]. The fluorescence data indicate that BSA shows a change in its space structure (Fig. 2B) in the pH range, 3.0 - 8.0. A recent study [12] reveals that BSA does not show an essential change in its tertiary structure in the pH range, 7.0 - 9.0, but shows a relevant decrease in tertiary constrains in the pH range, 9.0 - 11.2, without any significant change in the secondary structure. This may be due to the *molten globule* state that could appear in globular proteins, under certain milieu conditions.

Table 1. Emission maximum intensities and the emission maximum wavelengths of BSA, in the denaturation and renaturation processes, as a pH function, at 25 ºC, in 50 mM HEPES (data collected from Fig. 1).

pH	F_{max}^{denat}	F_{max}^{renat}	λ_{max}^{denat}	λ_{max}^{renat}
3.0	786.7	715.9	336.0	336.5
4.0	825.3	580.3	344.5	340.0
5.0	645.9	173.6	348.5	340.0
6.0	585.6	110.6	347.5	339.5
7.0	752.9	456.9	347.5	340.5
8.0	523.6	394.6	344.0	339.0

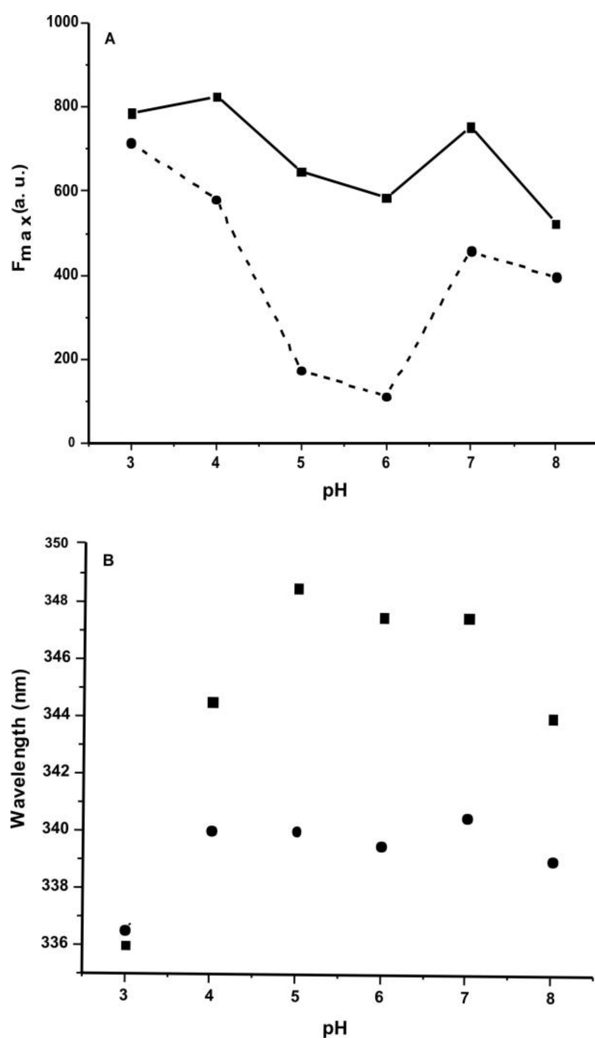

Fig. 2. Emission maximum intensity (A) and emission maximum wavelength (B) of BSA, as a pH function, in the denaturation (squares) and renaturation (circles) processes (λ_{ex} = 290 nm). BSA (8 μM) was dissolved in 50 mM HEPES, at 25 ^{0}C.

The denaturation of BSA was also monitored in 50 mM Tris-HCl buffer, in the same experimental conditions. The pattern of the unfolding process is the same as in 50 mM HEPES (as it is showed for pH 3.0 in Fig. 3), but the intensities are smaller when the protein is dissolved in Tris-HCl buffer. This could be an indication that, in Tris-HCl, the Trp residues exhibit a smaller exposureto the environment than in HEPES buffer.

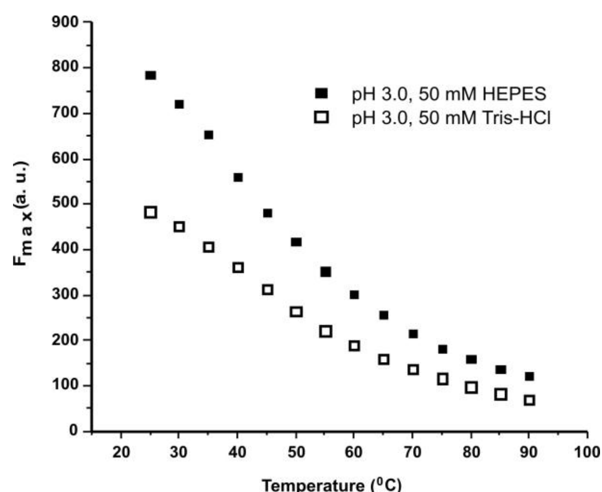

Fig.3. Buffer influence on thermal induced unfolding of BSA, monitored by fluorescence emission (λ_{ex} = 290 nm). Protein (8 μM) was dissolved in two buffers: 50 mM HEPES (■) and 50 mM Tris-HCl (□), respectively, at pH = 3.0.

This information can be useful when interactions of BSA with ligands are investigated by emission fluorescence because the buffers used for proteins and their complexes with ligands could have profound effects on the protein tertiary structures [27].

3.2. Thermal denaturation of HSA

Human serum albumin denaturation is also an interesting process. Many studies put in evidence this process, triggered by different denaturation agents: urea, guanidine chloride, temperature, and pH [26]. The albumins bind reversibly different molecules and drugs. Therefore the stability of albumin structures is an important factor that may affect the binding parameters or the pharmacokinetics/pharmacological effects of these molecules and drugs. Our aim was to better understand the spatial structure transformations occurring when HSA unfolds, with temperature, and the behaviour of the protein structure when the refolding process is taken place.

Emission fluorescence was used to characterize the unfolding and refolding of HSA and the behaviour of this protein to several denaturation (•) and renaturation (-----) cycles (Fig. 4). Like BSA, HSA, dissolved in 50 mm HEPES, changes its spatial conformation, when pH is increased from acid to basic values (data not shown). The microenvironment exposure of the Trp-214, as a function of temperature, was also monitored by fluorescence spectroscopy. We found that HSA has the same fluorescent behaviour like BSA, when the protein is in the spatial forms *E*, *F*, *N* and *B*.

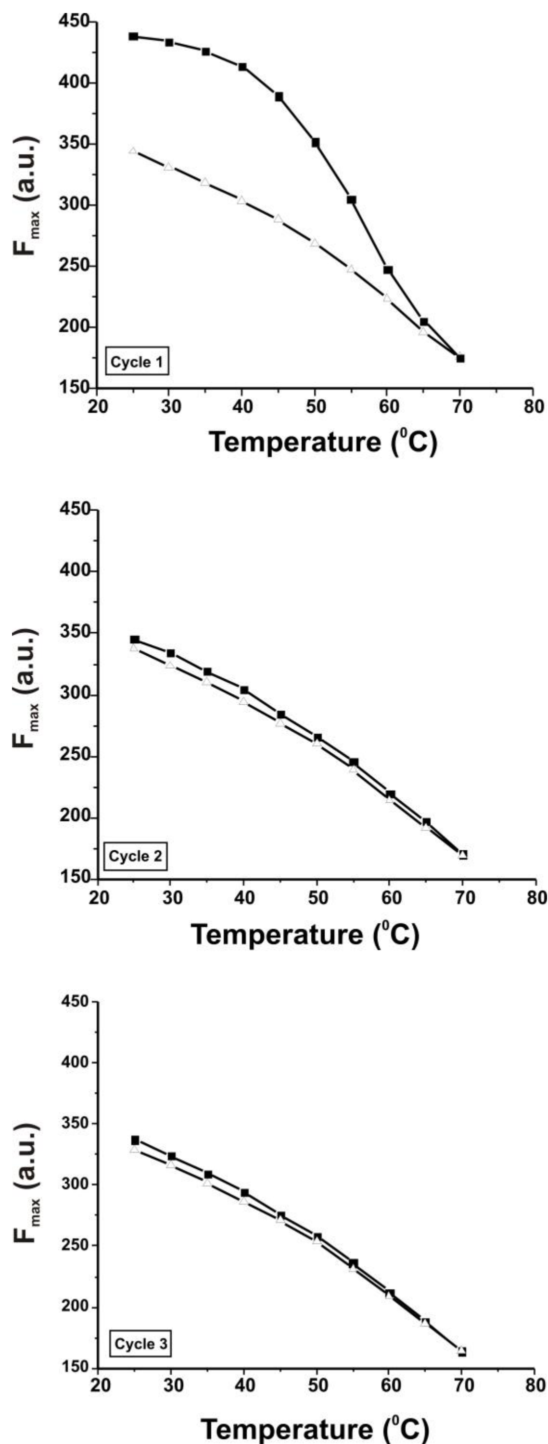

Fig.4. Thermal denaturation (■) and renaturation (Δ) cycles for of HSA, monitored by fluorescence emission (λ_{ex} = 290 nm). HSA (3.5 μM) was dissolved in 50 mM HEPES, at pH = 3.5.v

In order to evaluate the degree of serum albumin "resistance" to the unfolding - refolding cycles, we performed an experiment in which HSA behaviour was subjected to three thermal denaturation - renaturation cycles. In the first cycle, the temperature increase leads to HSA denaturation, Trp-214 being more and more hidden to the buffer. It is noticed that under temperature influence, the HSA denaturation is not totally reversible, a part of the spatial structure not being recovered (cycle 1). Further, in the second and the third cycles, the curves of the two processes are superimposed. This is an indication that once HSA is denatured, it recovers only partially its initial space structure, but any further cycles have the same thermal evolution.

4. Conclusions

In this paper, fluorescence spectroscopy was used to study the pH and temperature-dependent folding of HSA and BSA. pH is an important parameter that influences serum albumin spatial forms. At strong acidic pH > 3.0 and around physiological pH, serum proteins undergo partially reversible conformational changes when temperature is modified. The fluorescence allows analyzing the spatial structures of human and bovine serum albumins. The results on the unfolding of human and bovine serum albumins are important for understanding some aspects of albumin behaviour, being thus useful for further proteomic analysis.

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