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EFFECT OF SOLVENT COMPOSITION ON DSC EXOTHERMIC PEAK OF HUMAN SERUM ALBUMIN SUSPENDED IN PYRIDINE–*n*-HEXANE MIXTURES

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Abstract

Human serum albumin (HSA) immersed in pyridine–*n*-hexane mixtures was analyzed using differential scanning calorimetry (DSC). State of the solid HSA in organic solvent mixtures is the non-equilibrium state which is seen as the exothermic peak on the DSC curves. The enthalpy change corresponding to this exothermic peak approaches zero when going from pure pyridine to pure *n*-hexane. Dependence of the enthalpy change on the pyridine concentration is suggestive that the non-equilibrium state of the immersed HSA results from the HSA-pyridine interactions 'frozen' at the lower temperature. Most likely the temperature-initiated exothermic peak observed on the DSC curves reflects the swelling of HSA by pyridine.

Keywords: DSC, exothermic peak, human serum albumin, non-equilibrium state, pyridine–*n*-hexane mixtures

Introduction

Thermostability is an important characteristic of proteins placed in non-conventional environments like reverse micelles and/or organic solvents in which there is no bulk water stabilizing the protein structure through the hydrophobic effect. In particular, interest in data on thermostability of proteins in non-aqueous media is caused by use of 'non-conventional environments' in the enzymatic catalysis [1–5] and by the known phenomenon of increased thermostability of proteins suspended in organic solvents [6–8].

Differential scanning calorimetry (DSC) was well used for examining the thermostability of proteins in aqueous solutions [9, 10], or in reverse micelles in

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organic solvents [11, 12]. However, less work has been done concerning the DSC study of solid proteins suspended in organic solvents. So, endothermic peaks reflecting the denaturation of the protein molecules were observed on the DSC curves for solid preparation of ribonuclease immersed in organic solvents [8]. Reported effect of hydration [8] on the denaturation peak of the suspended protein was similar to data for chymotrypsinogen and lysozyme in their pure solid state [13,14]. Denaturation peak and its hydration dependence were found also for ribonuclease immobilized on Celite in organic solvents [15] and for human serum albumin (HSA) immersed in organic solvents [16].

Recently we observed, most likely for the first time, exothermic peaks on the DSC curves for the HSA preparation immersed in organic solvents [16]. Such exothermic peaks were detected for relatively low temperature region (65÷75°C for suspensions in dimethyl sulfoxide and 55÷90°C for suspensions in pyridine with water additions). Hence, they should not be related to the chemical destructive processes.

Temperature-initiated exothermic peaks on the DSC curves of protein suspensions showed that the state of HSA immersed in organic solvents is not the equilibrium state at the near room temperature. One possibility was that the non-equilibrium state was caused by the properties and structure of a protein preparation (or protein molecules) itself. Since such low temperature exothermic peaks were not observed for solid HSA immersed in *n*-hexane or exposed on air [16], then it might mean that there are kinetic constraints for this exothermic process in *n*-hexane or on air. Active organic solvents (like pyridine or dimethyl sulfoxide) might diminish a potential barrier for the temperature-initiated eliminating of the non-equilibrium status. Second possibility is that the exothermic DSC peaks reflect the organic solvent–protein interactions 'frozen' at the room temperature.

In the present work we have reported the DSC data for HSA immersed into pyridine–*n*-hexane mixtures of a variable composition. The negative enthalpy change extrapolated from the mixture to pure *n*-hexane would show that the non-equilibrium state is caused by internal features of the preparation and does not reflect the only solvent–protein interactions.

Experimental

Organic solvents were purified by standard techniques [17]. *n*-Hexane was distilled over metallic sodium. Pyridine was dried over sodium hydroxide and distilled over calcium hydride. The water content in the solvents was checked by the Karl Fischer titration method. It was found as 0.001 mol l^{-1} in *n*-hexane, and 0.12 mol l^{-1} in pyridine. The HSA preparation (A-1887, >96% albumin, essentially fatty acid free) was purchased from Sigma. To dry HSA, protein samples were kept for several hours at 298 K and at pressure $<10^{-3}$ mm Hg until the mass of sample was found constant.

The calorimetric experiments were performed on the differential scanning calorimeter (DSC-111 Setaram) which had been significantly modernized [16]. To calibrate calorimeter, DSC curve for corundum was measured in the temperature interval of interest.

The recording of the DSC curves for HSA suspensions was performed in a tightly closed titanium cell with the Teflon liner. Total volume of this titanium cell was 120 μl. Typically, 5÷8 mg of a dried HSA and 50÷100 μl of an organic solvent were placed in this titanium cell. DSC curves of heterogeneous mixtures were determined in the temperature range 25–120°C with the heating rate 2°C min^{-1}. Heating rate was chosen slow enough to minimize the initial step of the thermal stabilization of the DSC instrument. Reference material was the same organic solvent. The baseline for each solvent composition was measured by scanning a solvent itself against the same reference material that was used in the heterogeneous mixture scanning. Final DSC curves were obtained by subtracting the baseline curve from the HSA sample curve. Such a subtracted curve as a small difference of great values has a significant inaccuracy which does not allow estimating the confident absolute heat capacities of HSA and its variation upon the solvent composition. Nevertheless, the effect of the solvent composition on the peak areas may be estimated from the subtracted curve.

In order to determine the peak area, the final subtracted DSC curve was shown on the computer display. The beginning and the end of the observed peak were determined visually and connected by a straight line. Then, the area enclosed by the curve line and this straight line was found by numerical integration. Final enthalpy changes corresponding to the DSC peaks were reproducible within ±250 kJ mol^{-1}. Reproducibility of temperatures of the observed exothermic peak maximum was ±4°C. Some second runs of the HSA suspensions were also performed. No reversible phenomena were found. Since the heat evolution of most interest was exothermic, and therefore, the HSA sample state preceding this heat event was non-equilibrium, no extrapolations of the DSC exothermic peak characteristics toward smaller scanning rates were performed.

Results and discussion

Some of the obtained DSC curves for a HSA sample in pyridine–*n*-hexane mixtures of various composition are shown on Fig. 1. As one can see from Fig. 1, the thermal scanning of the HSA suspensions in *n*-hexane (curve 1) does not demonstrate any features. According to the previous results [16], when the dried HSA sample is immersed into *n*-hexane, endothermic denaturation peak is suppressed. Since the DSC curves for HSA immersed in *n*-hexane and exposed on air were found to be similar [16], we considered that the curve (1) in Fig. 1 does not include the significant contributions from the HSA–*n*-hexane interactions. Adding pyridine to *n*-hexane causes the broad exothermic peak on the DSC

curve. Increase of the pyridine concentration in the mixture changes both the position of the peak maximum and its area. Such exothermic peaks in Fig. 1 show that the state of HSA immersed in pyridine–*n*-hexane mixtures is the non-equilibrium state.

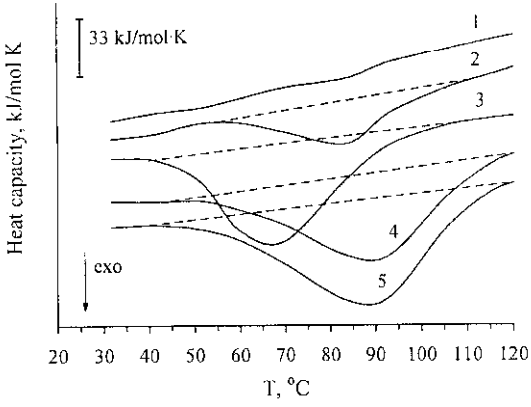

Fig. 1 DSC curves of the HSA immersed in pyridine–*n*-hexane mixtures of different composition. Pure *n*-hexane (1); 0.15 mol fraction of pyridine (2); 0.33 mol fraction of pyridine (3); 0.62 mol fraction of pyridine (4); pure pyridine (5). Enthalpy change for each exothermic peak was found from the area enclosed by the DSC curve and the dashed straight line

In Fig. 2 the negative enthalpy changes ΔH corresponding to the exothermic DSC peaks are plotted against the mol fraction x of pyridine in binary mixtures with *n*-hexane. As it can be seen from Fig. 2, the dependence for the enthalpy changes demonstrates apparently the saturation. Concentration of pyridine exceeding mol fraction 0.5 does not influence significantly on the ΔH values. However, when the mol fraction of pyridine is less than 0.2, the enthalpy changes are significantly more positive. When going to the HSA suspension in a pure *n*-hexane, the enthalpy change ΔH for this exothermic heat evolution is thought to tend to zero. General tendency in Fig. 2 is similar superficially to the well-known Langmuir sorption model. With the only goal to illustrate the tendency to the Langmuir-like saturation, the curve was drawn in Fig. 2 according to the empirical equation $\Delta H=abx/(1+bx)$. No any additional meaning was ascribed to the parameters a and b.

Evidently, such a strong tendency is not consistent with the assumptions that a) the non-equilibrium state of a protein sample in pyridine mixtures is caused by the internal properties of the HSA preparation, and b) pyridine only reduces the potential barrier hindering the elimination of this non-equilibrium status at lower temperatures.

Earlier we examined the enthalpy changes corresponding to the isothermal immersion of HSA into water–pyridine mixtures at 298 K [18, 19]. Sharp drop of

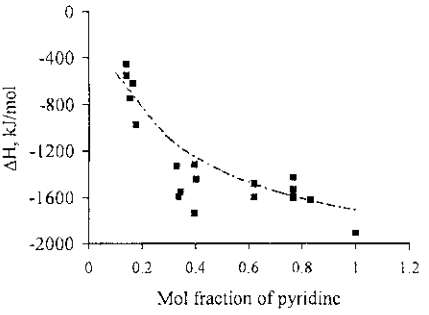

Fig. 2 Enthalpy changes ΔH corresponding to the exothermic DSC peaks plotted against the mol fraction x of pyridine in binary mixtures with n-hexane. Curve was drawn according to the equation: $\Delta H=abx/(1+bx)$, where a=–2260±208 kJ mol^{-1}, b=3.7± 1.0 (mol fraction)$^{-1}$

the enthalpy values was observed when some critical water content was reached. Considering calorimetric and water sorption data for HSA in water–organic mixtures of various nature, this enthalpy drop was interpreted by us as indication of the disruption of protein–protein contacts in a solid phase caused by water and/or an organic solvent [19, 20].

Water acts as the 'lubricant' that makes the protein immersed in organic environment less rigid [21, 22]. The rise of temperature has the capability also to increase the mobility of the protein fragments. Probably, the exothermic peaks observed during the thermal scanning of the HSA suspensions in pyridine (Fig. 1) and the sharp negative changes of enthalpy corresponding to the immersion of HSA into water–pyridine mixtures [18, 19] include the components of the similar nature. Both kinds of the exothermic enthalpy changes are assumed to reflect the swelling of the biopolymer preparation involving the disruption of protein–protein contacts (or solution of a solvent in the solid protein phase). Negative values of the measured enthalpy changes in Fig. 2 and their Langmuir type saturation upon the pyridine mol fraction are suggestive that the temperature-initiated protein swelling is followed with the specific interactions (hydrogen bonding) between HSA and pyridine.

It is worth to mention that HSA placed in the solvent mixtures may be spontaneously hydrated with the small residual amounts of water in pyridine. Using the water sorption constant for HSA in pyridine and the capacity of the water monolayer on HSA [18, 19], the maximally possible amount of water on HSA immersed in pure pyridine was estimated as not exceeding 3% for given experimental conditions at the room temperature. Since the water sorption by HSA in pyridine is the slightly exothermic process [19], this sorbed water amount has the potential to be endothermically desorbed when the temperature rises.

It should be mentioned also that the DSC curves for the suspension in pure pyridine measured in the present study for HSA (Sigma) and examined earlier

for HSA (Reanal, Hungary) [16] have the differences. Exothermic effects for the Sigma preparation are observed at lowered temperatures and in a more narrow temperature range in comparison with the DSC curves for the Reanal preparation. It is known that the solid state albumins are able to form aggregates [23, 24]. Such an aggregation of the protein is likely responsible for differences in the DSC curves of different preparations. This is confirmed by the fact that after maintaining the pure solid Sigma sample for 3 months at 25°C the DSC curve of the HSA suspension in pyridine approached that observed for the Reanal preparation in [16]. Such 'aging' of the HSA (Sigma) preparation resulted in the broadening of the exothermic peak and in the rise of temperature of the peak maximum.

This 'aging' effect corresponds also to the consideration of the HSA–pyridine interactions in terms of swelling of the biopolymer. Swelling of the 'aged' (and more aggregated) HSA sample should be more complicated and needs higher temperature.

In Fig. 3 we have plotted the temperatures (T_{max}) of the maximum of the exothermic peak against the pyridine mol fraction in the solvent. T_{max} may be considered as the kinetic characteristic of the exothermic swelling process. As shown in Fig. 3, this dependence demonstrates the minimum of kinetic stability at mol fraction 0.3÷0.35 of pyridine in the solvent mixture.

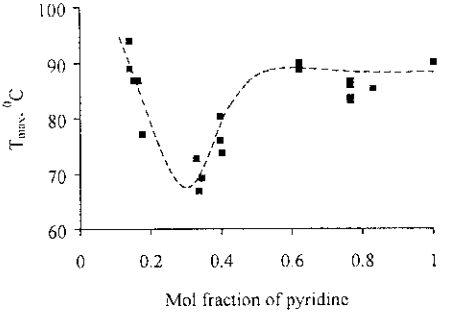

Fig. 3 Temperatures T_{max} of the exothermic peak maximum plotted against the mol fraction x of pyridine in binary mixtures with *n*-hexane

Non-equilibrium state of HSA in pyridine–*n*-hexane mixtures should increase the free energy of the HSA preparation. From consideration of the exothermic DSC peaks in terms of the Langmuir-like swelling of HSA it follows that this free energy increase should be bigger for HSA suspensions in more concentrated pyridine solutions. Then, the different shape of the dependences in Figs 2 and 3 indicates that the solvent composition influences on the thermodynamic and kinetic stability of HSA not in parallel. Thermodynamically less stable (at the room temperature) suspensions in more concentrated pyridine solutions may have greater kinetic stability (i.e. non minimal T_{max}).

Conclusions

DSC demonstrates the temperature-initiated exothermic heat evolution due to the solid human serum albumin (HSA) immersed into *n*-hexane–pyridine mixtures. This exothermic process clearly shows that HSA suspended has the non-equilibrium status. Non-equilibrium status of the HSA suspensions is strongly solvent-dependent. Both the enthalpy change corresponding to the exothermic heat evolution and the heat evolution maximum temperature vary significantly upon the composition of the *n*-hexane–pyridine mixtures. Exothermic peak disappears eventually when going from the binary mixtures to the *n*-hexane suspension. Extrapolation of the corresponding enthalpy change from the binary mixtures data to pure *n*-hexane gives the zero value. These facts lend support to the idea that non-equilibrium state of the suspended HSA results from the HSA–active organic component (pyridine) interactions 'frozen' at the near room temperature.

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