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Spectral demonstration of structural transitions in albumins

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ABSTRACT

Several spectral methods (UV absorbance, fluorescent and FTIR spectroscopy) were applied to reveal the alterations in the secondary and tertiary structures of human serum albumin (HSA) in water solutions under various conditions (pH, protein concentration, ethanol and n-propanol addition). The structural transitions well known for defatted HSA at $1.5 < pH < 13$ are observed for HSA in a complex with long-chain fatty acids in the molar ratio about 1:1. The changes of HSA ζ-potential during the protein isomerizations induced by the variation of pH are traced. It is shown that HSA does not completely lose its secondary structure at extreme alkaline or acidic solutions, which indicates that at these conditions HSA takes the "molten globule" conformation. A comparison of aggregation processes of HSA and ovalbumin in neutral water and 0.15 M NaCl solutions reveals that OVA aggregation is preceded by a partially denaturated state of the protein, whereas an intermediates of HSA aggregates are close to the native state of the protein. The aliphatic alcohols disturb the tertiary structure of HSA, but stabilize its secondary structure. This effect increases with the rise of the alcohol hydrophobicity.

1. Introduction

Albumins are a family of single-chain globular water-soluble proteins. They are commonly used as model proteins in a laboratory practice for their wide accessibility. Albumins remain an important objects of research in spite of their long-term study. Serum albumin is the most abundant soluble protein in the body of all vertebrates. It is the main transporter of multiple low-molecular ligands, both hydrophilic and hydrophobic, and a regulator of colloid osmotic pressure in blood [1]. The investigations of serum albumins interactions with biologically active agents and drugs are of great importance for medicine and pharmacy [2,3]. Ovalbumin (OVA) is the major protein in egg-white. OVA belongs to the serpin superfamily but it is not an inhibitor of serine proteinases. Despite the fact that it was one of the first proteins to be isolated in a pure form, its functions (except the bank of nutrients) are still unknown [4,5].

Human serum albumin (HSA) consists of a nonglycosylated chain of 585 amino acids (with a single Trp and 18 Tyr among them), with the molecular mass of 66,5 kDa. It is tightened by 17 intrachain disulfide bonds and has a heart-like shape [1,2]. In the HSA structure three domains are distinguished. The conformation of the bovine serum albumin (BSA) is thought to be similar to HSA. 67% α helix, no β sheet, 10% β turn and 23% extended chain were found by the X-ray crystallography for HSA, and close values for BSA [1]. At the same time the secondary structure data revealed from circular dichroism and IR spectroscopy studies sometimes differ from the above-mentioned volumes, for example: 52% α helix and 7% β sheet [6], 52% α helix and 40% β sheet [7], 52% α helix and 14% β sheet [8], 65% α helix and 7% β sheet [9]. Such a discrepancy can be explained in some extent by the different experimental conditions, such as the protein concentration and the sample purification, in particular, from fatty acids. In an organism the serum albumin is the main carrier of unesterified fatty acids. The long-chain fatty acids (LCFAs) – oleic (C18:1), palmitic (C16:0), linoleic (C18:2), stearic (C18:0), arachidonic (C20:2) and palmitoleic (C16:1) – are important intermediates in lipid metabolism, but they can not circulate in blood in a free state because of their poor solubility. Almost all of the total amount of LCFAs in the plasma (over 99.9%) exists in a complexes with the serum albumin [1]. In HSA there are seven fatty acid binding sites with different affinities [1,2]. Under the normal physiological conditions one HSA molecule carries about 1–2 molecules of LCFAs. Fatty acids are strongly bound by serum albumin (the binding constants are $K \sim 10^7$–10^8 M^{-1} for various binding sites [1]), they immerse in its structure and augment its

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stability [1,10,11]. In the present study HSA is used in the native form, as it is contained in blood in a complex with LCFAs in the molar ratio $\frac{[LCFA]}{[HSA]} \cong 1$.

The amino acid sequence of hen egg-white ovalbumin consists of 386 amino acids (including 3 Trp and 10 Tyr), the molecular mass is 45 kDa. A single carbohydrate side chain is covalently linked to the amide nitrogen of Asn293 [5,12]. There are six cysteines in OVA polypeptide chain and only one disulfide bond. During the egg storage the native OVA gradually converts to the so-called "S-ovalbumin" – more stable, compact and hydrophobic form owing to the formation of one additional S–S bond. *S*-Ovalbumin also forms in vitro by a 20 h incubation of OVA at 55 °C in 100 m*M* sodium phosphate, pH 10. The biological meaning of this transformation is still unknown. The fraction of the native ovalbumin may range from 20 to 80% in commercial preparations, and from 20 to 40% in fresh preparations from old eggs [4,5,12,13]. The secondary structure of native ovalbumin derived from the X-ray crystallography includes 30,6% α helix, 28,5% β sheet, 16,6% β turn [14], conformational change into *S*-ovalbumin leads to a 2–5% loss of a-helix content and a concomitant increase in anti-parallel b-sheet [4]. Similar values were obtained by ATR FTIR spectroscopy for the untreated hen egg white [15].

The aim of the present study is to bring together the data obtained by different methods, mainly spectral, to reveal the changes in the protein secondary and tertiary structures under different conditions: variations of pH and protein concentration, in the presence of NaCl and alcohol, and so to demonstrate the complementarity of these methods. Such an investigation can help us to recognize different non-native states of proteins, which may be of a biological relevance, for example, be the intermediates of the protein folding pathway, or give rise to the intermolecular aggregation. Many facts about conformational transitions of HSA and BSA at different pH and in the presence of non-electrolytes are well-established (see, for example [1,9,16–20]. But the most part of these researches deal with the so-called defatted serum albumin, purified from LCFAs. In the present study HSA is used in the native form, as it is in blood in a complex with LCFAs in molar ratio $\frac{[LCFA]}{[HSA]} \cong 1$. We will make an attempt to trace the conformational changes of this form of HSA and, if possible, to compare them with the known results for defatted albumins.

2. Materials and methods

A commercial preparation of crystalline OVA was purchased from Reanal (Hungary). HSA was used in the form of 10% solution for infusion (Microgen, Russia) without purification. A stock water solution of HSA was prepared by thrice-repeated dialysis. All solutions were prepared using the double distilled water. NaCl, NaOH, HCl, ethanol and n-propanol were reagent-grade. The protein concentration in the solution was determined spectrophotometrically (see below). The preparation of the solutions with the specified concentrations were carried out via a direct mixing of equal volumes of a protein solution and a corresponding solvent, both with double concentration. The solutions of different pH were prepared by the titration of a protein solution with 0.1 M or 0.01 M HCl or NaOH solutions. For the IR experiments all prepared solutions (except alcohol) were brought to the protein concentration $C_{pr} = 6 \cdot 10^{-4}$ M in the Eppendorf Concentrator (Germany) at 25 °C. The water-alcohol protein solutions for the IR experiments with $C_{pr} = 6 \cdot 10^{-4}$ M were prepared via the direct mixing of water protein solution with $C_{pr} = 8 \cdot 10^{-4}$ M and alcohol solution with $C_{alc} = 3 \cdot C_{alc0}$, where $C_{alc0} = 4$ vol% is the requiring alcohol concentration.

UV absorbance spectroscopy and the Beer–Lambert law testing. The UV absorbance spectra were obtained on SF-56 (Russia) in rectangular quartz cells of 0.1 cm or 1 cm optical path at the room temperature. The absorbance of a corresponding solvent was subtracted from the protein spectrum. HSA solutions reveal no significant optical density at 340 nm (out of the absorption band), in contrast to the OVA solutions (see Fig. 1), so we used the correction on dispersion (turbidity) for the OVA spectra [21].

For the Beer–Lambert law testing the UV absorbance spectra of the series of protein solutions of different concentrations (diluted gradually from the stock solution) at pH = 6.0 ± 0.2 were measured at λ = 230–350 nm in rectangular quartz cells of 0.1 cm or 1 cm optical path at the room temperature. The solution of minimal protein concentration was used for the determination of concentration taking the following values for molar extinction coefficients: 32000 $M^{-1}cm^{-1}$ for OVA and 34400 $M^{-1}cm^{-1}$ for HSA [22].

Heat denaturation. The melting curves of HSA samples were obtained by measuring the dependence of the optical density of a protein solution at λ = 222 nm on the temperature ($D_{222}(T)$). The experiment was carried out on Specord 210 Plus (Analytik Jena, Germany) with the Peltier equipment in a 0.5° step mode, the velocity of heating was 1° per minute. The temperature in the cell was within the range of 30–97 °C, $C_{HSA} = 1 \cdot 10^{-6}$ M. The data were collected with the help of the software WinASPECT (Analytik Jena, Germany) supplied with the instrument, a further data processing was carried out via OriginPro. The melting curves $D_{222}(T)$ were smoothed by the Savitzky-Golay method. The normalized melting curves $f(T)$ for HSA solutions were obtained using the following equation [23]:

$$f(T) = \frac{D_{222}(T) - D_{222}^{min}}{D_{222}^{max} - D_{222}^{min}},$$

where D_{222}^{min} and D_{222}^{max} are the minimal and maximal values of the optical density at 222 nm, corresponding to the native and denaturated states of HSA in the samples. The melting temperature T_m of the protein is determined as the point of maximum of the first derivative of the melting curve $df(T)/dT$.

The intrinsic protein fluorescence. The emission spectra of HSA were measured with the fluorescence spectrometer Lumina (Thermo Fisher Scientific) at the excitation wavelength 278 nm, at which both Tyr and Trp are exited [1], in rectangular quartz cells of the 1 cm optical path at 90°. The optical density of the solutions at 278 nm was $D \approx 0.1$. For all solvents used the emission and excitation spectra were measured, these spectra do not differ from the spectra of double distilled water.

FTIR spectra measurement. The spectra were obtained using a FTIR spectrometer Nicolet 8700 by the ATR method [24]. The spectral data within the range of 4000 to 500 cm^{-1} were recorded, and 512 scans were averaged for each spectrum with a spectral resolution of 2 cm^{-1}. The samples were covered with a cap to prevent evaporation. The protein concentration in IR experiments was $6 \cdot 10^{-4}$ M. The spectrum of the background was recorded and subtracted from the spectra of the samples automatically. The spectrum of the corresponding solvent was registered for further subtraction from the spectra of protein solutions. The software supplied with the spectrometer and OriginPro were used for data processing.

ζ-*Potential* values were collected using the Horiba SZ100 instrument (Japan). The data were processed using the software supplied with the instrument. The measurements were performed in a thermostated chamber (at 25 °C), at a laser radiation wavelength of 532 nm (laser power was 10 mW). The ζ-potential value was determined according to the theory of Smolukhovskii [25]: