SPECTROSCOPIC STUDY OF SOME HUMAN AND BOVINE SERUM ALBUMIN CONFORMERS OBTAINED BY RENATURATION

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abstract: From various folding pathways of thermally denatured human and bovine serum albumin, a mixture of different conformers was obtained by different cooling procedures, the aim of the present paper being the study of their spectroscopic properties. Spectral study of eosin (EOS) and bromphenol blue (BSP) association with some albumin conformers (AC) was performed in order to determine their molecular interaction parameters. The slope of eosin absorbency (in the region $\lambda \sim 530$ nm) differentiates the conformers among them, as well as in respect with the natural albumin. The association constants, obtained by various methods and the number of binding sites are different between AC and the non-denatured albumin, as well as among them, having the order of magnitude of $10^5 M^{-1}$. The results are in agreement with the hypothesis that, corresponding to various refolding conditions, the albumin molecule acquires new local secondary structures, different among them and from the one in the initial unfolding conditions.

Introduction

The interaction between albumins and several ligands has been largely studied in the last decades [1], in order to determine parameters of the interaction, as well as the conformational changes upon binding. The aim of the present paper is to study, using the absorption spectra, the influence of the refolding process of the thermally denatured BSA conformers. In its native state, the albumin macromolecule has probably a conformation in which the polar aminoacid residues are especially directed towards the outside of the macromolecular surface and the non-polar ones are directed prevalently inside the surface. The thermal denaturation, followed by particular refolding conditions, leads to conformers in which the ratio between the hydrophobic and hydrophilic moieties is gradually modified in respect with the natural albumin. Previously, results on the same conformers, obtained by chromatography [2] and electrophoresis [3] had been reported. In the present study we propose an alternative way of investigating modifications in the protein conformation, using a dye (eosin, bromphenol blue) as a tool, by following the effect produced on its spectral properties after adding in solution natural or denatured protein. Albumin conformers are supposed to regain only a part of their initial structure, presenting differences function of the renaturation pathway.

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Experimental

BSA, Sigma fraction V, was used without further purification. Buffer solutions (NaOH and H_3BO_3) have been prepared, to keep the *p*H at 7.4. The solutions of albumin have been obtained with natural (sample 1) and thermally denatured, for 15 minutes at 65°C, albumin. Samples 2, 3, 4 have been obtained with renatured albumin, after a slow refolding of ~1.5°C/min. (sample 2), refolding at 4°C (sample 3) and at –20°C (sample 4). Eosin (2',4',5',7'-tetrabromofluorescein) disodium salt, was purchased from Baker. Absorption measurements have been done with a Unicam-Helios α spectrophotometer (Vision software). Working solutions were obtained by adding variable volumes of eosin stock solution, at total concentration of albumin, kept constant during the experiment. In the case of BSP, dye concentration has been kept constant and its absorption measured in presence of different concentrations of albumin. The molecular formula of the two dyes is presented in Fig. 1.
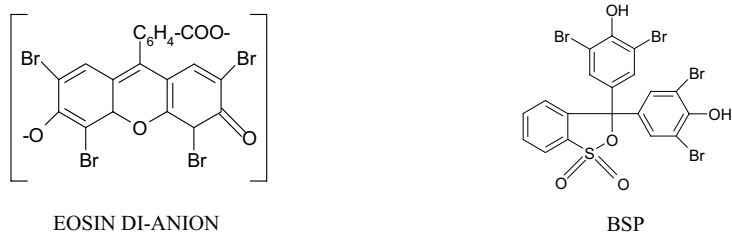

EOSIN DI-ANION   BSP

Fig. 1: *Molecular structure of the two dyes used in interaction with albumins*

Results and Discussion

Interaction between renatured HSA and eosin

In respect with the reference eosin molar absorption coefficient (noted ε_R) obtained in presence of native albumin at a constant concentration 2.875 μM, eosin presents different absorption molar coefficients when albumin conformers obtained after denaturation-renaturation are added in solution, in the same constant concentration.

Table 1. Slopes of eosin absorption as a function of concentration in the range (0.536-5.490) μM, in presence of denatured-renatured HSA (probes 1-4) at constant concentration 2.875 μM

Sample No.	Renaturation	λ max / nm	Slope (m)	Δ m	r
1	Natural albumin	530	46910	-	0.9969
2	Isothermal	528	**42271**	4639	0.9986
3	At constant t = 4°C	528	43854	3056	0.9977
4	At constant t = -20°C	528	**49531**	2621	0.9977

In Table 1 we could observe comparatively, for probes 2 and 5, a decreasing, respectively increasing of ε, from the reference probe $\varepsilon_R = 46910 M^{-1} cm^{-1}$. We assumed that the absorption results from the Lambert-Beer law (the length of the spectrocell is 1cm):

$$A = \varepsilon_{\text{free}} C_{\text{free}} + \varepsilon_{\text{bound}} C_{\text{bound}} \tag{1}$$

where A is the absorbance experimentally measured, $\varepsilon_{\text{free}}$ and $\varepsilon_{\text{bound}}$ are the molar absorption coefficients of the free and bound eosin in solution, C_{free} and C_{bound} are the concentrations of these two forms, respectively. Thus, the value of apparent molar absorption coefficient $\varepsilon_{\text{app}} = A/C_0$, ($C_0 = C_{\text{free}} + C_{\text{bound}}$) results from a different contribution of the free and bound form. The value of the maximum wavelength confirms these results, being shifted with $9 \div 14$ nm from $\lambda_{\text{free}} = 516$ nm. The plot of eosin absorption as a function of its concentration is presented in Fig. 2. The relative position of slopes obtained in presence of different constant concentration of albumin conformers differentiates the interaction of all these conformers and the natural albumin.
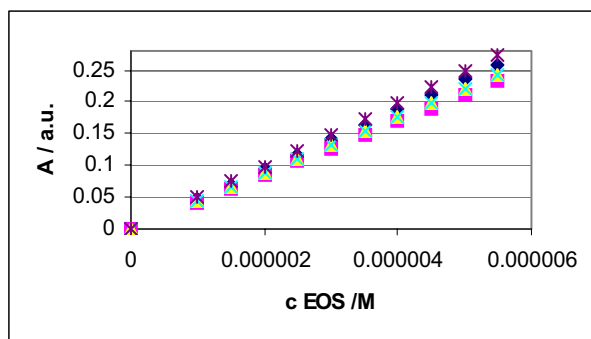

Fig. 2: *Eosin absorption at λ = 525-530 nm as a function of its concentration, in presence of natural and denatured human serum albumin (where * is sample 4; ♦ is sample 1; Δ and ■ are samples 2 and 3).*

In the region of the ratio between protein and eosin concentrations, P/D = $0.52 \div 5.39$, a more quantitative approach of this interaction has been made using Scatchard-type relation [4,5]:

$$\frac{v}{C_{\text{free}}} = nK - vK \tag{2}$$

where v represents the ratio between the concentration of bound eosin and the concentration of protein, n is the number of binding sites and K is the association constant; the results are presented in Table 2. In all cases, a strong affinity of albumin for eosin has been noticed, of the order of magnitude $K \sim 10^5 M^{-1}$ for the association constant, the bigger value being obtained with natural HSA. The correlation coefficients are relatively good for the number of experimental points used in the fitting. Regarding the conformers, we observe that a relatively large affinity still exists for conformer 4 and that the smaller affinity presents conformer 2. Another observation is that, whereas all Scatchard representations could have been fitted only with one type of binding sites, in the case of conformer 2, relatively good results have been obtained with two types of binding sites.

It could be possible that some of the sites, which have been buried in the hydrophobic core, became exposed at the solvent during the denaturation and have a small probability to regain their initial position in the renaturation time.

Table 2. Scatchard calculation of v/cf = f (cf) for C_{HSA} = 2.857 µM (constant).

Sample No	P/D	$K*10^{-5}$ / M^{-1}	n	r	No. of points
1	0.52-5.33	11.313	0.94	0.9553	14
2	0.63-0.77	0.113	21	0.9752	4
	0.83-1.54	1.356	0.5	0.9888	5
3	0.52-5.33	4.035	0.83	0.9539	17
4	0.52-5.33	6.735	1.27	0.9828	16

Interaction between renatured BSA and eosin

By comparison with HSA, the first observation is that the absolute value of the slope for BSA is almost two times bigger for all conformers. The correlation coefficients are very good, the fitting being made for 20 experimental points in all cases. The maximum of the absorption band is placed at the same value, presenting also the contribution of free and bound eosin in solution. The main difference consists of the fact that all slopes are smaller for conformers in respect with the natural protein and that as conformer 2 has the same behaviour as in HSA, the conformer 4 has a completely different behaviour, presenting the smaller value of the slope (Table 3).

Table 3. Slopes of eosin absorption as a function of concentration in the range (0.536-5.490) µM, in presence of denatured-renatured BSA (probes 1-4) at constant concentration 2.875 µM.

Sample No.	Renaturation	λ max / nm	Slope (m)	r
1	Natural albumin	530	90701	0.9997
2	Isothermal	528	87624	0.9991
3	At constant t = 4°C	528	85656	0.9991
4	At constant t = -20°C	528	79472	0.9995

Table 4. Scatchard calculation of v/cf = f (cf) for C_{BSA} = 2.857 µM (constant).

Sample No	P/D	Method	$K*10^{-5}$ / M^{-1}	n	r
2	0.086 - 0.877	Scatchard	3.048	33	0.9198
		Gentile	4.059 ± 0.730	26 ± 3	0.9858
4	0.224 - 0.740	Scatchard	4.362	25	0.9314
		Gentile	4.757 ± 0.440	22 ± 1	0.9896

A possible interpretation could be the following: it is well known that HSA contains only one tryptophan residue (Trp 214), whereas BSA has two Trp residues in the molecule (Trp 134 and Trp212), one in the polar environment and the other in the hydrophobic core [6÷8]. Supposing the interaction eosin-Trp, it is possible that electrostatic binding with charged residues in near vicinity of tryptophan occurs. If we consider Trp 212 and Trp 214 as having similar environment in the two albumins, in the conformer 4 of BSA the binding could occur at Trp 134. Conformer 2, having similar behaviour in both albumins, may give an indication of the interaction in the vicinity of Trp 212 and Trp 214, which are in the polar environment, more exposed and perturbed by the interaction with the ionic atmosphere. This hypothesis is also supported by Scatchard and Gentile calculation (Table 4). In the range of P/D = 0.086-0.877 there is also a high affinity of BSA for eosin, the association constant having the same order of magnitude $K \sim 10^5 M^{-1}$ with both methods.

Very different is the number of binding sites, 22-33 for eosin to BSA. This result has been found in literature only concerning small ions interacting with BSA [9,10].

Interaction between denatured BSA and bromphenol blue

In order to verify our results, we used a dye for which the association constant with proteins is well known from the literature [11] as being $1.5*10^6 M^{-1}$ and studied its interaction with natural bovine albumin, conformer 2 and conformer 4 only (which presented the most different behaviour in BSA and HSA).
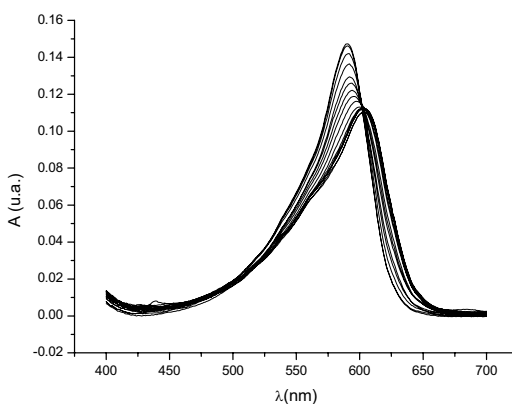

Fig. 3: *Absorption spectrum of 2.06 μM BSP in presence of renatured BSA (sample 4) at P/D between 0.029 and 2.2, in buffer at pH=7.4.*

The absorption spectrum of BSP in the absence (the most intense curve) and in presence of BSA is given in Fig. 3. An important shift occurs in solution containing increasing concentrations of BSA. Four calculation methods have been used in order to determine the association constant (Table 5).

Table 5. Scatchard calculation of v/cf = f (cf) for C_{BSA} = 2.857μM (constant)

Sample No	$K*10^{-6}$ / M^{-1}				K_{mean} $*10^{-6}$ / M^{-1}
	Scatchard[4]	Gentile[5]	Chaires[12]	Molec.model[9]	
1	0.628	-	0.439	1.989	1.019
2	0.152	-	0.092	0.053	0.099
4	3.020	2.910	0.742	0.579	1.813

A relatively large difference between natural and the two albumin conformers is noticed. A relatively small affinity for the conformer 2 and a very big affinity for the conformer 4, by respect with the normal albumin. It is the same result as for the previous cases.

Conclusions

1. Absorption spectra of eosin and BSP in presence of BSA and HSA have proved to be useful tools in order to differentiate conformers obtained after 15min. thermal denaturation

of albumin at 65°C. In respect with the reference eosin molar absorption coefficient in presence of native albumin (HSA or BSA) at a constant concentration of 2.857 μM, eosin in presence of different albumin conformers obtained after denaturation followed by refolding showed different molar absorption coefficients.

2. In all cases the absorption maximum of dye appears to a different value from that of the free dye in buffer solution, indicating the presence of bound dye (previous studies showed that during binding a red shift of approximately 10 nm is noticed). By respect with ε_R, conformer 2 presents a smaller value for both albumins. This conformer corresponds to a long time refolding process. On the contrary, conformer 4 obtained after fast cooling at $t \sim -20°C$ gives a value of molar absorption coefficient $\varepsilon_4 < \varepsilon_R$ for BSA and $\varepsilon_4 > \varepsilon_R$ for HSA, differentiating between binding sites on the macromolecular surface: as we consider Trp 212 and Trp 214 as having similar environment in the two albumins, than in BSA the binding could occur at Trp 134.

3. Quantitative parameters, association constants and number of binding sites have been obtained using Scatchard and Gentile methods for BSA-EOS, HSA-EOS and BSA-BSP interactions, as having the order of magnitude $K \sim 10^5 M^{-1}$ in the first two cases; the affinity of conformer 2 for eosin is smaller than that of conformer 4, both smaller than the native one, showing that the macromolecule does not regain the entire binding affinity by renaturation. BSP has the same behaviour, the order of magnitude being $K \sim 10^6 M^{-1}$.

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