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DSC STUDY OF THE ASSOCIATION OF ETHANOL WITH HUMAN SERUM ALBUMIN

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Human serum albumin unfolding in ethanol/water mixtures was studied by use of differential scanning calorimetry. Ethanol-induced changes in DSC curves of defatted and non-defatted albumin were markedly different. In the presence of ethanol, bimodal denaturation transition for fatty acid free albumin was observed while that for albumin containing endogenous fatty acids was single and more sharpen than in aqueous solution. Ethanol was found to decrease the thermal stability of albumin due to the binding to the unfolded state to a higher degree than to the native state, thus favouring unfolding. The binding with different affinities has been suggested depending on ethanol concentration range.

Keywords: *DSC, ethanol binding, human serum albumin, thermal stability*

Introduction

The recent years has brought the dramatic advances in the area of the identification and characterization of human serum albumin (HSA) binding sites location and structure [1–4]. Binding of ligands at these sites may alter the local protein structure. Alcohols do not form probably any kind of strong complexes with protein macromolecules. It was reported however, that ethanol directly binds to proteins (e.g. ferrihemoglobin, bovine serum albumin (BSA)) in a site-specific manner [5–7]. In current opinion alcohols act mainly as hydrogen bond donors whose binding to the polypeptide chain is stabilized by hydrophobic interactions. Dwyer & Bradley [8] took an attempt to formulate the general motifs of alcohol binding to proteins.

Additionally, ethanol may induce changes in native protein structure via replacement of hydrogen-bonded water [9] and/or displacement of endogenous ligands from their binding sites. It was reported that ethanol inhibits binding of *cis*-parinaric acid and another hydrophobic compound, 1,8-ANS, to BSA [7]. Ethanol may also affect the HSA/drug interactions by displacement of the drug due to competition at the binding site or through an allosteric mechanism by changing the overall conformation of the protein [10].

The present study was undertaken to demonstrate the binding of ethanol to HSA by differential scanning calorimetry (DSC). The paper reports the effect of ethanol on albumin thermal unfolding and documents a prominent ethanol-induced changes in protein DSC curves. Because the interaction of ethanol with hydrophobic fatty acid-binding sites on fatty acid free BSA was found [7], it would be interesting to compare the binding of ethanol to non-defatted and fatty acid free albumin. These two species of albumin are well distinguishable in DSC experiment [11, 12], thus DSC technique seems to be suitable to this purpose.

Materials and methods

Two kinds of human serum albumin: 1) containing endogenous fatty acids (HSA, lot 111K7612) and 2) defatted (with fatty acid content below 0.0005%; HSAf, lot 113K7601), essentially globulin free (purity minimum 99%) were purchased from Sigma. DSC measurements were carried out in the temperature range 20–100°C with the scan rate 1 K min^{-1} by using the ultrasensitive microcalorimeter (VP DSC, MicroCal Inc., Northampton, MA). All experiments were performed for two albumin concentrations: 1.5 and 3 mg mL^{-1}, hereafter referred to as (l) and (h), respectively. Albumin solutions were prepared by direct dilution of the lyophilised protein with sterile water or ethanol-water solutions (with ethanol concentration ([*et*]) ranging from 1 to 20% (*v*/*v*)). pH of the albumin solutions was 6.0±0.5. All other DSC experimental details were the same as described previously [11, 12]. The measurements for every combination of albumin and ethanol concentration were repeated a few times.

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DSC curves were analysed with MicroCal Origin software. The signal obtained with the albumin solution was corrected for by the signal of an identical solution without the protein and for the difference in heat capacity between the initial and the final state by using a sigmoidal baseline.

Results and discussion

Ethanol-induced effects on albumin thermal unfolding

Figures 1–3 show the influence of increasing ethanol concentration on HSA and HSAf DSC curves. The observed endothermic peak connected with albumin denaturation in aqueous solution depends on the kind of albumin [12]. HSAf unfolds in lower temperature than HSA. The shoulders at the left and right side of the peak are visible for non-defatted and fatty acid free human albumin respectively. Ethanol causes a decrease of the protein transition temperature T_m (temperature at the maximum excess molar heat capacity $C_{p,max}$). Additionally, the DSC curves corresponding to defatted albumin (Figs 2, 3) become bimodal in the presence of ethanol. In the case of protein-ligand interactions, the biphasic endotherms are usually associated with the separate unfolding of ligand-poor and ligand-rich forms of protein [13, 14]. The temperature of the maximum of lower-temperature peak T_{mI} decreases successively with increasing ethanol concentration while that of the higher temperature peak T_{mII} shifts upwards when ethanol concentration increases from 0 to 3% *v*/*v* and next remains practically constant up to 10% *v*/*v*. The mean values of T_m (± standard error of the mean (SEM)) listed in Table 1 for HSA and HSAf confirm trends pointed above.

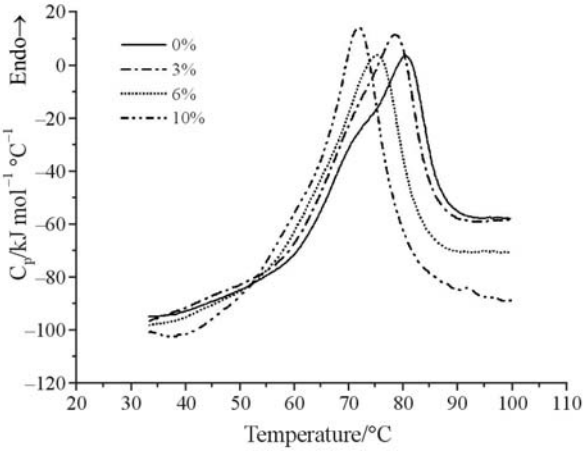

Fig. 1 DSC curves of HSA unfolding in the presence of various concentrations of ethanol (in % (*v*/*v*)); albumin concentration 0.0225 mM L^{-1}

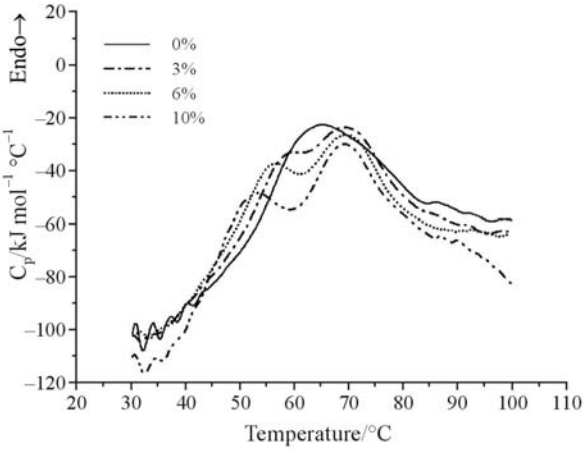

Fig. 2 DSC curves of HSAf unfolding in the presence of various concentrations of ethanol (in % (*v*/*v*)); albumin concentration 0.045 mM L^{-1}

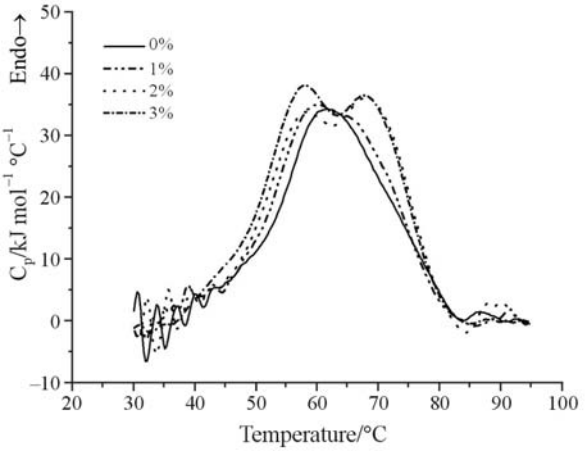

Fig. 3 Excess heat capacity function for HSAf (concentration 0.045 mM L^{-1}) in the presence of various concentrations of ethanol (in % (*v*/*v*))

A change in the apparent T_m of the protein unfolding may be a manifestation of ligand binding effects. In cases where ligand binding is to the native form of protein, T_m increases, whereas binding to the unfolded polypeptide causes decrease of T_m [15]. The results presented above indicate ethanol binding mainly to the unfolded state of albumin. However, in the case of HSAf at low ethanol concentration, the binding to both folded and unfolded states should be considered.

DSC curves presented in Figs 1 and 2 indicate that addition of ethanol to the aqueous albumin solution reduces ΔC_p value observed when the protein unfolds (the post-transition heat capacity baselines move down with increasing [*et*]). Similar diminution in ΔC_p has been observed for unfolding of ubiquitin upon the addition of methanol [16], where a ΔC_p value fell close to zero above about 30% (*v*/*v*) methanol.

The effect of ethanol seems to be opposite for HSA and HSAf taking into account the widths of

Table 1 The influence of ethanol concentration on the mean T_m values of HSA and HSAf in 0.0225 mM L^{-1} (l) and 0.045 mM L^{-1} (h) solutions

Ethanol concentration/ % *v*/*v*	T_m/K					
	HSA		HSAf			
			I		II	
	l	h	l	h	l	h
0	353.3±0.1	353.8±0.1	334.7±0.7	335.6±0.3	334.7±0.7	335.6±0.3
1	351.9±0.3	352.9±0.1	331.8±0.4	334.5±0.4	338.4±0.4	338.1±0.1
2	351.9±0.1	352.1±0.1	331.1±0.3	331.5±0.1	342.2±0.2	339.7±0.4
3	351.4±0.1	351.7±0.1	328.6±0.3	330.6±0.3	342.6±0.2	340.8±0.1
6	348.1±0.1	348.9±0.1	326.2±0.1	328.2±0.1	341.8±0.1	341.5±0.3
10	344.8±0.1	345.6±0.1	322.1±0.2	324.0±0.1	342.3±0.1	341.4±0.2
20	335.4±0.3	336.5±0.2	314.4±0.9	315.9±0.2	337.6±0.3	336.3±0.1

Table 2 The denaturation enthalpy (mean±SEM) for HSA and HSAf at 0.0225 mM L^{-1} (l) and 0.045 mM L^{-1} (h) albumin and different ethanol concentrations

Ethanol concentration/ % *v*/*v*	ΔH/kJ mol^{-1}			
	HSA		HSAf	
	l	h	l	h
0	1212±36	1046±20	910±23	809±25
1	1168±42	1230±45	1063±31	1053±63
2	1193±36	1007±17	1151±42	1004±52
3	1242±44	1097±50	1135±37	1141±21
6	1488±24	1270±42	1293±18	1266±58
10	1535±37	1107±33	1355±52	1116±51
20	1269±91	1615±135	744±45	763±36

curves at half height – HHW. This is because for HSA contrary to HSAf the decrease of HHW with increasing ethanol concentration is visible (Fig. 4). The tendency observed for HSAf arises from bimodality of the transition. Additionally, HSAf peak broadening could be expected because alcohol-induced denaturation leads to destabilization of protein tertiary structure. The reason of HSA peak sharpening is unclear. It may be connected with an increase of HSA transition cooperativity in ethanol solution as well as the change in compactability of HSA molecule. It was reported that ethanol might induce a transition in HSA conformation from β-sheet to α-helical structure [17]. Such alterations may lead to more compact structure of albumin molecule.

The values of mean denaturation enthalpy (ΔH, calculated from the area under calorimetric peak) are presented in Table 2. It is interesting to note the tendency of ΔH increasing with increasing ethanol concentration for both kinds of albumin, but only at low (l) concentration. An attempt of explanation of this observation may be done taking into consideration the aggregation process, which always accompanies the thermal protein unfolding. This process is stronger when the protein concentration is higher thus probably small net effect of ΔH increasing may be shaded. At the highest studied ethanol concentration, 20% *v*/*v*, ΔH decreases and HHW increases (besides the case of higher (h) HSA concentration). It is proba-

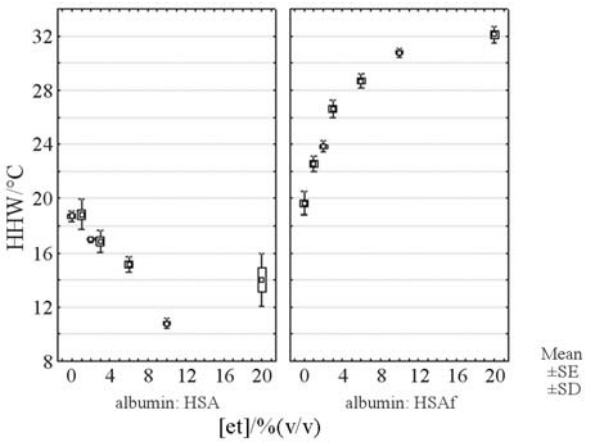

Fig. 4 Effect of ethanol on HHW of albumin unfolding transition

bly connected with the greater extent of destabilization and loosening of albumin structure in solution with high ethanol concentration.

Ethanol binding to unfolded albumin

The application of reversible thermodynamic to the analysis of protein denaturation process should be preceded by checking the reversibility and scan rate independence of this process. We examined earlier that human serum albumin denaturation process is only slightly dependent on scan rate. The effect of protein concentration on the shape of albumin DSC curves was also reported [12]. The partial reversibility of the investigated process noticed in aqueous ethanol solutions has been discussed in parallel submitted paper and the permissibility of equilibrium approach has been concluded [18].

In an equilibrium model of protein unfolding with simultaneous ligand binding or dissociation, the shift in T_m with increasing ligand concentration is related to additional Gibbs energy of stabilization ($\Delta\Delta G$) due to ligand binding to the native protein or destabilization ($-\Delta\Delta G$) when ligand binds to the unfolded polypeptide [15].

Assuming that on albumin molecule there are n identical and independent ethanol binding sites with identical association constants K_b, the Gibbs free energy of unfolding at any temperature (T) is given by

$$\Delta G = \Delta G^0 - nRT\ln(1+K_b[et]) \quad (1)$$

where R is the gas constant, [*et*] is the free ethanol concentration (in this study [*et*] is taken as the total ethanol concentration because there is the great excess of ethanol) and ΔG^0 is the free energy of unfolding in the absence of ligand.

The transition midpoint temperature in the presence of ethanol, $T_{1/2[et]}$, is the temperature where ΔG for unfolding is zero. Thus the change of Gibbs energy due to ethanol binding may be expressed as:

$$\Delta\Delta G = nRT_{1/2[et]}\ln(1+K_b[et]) \quad (2)$$

and further from

$$\Delta\Delta G = \Delta G^0(T_{1/2[et]}) = \Delta H(T_{1/2[et]})\frac{T^0_{1/2}-T_{1/2[et]}}{T_{1/2[et]}} - \\ -\Delta C^0_p\left\{T_{1/2[et]} - T^0_{1/2} + T^0_{1/2}\ln\left(\frac{T^0_{1/2}}{T_{1/2[et]}}\right)\right\} \quad (3)$$

where $T^0_{1/2}$ is the midpoint temperature of the protein transition without ethanol, $\Delta H(T_{1/2[et]})$ is the enthalpy of the transition at the midpoint temperature $T_{1/2[et]}$. $T_{1/2[et]}$ refers to the transition temperatures in the presence of the various ethanol concentrations. The value for ΔC^0_p, can be estimated from a plot of $\Delta H(T_{1/2[et]})$ *vs.* $T_{1/2[et]}$. Unfortunately, such ΔC^0_p estimation may be associated with great errors, up to 50% [19].

From the slope of plots of $\Delta\Delta G$ *vs.* ln[*et*] and [*et*], the lower limits for n and nK_b values respectively, at the transition temperature, can be estimated according to dependencies:

$$\frac{\partial(\Delta\Delta G)}{\partial\ln[et]} = nRT_{1/2[et]}\frac{K_b[et]}{1+K_b[et]} < nRT_{1/2[et]} \quad (4)$$

and

$$\frac{\partial(\Delta\Delta G)}{\partial[et]} = nRT_{1/2[et]}\frac{K_b}{1+K_b[et]} < nRT_{1/2[et]}K_b \quad (5)$$

In similar way Tanner *et al.* [20] obtained binding parameters for halothane to native BSA.

Using the experimental data the values of $\Delta\Delta G$ for various ethanol concentrations were calculated according to Eq. (3). Figures 5 and 6 show the dependencies of $\Delta\Delta G$ *vs.* ln[*et*] and [*et*], respectively. The ΔC^0_p values (in kcal mol^{-1} °C^{-1}) estimated from the slope of the line best fitted of $\Delta H(T_{1/2[et]})$ *vs.* $T_{1/2[et]}$

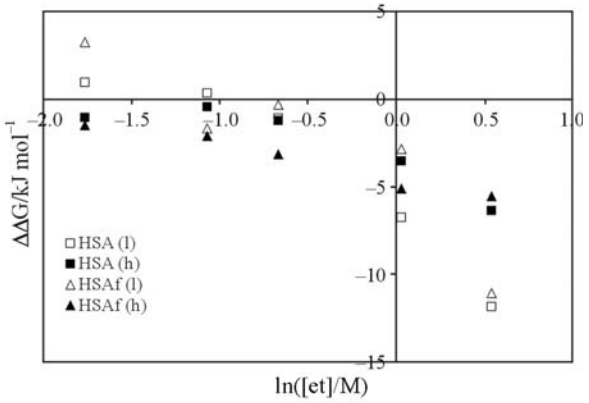

Fig. 5 Additional Gibbs energy of albumin destabilization *vs.* natural logarithm of ethanol concentration

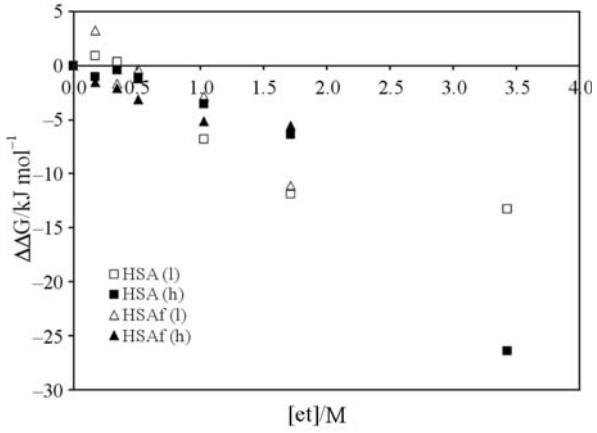

Fig. 6 Additional Gibbs energy of albumin destabilization *vs.* ethanol concentration

Table 3 The minimal n and K_b values estimated according to dependencies (4), (5)

Range of ethanol concentration/ % (v/v)	n				K_b/M^{-1}			
	HSA		HSAf		HSA		HSAf	
	l	h	l	h	l	h	l	h
0–3	0.7			0.5	1.2			4.4
0–6	1.7	0.5	1.1	0.7	1.5	2.2		2.6
0–10	2.2	0.8	1.9	0.7	1.0	1.5	1.3	2.0
0–20	3.0	2.6			0.5	0.9		
3–10	3.4	1.5	3.3	0.7	0.7	0.8	1.1	1.9

for data from the [*et*] range 0–10% (v/v) were: –6.6 (R^2=0.96), –4.7 (R^2=0.77), –5.4 (R^2=0.56), –10.4 (R^2=0.63) for (l) and (h) HSA and (l) and (h) HSAf, respectively. The poor precision of ΔC_p^0 estimation for HSAf is connected with difficulties of exact $T_{1/2[et]}$ determination. This temperature lies at some point midway between temperatures of maxima of two peaks in bimodal HSAf DSC curves. The experimental results obtained for HSAf at 20% (v/v) [*et*] were not used for n and K_b estimation because of poor repeatability of DSC curve in this case.

From the line best fitted to the points in selected [*et*] range, the smallest values of n, nK_b and next K_b (presuming calculated n) were estimated according to dependencies (4) and (5), taking as $T_{1/2[et]}$ the temperature corresponding to the higher limit of considered [*et*] range. The obtained binding parameters are listed in Table 3. At the lowest [*et*] range the minimal n values are below 1 while K_b above 1.2 and 4.4 M^{-1} for HSA and HSAf, respectively. At higher [*et*] more ethanol molecules are associated with each albumin macromolecules but the binding is weaker. The results suggest the existence of binding sites with different affinity. The low-affinity binding site has higher capacity and may adopt more than 3 ethanol molecules. No essential differences can be noted in binding of ethanol to the unfolded state of HSA and HSAf, however the binding constants per site, K_b, are slightly higher for HSAf than for HSA. The estimated K_b values are significantly lower from 19.3 M^{-1} obtained by Avdulov *et al.* [7] for BSA. However, they studied the affinity of ethanol for BSA at 36.5°C and very low [*et*] (0.025–0.2 M) while the K_b values in this study refer to the transition temperature and [*et*] range 0.2–3.4 M.

Calorimetric experiment reported here indicates that ethanol reduces the mean unfolding transition temperature T_m for both albumins examined. It means that ethanol promotes protein unfolding and reduce its thermal stability. The binding of alcohol molecules to the exposed residues may destabilize protein native conformation by shifting the equilibrium in favour of the unfolded polypeptide chain. However, in the case of HSAf the higher-temperature peak in bimodal DSC curve shifts to higher temperatures with [*et*] increase from 0 to 3% (v/v). Such T_m shift might reflect ethanol binding to the native albumin form. Further work in this area is concentrating on the albumin-ethanol interactions in the lowest [*et*] range.

Conclusions

Ethanol promotes unfolding and its increasing concentration progressively reduces the thermal stability of the albumin. Essential differences exist between ethanol-induced changes in thermal unfolding profiles of fatty acid-free and fatty acid containing human serum albumin. Ethanol binds stronger to albumin molecule at low concentration, below 3% (v/v). In such conditions at least one from two albumin molecules forms complex with ethanol. In the case of defatted albumin, K_b at transition temperature was estimated to be higher than 4.4 M^{-1}. The binding affinity of ethanol to non-defatted albumin seems to be slightly lower. At higher ethanol concentrations (above 3% (v/v)) more than 3 ethanol molecules may be weakly associated with the unfolded albumin molecule.

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