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COMPARATIVE STUDY OF THERMAL STABILITY OF HEALTHY AND FOCAL SEGMENTAL GLOMERULOSCLEROSIS PLASMA ALBUMIN

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The thermodynamic parameters calculated from measurements obtained by differential scanning calorimetry from healthy and focal segmental glomerulosclerosis albumin are reported. The same values were determined by fluorescence spectra and by the second derivative absorption spectra and they resulted in agreement with values obtained from the calorimetry technique. Nevertheless the unfolding mechanism seems to be completely altered when pathological albumin is compared with healthy albumin. The C_p values measured by calorimetry show an increase with mild slope with healthy protein; on the contrary the slope intensely increase with pathological protein. Furthermore the λ_{max} of this molecule is lower and drastically decrease with the increase of temperature when compared with healthy one.

Therefore the modification of cys 34 on pathological albumin is supposed to cause an alteration of the structure, the swelling and the unfolding mechanism.

Keywords: *albumin, differential scanning calorimetry (DSC), focal segmental glomerulosclerosis (FSGS), intrinsic fluorescence, plasma, second derivative UV spectrum*

Introduction

Primary focal segmental glomerulosclerosis (FSGS) is a glomerular disease of unknown etiology, responsible for non-responder proteinuria and the leading cause of renal insufficiency in children [1, 2]. In a previous work it was demonstrated that whole FSGS albumin (F-alb) shows a non accessible thiolic group of cys 34, furthermore a unique and highly homogeneous band is observed in native electrophoretic titration curve between pH 4–9 [3].

Albumin (alb) is the main protein in the healthy mammalians plasma. Its conformation, as well as the physical and chemical properties, have been largely reported in literature [4]. Indeed the presence of a free thiol group of cys 34 and its vicinity of an imidazole group as well as the presence of several pockets, play an important role in the biological property of the molecule as carrier, buffer system, antioxidant properties. In a recent paper it has been reported that several pathologies cause an oxidative process involving the transformation of thiol group [5]. In analogy for phosphatase Cdc25B protein [6] it can be suggested that the oxidation of cys 34 in alb to sulfonic group can form hydrogen bonds with adjacent amino acid. Therefore the enthalpy change as well as ΔG in the unfolding process of F-alb, are predicted to be larger than in the native alb: indeed the differential scanning calorimetry (DSC) technique can verify this assumption. The oxidation process can also modify partially the structure of the whole protein, therefore it is necessary to verify the unfolding process by mean of other techniques that are involved with other properties of the structure such as spectroscopy and spectrofluorimetry.

In the present paper we report a study of the unfolding of healthy and pathological alb performed by DSC, intrinsic tryptophan fluorescence and the second derivative absorption spectra in UV.

Experimental

Materials

Albumin was purified in native conditions from plasma of healthy donors and FSGS patients. Briefly one mL of plasma was applied to gel and electrophoresis was run in 80 mM Tris, 90 mM borate, 2.5 mM EDTA at pH 8.6 for 12 h with 16 mA at 12°C. Albumin was desorbed from acrylamide by gentle pestle and was maintained in phosphate buffer saline at 4°C for 24 h with 2 changes of the solution and further purified by gel-filtration at 4ºC using a Superdex 75 HR 10/30 column (Amersham Biosciences Europe GmbH, Uppsala, SE) in 150 mM NaCl and 20 mM Tris-HCl at pH 8.0. Protein concentrations were spectrophometrically determined (ε_{280}=35219 M^{-1} cm^{-1}) assuming the same coefficient for both proteins. Light scattering correc-

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tions of the solution have been done according to Leach and Sherage [7].

All chemical reagents were analytical grade (Fluka, Buchs, CH). Solutions were fresh prepared using Milli-Q water (Millipore, Billerica, MA, USA).

Methods

Differential scanning calorimetric

Degassed samples with a concentration ranging from 0.5 to 1.0 mg mL^{-1} were performed with a MicroCalorimeter VP-DSC and curves have been analyzed with Origin software (MicroCal, Northampton, MA, USA). The scan heating rate were 30, 60 and 90°C h^{-1}.

Independent instrumental baselines were carried out by scanning 50 mM phosphate buffer at pH 7.4, at the correspondent heating rate and subtracted from experimental runs.

Ultraviolet spectra

Unfolding transition have also been studied by UV spectra using a UV-VIS Cary BIO400 (Varian, Palo Alto, CA, USA) spectrophotometer equipped with thermospacers and thermal control in the cuvettes measured by a teflon coated microthermistor probe. Protein concentrations were 0.9 mg mL^{-1} for healthy and pathological macromolecules.

All the normal and second derivative spectrum were obtained at different temperatures. The two maxima measured were scored at 284 and 293 nm, and the two minima utilized were scored at 279 and 286 nm.

Variations in the dielectric microenvironment surrounding Tyr residues are dependent on ratio (r) of two peaks to through second derivate absorbance differences (a/b) in proteins containing Tyr and Trp. The value of r is defined by Ragone *et al*. [8] as:

$$r=\frac{a}{b}=\frac{A''(284\,nm)-A''(279\,nm)}{A''(293\,nm)-A''(286\,nm)}$$

where A" is the second derivative absorbance at wavelength λ. The degree of Tyr exposure, α, is obtained from the equation

$$\alpha=\frac{r_n-r_a}{r_u-r_a}$$

r_n and r_u are the numerical values of the ratio a/b determined for native and unfolded conformers of protein; r_a corresponds to the ratio obtained when the same molar ratio of Tyr and Trp model compounds as in the protein in the presence of ethylene glycol and represents a complete burial of all aromatic residues of protein. Albumin contains 18 Try and 1 Trp residues. The second derivate peak through ratios r_a and r_u were obtained for the molar ratio (18/1) with the second derivate molar extinction coefficients of Ragone *et al.* Thus for alb, the calculated values were r_a= –2.74 and r_u=17.82 for the complete burial and exposure of Try residues, respectively.

Fluorescence spectra

Fluorescence measurements were carried out with a LS50B Luminescence Spectrometer (Perkin Elmer, Wellesley, MA, USA). Excitation was used at 295 nm to minimize the contribution of tyrosine in intrinsic fluorescence spectra. Excitation and emission slit widths were 4.5 nm and scan speed was 300 nm min^{-1}. The temperature of sample cuvette was maintained constant by circulating water bath (Haake, Karlsruhe-Berlin, Germany) and monitored in cuvette by a microthermistor probe.

Protein concentrations were 0.6 mg mL^{-1} for both macromolecules.

Spectra were analyzed after subtraction of baselines. The position of middle of a chord draw at the 80% level of the maximum intensity (λ_{max}) was taken as the position of the spectrum [9].

Results and discussion

Calorimetric traces from experiments performed with different protein concentration, as well as at different heating scanning rate did not show significant variation indicating that no aggregation or precipitation occurred at these environmental conditions [10, 11]. Second runs showed slightly lower curves. Taking into account that the results obtained in this work were practically independent of the scan rate, we conclude that the data could still be quantitatively ana-

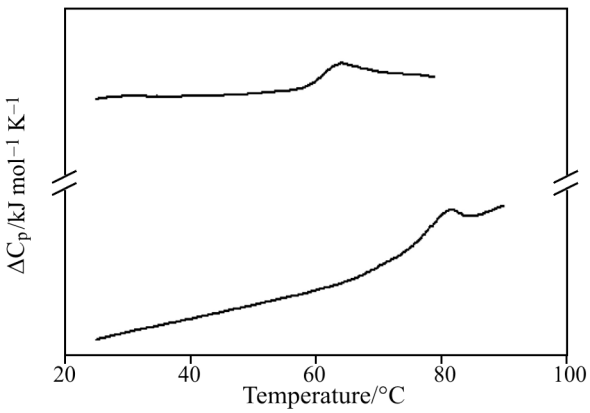

Fig. 1 Temperature dependence of partial heat capacity of H-alb (top) and F-alb (bottom) in 50 mM phosphate buffer at pH 7.4. The mass/mass base line has been subtracted

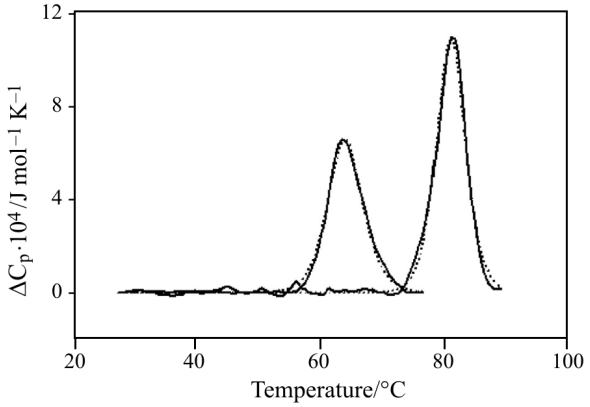

Fig. 2 Temperature dependence of partial heat capacity of H-alb (solid line , T_m at 65.6°C) and F-alb (solid line T_m at 81.2°C) after subtraction of baselines as reported in [12, 13] calculated by the Origin program. Dotted lines show the calculated curves according van't Hoff equation

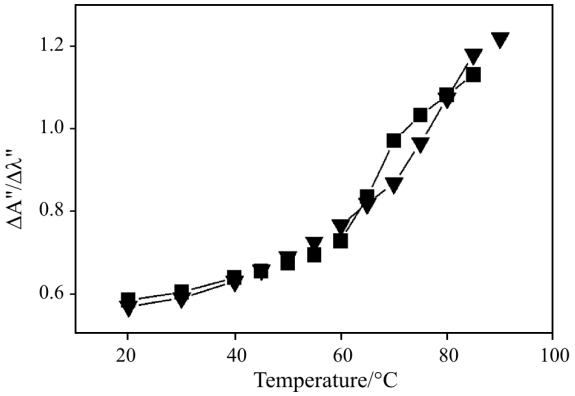

Fig. 4 Temperature dependence of degree of exposure of Tyr, ■ – H-alb and ▼ – F-alb monitored by second derivative absorption UV spectra according to Ragone *et al*. [8]

lyzed using a thermodynamic model in according to Freire and Biltonen [12].

The DSC curves obtained from both proteins are showed in Fig. 1. The top curve represents the healthy albumin (H-alb). Before the unfolding transition the slope of C_p corresponds to the values of 3074 J mol^{-1} °C^{-2} in analogy with the values reported by Ross and Shrake [13], while the bottom curve represents the F-alb. The slope of C_p *vs.* T is markedly emphasized of 10220 J mol^{-1} °C^{-2}. The same curves after subtraction of the base lines, according to Freire and Biltonen [12] and Krishinan and Brandts [14], are reported in Fig. 2.

The analytical procedure used to calculate the enthalpic change and T_m of the unfolding process provide the values reported as calorimetric ΔH which is obtained from the area of the peak and the van't Hoff ΔH which is calculated from the shape of the curve after subtraction of the baseline. According to the theoretical assumption reported by Freire and Biltonen [12] or by Pfeil and Privalov [15] if the two ΔH values are identical we can assume valid the two state approximation theory i.e. statistically the protein in solution can be either folded or unfolded. Our experiments indicate that both proteins, H-alb as well as F-alb, undergoes the unfolding process are in agreement with this theory. The ΔH is $510.8 \cdot 10^3$ J mol^{-1} for H-alb and $607.1 \cdot 10^3$ J mol^{-1} for F-alb and the unfolding T_m are 65.61 and 81.18°C respectively.

The second derivative spectra obtained from the absorption experiments for alb solution are shown in Fig. 3. The fractional change in Tyr exposure is represented in Fig. 4 for both proteins. The fraction of α in the function of temperature, before and after the transition, have been extrapolated and they have been used as baselines for folded and unfolded structures according the procedure reported by Brandts [16, 17] as reported in Fig. 5.

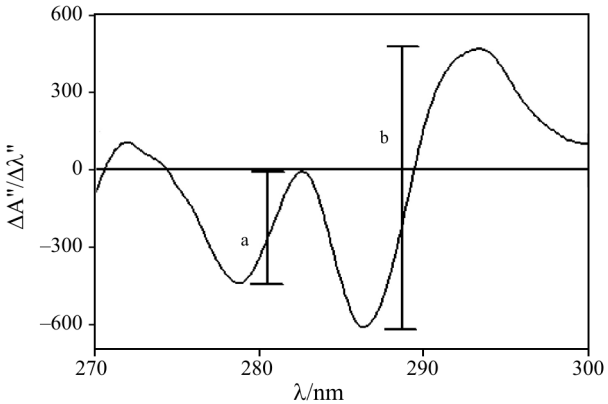

Fig. 3 Second derivative absorption UV spectrum and identification of the two arrow *a* and *b*, which indicate the peak to peak distance according to Ragone *et al.* [8] to calculate Tyr absorption variation in the presence of Trp

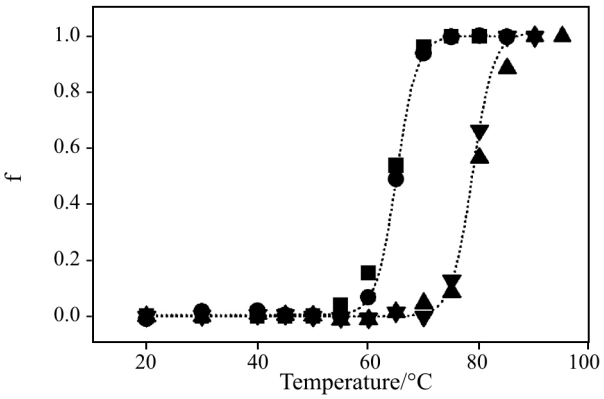

Fig. 5 Unfolding fraction of ■ – H-alb and ▼ – F-alb calculated from second derivative absorption spectra; unfolding fraction of ● – H-alb and ▲ – F-alb calculated from intrinsic fluorescence of Trp. Dotted lines show the calculated curves according van't Hoff equation

Table 1 Thermodynamic data for the unfolding transition of H-alb and F-alb determined by DSC, second derivative absorption UV spectrum and intrinsic fluorescence of Trp technique

	Healthy			FSGS		
	ΔH_{cal}/kJ mol^{-1}	ΔH_{vH}/kJ mol^{-1}	T_m/°C	ΔH_{cal}/kJ mol^{-1}	ΔH_{vH}/kJ mol^{-1}	T_m/°C
DSC	511±9	511±11	65.6±0.1	607±16	607±18	81.2±0.1
Spectrophotometer	–	505±40	65.1±0.3	–	621±45	79.5±0.3
Spectrofluorimeter	–	521±42	64.5±0.5	–	634±46	78.9±0.5
[19]*	372±5	–	63.2±0.4	–	–	–

∗ Human defatted albumin in 50 mM phosphate buffer at pH 7.0, $\Delta H_{cal}=\Delta H$ calorimetric, $\Delta H_{vH}=\Delta H$ van't Hoff

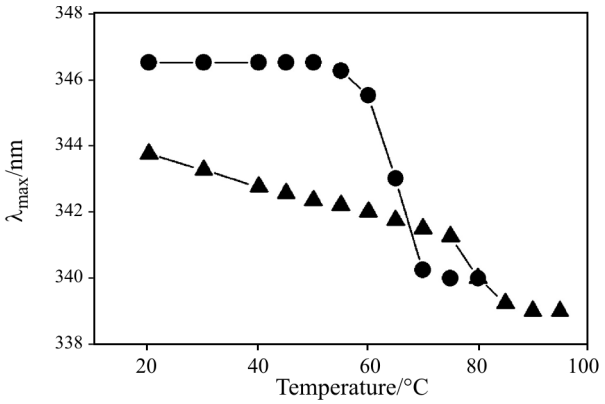

Fig. 6 Temperature dependence of exposure of Trp of ● – H-alb and ▲ – F-alb monitored by intrinsic fluorescence of Trp according to Ruiz-Arribas *et al.* [9]

The λ_{max} of both solutions measured at different temperature, is showed in Fig. 6, indicating the exposition of Trp to solvent. The aromatic group of F-alb is more exposed to solvent than the H-alb and the wetting is increasing with the temperature until the unfolding occurs. The subtraction of the extrapolations allow to have the transition curve alone [16, 17].

The T_m as well as the $\Delta H_{van't\ Hoff}$, dotted lines in Fig. 5, have been calculated for the last two analytical procedures and they are indicated in Table 1.

Conclusions

The difference of ΔH between the H-alb and F-alb can not be justified only by the presence of few hydrogen bonds formed in oxidized molecule [6] compared with alb with free thiol group. Kawakami *et al.* [5] have recently reported that molecular dynamic simulation of reduced and oxidized alb show conformationally changes in five regions, furthermore the presence of trace of bound fatty acids promotes the molecular oxidation [18] as well as the increase of enthalpy change and T_m of unfolding [19, 20].

Analyzing the three experimental processes we can suggest that at temperatures below the unfolding process, the exposition of Tyr do not indicate large discrepancies between the two molecules. Whereas the fluorescent determinations seem to indicate that the Trp residue located at the bottom of the mayor molecular pocket, modifies the λ_{max} with temperature, suggesting an increase of hydration of the aromatic group. Either the slope of ΔC_p values proposes a large exposition of bound water molecules easily released following the increase of temperature.

Further studies aimed to understand the molecular conformations which are necessary for eventual suggestion in therapeutic treatment.

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