Journal of Thermal Analysis and Calorimetry, Vol. 85 (2006) 3, 593–600

EFFECT OF SALT CONCENTRATIONS ON THE DISPLACEMENT ADSORPTION ENTHALPIES OF DENATURED PROTEIN FOLDING AT A MODERATELY HYDROPHOBIC SURFACE

X. P. Geng[1,4*], *Y. N. Wu*[1,2], *J. R. Song*[3], *X. D. Geng*[4], *J. W. Xing*[2] *and Z. M. Lei*[1]

[1]College of Environment & Chemical Engineering, Xi'an Politechnic University, Xi'an 710048, China
[2]College of Textile Engineering & Material, Xi'an Politechnic University, Xi'an 710048, China
[3]Institute of Chemical Engineering, Northwest University, Xi'an 710069, China
[4]Institute of Modern Separation Science, Northwest University, Xi'an 710069, China

The displacement adsorption enthalpies (ΔH) of the refolding of lysozyme (Lys) denatured by 1.8 mol L^{-1} guanidine hydrochloride (GuHCl) on a moderately hydrophobic surface at 298 K, pH 7.0 and various $(NH_4)_2SO_4$ concentrations were determined by using a Micro DSC-III calorimeter. The study shows that the effect of salt concentrations on the three fractions of the enthalpy is that with increasing $(NH_4)_2SO_4$ concentrations, the molecular conformation enthalpy of the adsorbed Lys has probably no distinct change at 1.8 mol L^{-1} GuHCl; the adsorption affinity enthalpy (exothermic) becomes more negative; and the dehydration enthalpy (endothermic) decreases. At lower salt concentrations, the dehydration, especially squeezing water molecules led by molecular conformation, which leads to an entropy-driving process, predominates over the adsorption affinity (also including the orderly orientation of molecular conformation), while at higher salt concentrations, the latter is prior to the former for contribution to ΔH and induces an enthalpy-driving process. Also, the optimal $(NH_4)_2SO_4$ concentration favoring refolding and renaturing of Lys denatured by 1.8 mol L^{-1} GuHCl was found.

Keywords: *adsorption, hydrophobic surface, lysozyme, microcalorimetry, protein folding, salt effect*

Introduction

The investigation of protein adsorption with simultaneously refolding on the surface of hydrophobic interaction chromatography (HIC) is a new research field of folding, renaturing and purification technology of protein, which has just risen recently. A moderate salt concentration becomes one of the important factors to improve the refolding efficiency and bioactive recovery of denatured protein. The effect of salt concentration on retention of protein in HIC has been intensively studied in the past decades [1–7]. Horvath and his coworkers [2] considered that the principal parameters which determine the effect of salt on the retention were salt molality and the molal surface tension increment of the salt as they investigated retention behavior in HIC with the solvophobic theory. According to the theory, increase in salt concentrations or using the salts enhancing surface tension facilitates retention of proteins. However, all those investigations, including folding, renaturing and purification technology of protein [8], are limited to the retention behavior of protein on a moderately hydrophobic surface. It is only the directly measured displacement adsorption enthalpy of protein folding on a moderately hydrophobic surface that can provide at an angle of thermodynamics the most reliable and direct method to explore the effects of salt concentration and other variations on the molecular mechanism of hydrophobic interaction during the process of protein refolding and renaturing. Chen and his coworkers [9] reported that adsorption enthalpy of myoglobin onto butyl- and octyl-sepharose were directly measured by using isothermal titration calorimetry (ITC) and the effect of salt concentration on the interaction mechanism was investigated. The measured adsorption enthalpies decreased with increasing salt concentrations and the result was attributed to the reduction in the dehydration heat and the enhancement of heat released by the hydrophobic interaction. Although the adsorption enthalpy was used to research the mechanism of hydrophobic interaction with altering salt concentrations, it has not been involved in the folding and renaturing of denatured protein. Recently, using a microcalorimetric method suitable for biological process, we directly measured the displacement adsorption enthalpies (ΔH) of guanidine-denatured lysozyme (Lys) adsorption with simultaneously refolding on the PEG-600 (a silica base – HIC packings, the end-group of polyethylene glycol) surface at 298 K, 2.1 mol L^{-1} $(NH_4)_2SO_4$ [10]. Since it is of great significance to further understanding the

* Author for correspondence: xinpenggeng@163.com

1388–6150/$20.00

Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

molecular mechanism of protein adsorption with refolding in HIC to take attention to investigation of salt concentration dependence of the displacement adsorption enthalpies of denatured protein folding at a moderately hydrophobic surface, in this paper, as first step of the investigation, we choose a fixed concentration of denaturing agent, 1.8 mol L^{-1}guanidine hydrochloride (GuHCl), at which the minimum displacement adsorption enthalpy existed in the previous study [10]. In order to analyze displacement adsorption enthalpy ΔH of protein for investigating the effects of salt concentrations, in theory, we can also follow the idea in the previous study [10] that the ΔH of denatured protein folding on a moderately hydrophobic surface may be divided into three fractions: (a) dehydration enthalpy ΔH_d (endothermic); (b) adsorption affinity or hydrophobic interaction enthalpy ΔH_a(exothermic); and (c) molecular conformation enthalpy ΔH_m (exothermic; the endothermic effect can be included in ΔH_d). The net consequence of ΔH affected by salt concentrations can be attained by analyzing the effect of salt concentrations on the three fractions of enthalpy. Moreover, the entropies are evaluated by combining the measured equilibrium adsorption amounts at various salt concentrations at 298 K so as to probe thermodynamically the molecular mechanism of protein adsorption with refolding on a moderately hydrophobic adsorbent surface with altering salt concentrations.

Experimental

Materials

PEG-600 packings made of a silica base-HIC packings (particle size, 6.5μm; pore diameter, 30 nm; the end-group of polyethylene glycol) was obtained from the Institute of Modern Separation Science, Northwest University, China.

Lysozyme (Lys, chicken egg white) was purchased from Sigma Co. (St. Louis USA). Guanidine hydrochloride (GuHCl) bought from Shanghai State-medicine Group Chemical Reagent Ltd. Co., ammonium sulfate ($(NH_4)_2SO_4$) obtained from Tianjin Nankai Chemical Reagent Co., potassium phosphate monobasic (KH_2PO_4) purchased from Tianjin Dengfeng Chemical Reagent Co. All the chemicals except Lys are analytic grade. The deionized water was prepared with Milli-Q Academic (Millipore Co. Ltd, USA).

Preparations of denatured and calorimetric Lys solutions

Partly denatured Lys solutions: 1.0 mg mL^{-1} Lys solutions with 0.05 mol L^{-1} KH_2PO_4 (pH 7.0) were prepared with 1.8 mol L^{-1} GuHCl and standing for 24 h at 298 K.

Calorimetric Lys solutions: 0.40 mg mL^{-1} Lys, separately 0.0, 0.3, 0.9, 1.5, 1.8, 2.1 and 2.4 mol L^{-1} $(NH_4)_2SO_4$, 1.8 mol L^{-1} GuHCl and 0.05 mol L^{-1} KH_2PO_4 (pH 7.0).

Methods

The concentrations of Lys solution were measured with a UV-Vis spectrophotometer (Model SHIMADZU UV-2450). A centrifugal machine (Type 800) for the separation of Lys solutions from PEG-600 packings and an isothermal vibrator (Type SHA-C/THZ-82) for adsorption of Lys were all made by Guohua Electromachine Co., Changzhou, China.

Microcalorimetric measurement

The calorimetric operations were carried out by a Micro DSC-III instrument (Setaram, Calurie, France), including a calorimeter, a controller, a computer and a printer-plotter.

Transfer 0.500 mL calorimetric Lys solutions with a syringe into the lower chamber of 'measurement' mixing vessel and 'reference' mixing vessel, respectively. Put 20±0.01 mg PEG-600 packings in the upper chamber of the 'measurement' vessel, the corresponding 'reference' one being empty. The calorimetric operation of Lys solution sample at 298±0.001 K is as same as that in the previous study [10]. The detection limit and calorimetric resolution of signal were 0.2 μW and 40 nW, respectively.

The procedure to measure the blank heats, Q_{blank}, which include both the mixing (wetting) heats of PEG-600 packings with solutions with absent Lys, Q_{mix}, and the rubbing heats produced due to the rod moving, Q_{rub}, was as same as that of Lys solution sample except Lys absent in the liquid sample (1.8 mol L^{-1} GuHCl, x mol L^{-1} $(NH_4)_2SO_4$, 0.05 mol L^{-1} KH_2PO_4, pH 7.0).

Adsorbed amount determination

In order to determine the adsorbed amounts of Lys at the surface of PEG-600, which correspond to the calorimetric processes, the mixtures of PEG-600 packings and the Lys in various $C_{(NH_4)_2SO_4}$ solutions with the same ratios (W/V) as that in the calorimetric mixing batch vessel employed were taken to shake for 5 h at 298±0.5 K in the SHA-C/THZ-82 isothermal vibrator. The obtained supernatants by centrifuging the mixtures at 2000 rpm were detected at 280 nm by SHIMADZU UV-2450 Spectrophotometer to determine the concentrations of Lys. Based on the differences of equilibrium concentra-

tion between before and after adsorption, the adsorbed amounts of Lys corresponding to the systems in mixing batch vessel were calculated.

Results and discussion

Typical calorimetric thermograms

In order to obtain the displacement adsorption enthalpies, Q_i, the heats directly measured by the microcalorimeter during contacting the partly denatured Lys solution samples with PEG-600 packings in the mixing batch vessel, Q_{obs}, should be corrected by the blank heats, Q_{blank}, which include both the mixing (wetting) heats of PEG-600 packings with solutions with absent Lys, Q_{mix}, and the rubbing heats produced due to the rod moving, Q_{rub}. That is:

$$Q_i = Q_{obs} - Q_{mix} - Q_{rub} \quad (1)$$

while $$Q_{blank} = Q_{mix} + Q_{rub} \quad (2)$$

then $$Q_i = Q_{obs} - Q_{blank} \quad (3)$$

The calorimetric curves of Q_{obs} (contained 0.4 mg mL^{-1} Lys) and Q_{blank} (absent Lys) can be determined as the solution (1.8 mol L^{-1} GuHCl, x mol L^{-1} $(NH_4)_2SO_4$, 0.05 mol L^{-1} KH_2PO_4, pH 7.0) mixes with PEG-600 packings. As an example, the obtained curves for x being zero (no salt) are illustrated in Fig. 1.

Each area under the two curves represents the heat effect of the individual operation and the exact data of each area can be calculated with the multitask (simultaneous acquisition and processing) and multimodal software package offered by Setaram Micro DSC-III. Every calorimetric test in this presentation was performed more than four times. The individually measuring results of blank heats for 0 mol L^{-1} $(NH_4)_2SO_4$ are listed in Table 1.

The mean value of Q_{blank} is –187.9±1.5 mJ, and the corresponding relative average error of it is 0.8%, indicating the measuring results to be very satisfactory. Notably, unlike the measuring blank heats in other studies [10, 11], in which Q_{blank} was constant with changing concentrations of GuHCl under the same other conditions, the measuring blank heats change with altering concentrations of ammonium sulfate. The determined data are listed in Table 2.

The Q_{blank} values are all negative and their released heats decrease with increasing $(NH_4)_2SO_4$ concentration. The results exhibit that the solvation of

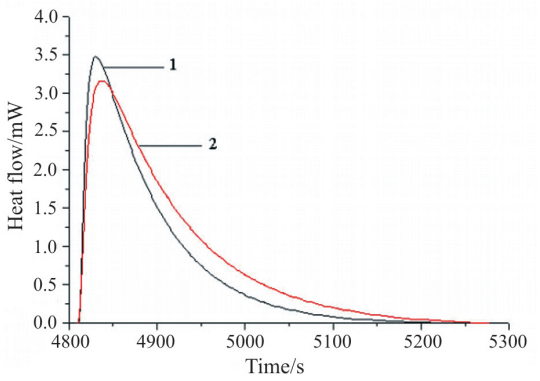

Fig. 1 Typical calorimetric curves of the involving fractions during mixing Lys solution with PEG-600 at 298±0.001 K, 1–Q_{blank} (including the Q_{rub} and Q_{mix} of mixing the PEG powder with blank solution without Lys), 2-Q_{obs}(at 1.8 mol L^{-1} GuHCl, 0 mol L^{-1} $(NH_4)_2SO_4$, 0.4 mg mL^{-1} Lys)

Table 1 Q_{blank} in solutions (1.8 mol L^{-1} GuHCl, 0.05 mol L^{-1} KH_2PO_4, pH 7.0) at 298±0.001 K

No.	Q_{blank}/mJ
1	–183.1
2	–191.1
3	–184.5
4	–189.3
5	–191.5
mean	–187.9±1.5

Table 2 The displacement adsorption enthalpies of partly denatured Lys at the PEG-600/solution(1.8 mol L^{-1} GuHCl, x mol L^{-1}$(NH_4)_2SO_4$, 0.05 mol L^{-1} KH_2PO_4, 0.4 mg mL^{-1}Lys, pH 7.0) interface at 298±0.001 K

$C_{(NH_4)_2SO_4}$/mol L^{-1}	Q_{blank}/mJ	Q_{obs}/mJ	Q_i/mJ	ΔH_i/kJ mol^{-1}
0.0	–187.9	–181.0±5.4	6.9	496.8
0.3	–181.9	–181.1±2.6	0.8	57.6
0.9	–170.0	–172.8±1.8	–2.8	–201.6
1.5	–158.0	–164.2±3.6	–6.2	–446.4
1.8	–152.1	–160.5±4.7	–8.4	–604.8
2.1	–146.1	–155.4±2.0	–9.3	–669.6
2.4	–140.1	–159.2±5.3	–19.1	–1375.2

Q_{obs}, observed value; Q_{blank}, including the rubbing heats produced due to the rod moving Q_{rub} and Q_{mix} of mixing the PEG powder with blank solution without Lys; Q_i, total heat of displacement adsorption of Lys, attained by Eq. (3). In the quantification of the ΔH_i, only the initial concentration (0.4mg mL^{-1}) of the Lys were used.

hydrophobic PEG-600 surface (the mixing or wetting heat dominates over almost ninety percent of the blank heat, data not shown) reduces with rise of salt concentrations, showing the hydrophobicity of PEG-600 surface to be increasing with the increment of $(NH_4)_2SO_4$ concentrations. The effect of hydrophobicity of PEG-600 surface on the measured enthalpies will be discussed later.

Equilibrium adsorption isotherms

The equilibrium adsorption isotherms of Lys molecules denatured by 1.8 mol L^{-1} GuHCl are illustrated in Fig. 2.

Under the conditions of salt absence or lower concentrations (less than 1.5 mol L^{-1}) of ammonium sulfate, the adsorbed amounts are very little, especially the ones corresponding to the lower equilibrium concentrations of Lys may be almost negligible. It is indicated that the lower salt concentrations have little effect on the adsorption of partly denatured Lys at the PEG-600 surface. This is because the hydrophobicity of adsorbent surface is weak at lower salt concentration, as discussed above, and the electrostatic repulsion and van der Waals force between the protein molecules are stronger [9, 12], which leads to lower adsorption affinity of protein at the hydrophobic surface. Whereas, the adsorbed amounts of partly denatured Lys increase significantly at higher salt concentrations (more than 1.8 mol L^{-1} $(NH_4)_2SO_4$) and are greater than that at lower salt concentrations as the salt concentrations increase. This is because the addition of $(NH_4)_2SO_4$ makes surface tension increment of an aqueous salt solution and the energy of cavity formation in solution increasing [9]. This leads to a more hydrophobic aqueous environment for both the partly denatured Lys molecules and ligands of PEG-600 surface to bind protein.

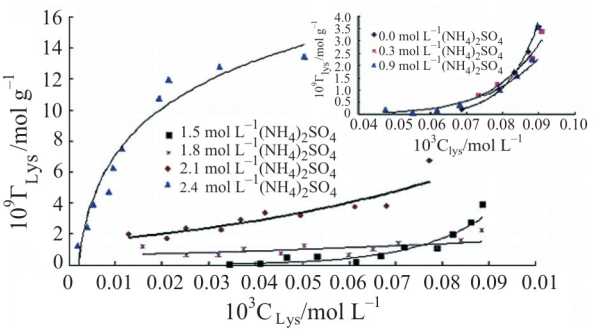

Fig. 2 The adsorption isotherms of partly denatured Lys from the solutions (x mol L^{-1} $(NH_4)_2SO_4$, 1.8 mol L^{-1}GuHCl, 0.05 mol L^{-1} KH_2PO_4, pH 7.0) by PEG-600 surface at 298 K

In order to explore the molecular mechanism of hydrophobic interaction of protein with a moderately hydrophobic surface with altering salt concentrations, the equilibrium adsorption amount data are measured under the same conditions as the microcalorimetric determination. According to the reverse equilibrium thermodynamics of adsorption in our previous studies [13–17], some important consequence can be obtained. Herein, under the investigation of stoichiometric displacement theory (SDT) [18] for partition coefficient P_a of adsorbate, which was derived from a true surface concentration based on the correct volume of adsorbed layer [13] (P_a=n^s/(CN_0V_0 n^s_{max}), n^s and n^s_{max} are the adsorbed amount and the maximum one, respectively, of adsorbate corresponding to equilibrium concentration C, N_0 is Avogadro constant, V_0 represents a molecular volume of adsorbate), and under the calculation of free energy ΔG from P_a: ΔG =–2.303RTlogP_a (R is the universal gas constant, T stands for Kelvin temperature) [14–17], we obtained P_a and ΔG according to equilibrium adsorption data. Then, combining the calorimetric enthalpies measured directly ΔH, the entropies can be evaluated. The results are listed in Table 3. The relative average error of Γ in Table 3 is about 2.03%, and that of P_a is 10.84%.

It should be pointed out that the data at the concentrations less than 1.5 mol L^{-1} $(NH_4)_2SO_4$ are not listed in Table 3 because the corresponding very little adsorbed amounts of Lys, as mentioned above, make the calculating results unreliable. It is shown that the calculated free energies decrease with salt concentration increment. This can be explained by the enhancement of surface tension of the aqueous solution by the addition of salts. That is, a higher surface tension increment of an aqueous salt solution can make the energy of cavity formation in solution increase and the

Table 3 Adsorption thermodynamic functions of 0.4 mg mL^{-1} Lys adsorbed by PEG-600

$C_{(NH_4)_2SO_4}$	10^5C_{Lys}	$10^8\Gamma$	LogP_a	ΔG	ΔH	$T\Delta S$
mol L^{-1}	mol L^{-1}	mol g^{-1}		kJ mol^{-1}	kJ mol^{-1}	kJ mol^{-1}
1.5	2.620	3.94	2.1118	–12.05	–446.4	–434.4
1.8	2.519	6.453	2.3433	–13.73	–604.8	–591.4
2.1	2.102	16.892	2.8396	–16.20	–669.6	–653.4
2.4	0.871	47.668	3.6729	–20.96	–1375.2	–1354.2

solution environment more hydrophobic, which provides a more favorable environment for both the hydrophobic interaction (adsorption affinity) between protein and a moderately hydrophobic adsorbent and the dehydration for protein folding. Therefore, the pushing force ($-\Delta G$) of protein adsorption and refolding on a moderately hydrophobic adsorbent surface can be enhanced as $(NH_4)_2SO_4$ concentration increase. The discussion of enthalpy and entropy will be seen in the next section.

Displacement adsorption thermodynamics of protein

Table 2 listed the micro-calorimetric determination of the displacement adsorption enthalpies, ΔH, of the refolding of Lys which originally had been partly denatured by 1.8 mol L^{-1} GuHCl on the surface of HIC packings (end group PEG-600) at 298 K, pH 7.0 and various $(NH_4)_2SO_4$ concentrations. It should be noted that in the quantification of the ΔH_i, although the equilibrium concentration of protein adopted should be reasonable theoretically, only the initial concentrations (0.4 mg mL^{-1}) of the partly denatured Lys were used in the prevention of both the difficulty of calculating lower equilibrium adsorption amounts and the interactions between bounded protein confronted in the high binding capacity for calorimetric measurements. In fact, it is in accordance with the literature [9] .

It is necessary to elucidate briefly the three fractions of enthalpy mentioned above before the effect of salt concentrations on ΔH of partly denatured protein refolding on a moderately hydrophobic surface is discussed. Dehydration enthalpy ΔH_d induced by the dehydration occurred both at the contacted region between the hydrated protein molecules and the hydrated hydrophobic ligands of surface as the protein molecules approaching the surface and between the hydrated protein molecule residues during the formation of the microdomains or intermediates of protein behaves an endothermic effect because of being an energy-requiring and entropy gain process. Adsorption affinity or hydrophobic interaction enthalpy ΔH_a led by the adsorption affinity process of protein molecules onto hydrophobic surface should be exothermic due to the entropy loss effect. Molecular conformation enthalpy ΔH_m produced by conformation of forming orderly domain of polypeptide amino acid residues behaves an exothermic. In order to discuss conveniently the effect of the enthalpy fractions, the endothermic effect induced by dehydration between the hydrated protein molecule residues, i.e. squeezing water molecules between the residues as the protein molecules refolding from its partly denatured state to native or intermediate state can be included to ΔH_d.

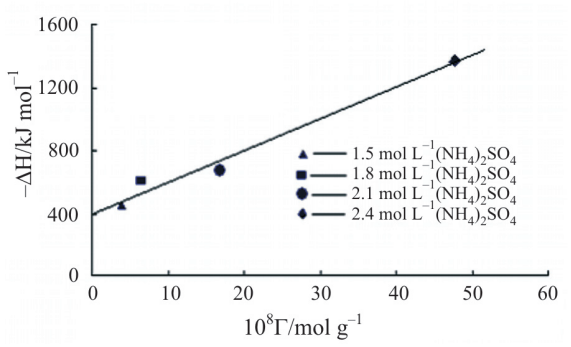

Fig. 3 The correlation of ΔH with Γ of Lys (C_0 = 0.4 mg mL^{-1}) denatured by 1.8 mol L^{-1} GuHCl on PEG-600 surface at 298 K at higher $C_{(NH_4)_2SO_4}$

Since both ΔH_a induced by hydrophobic interaction and ΔH_m produced by orderly domain of polypeptide amino acid residues are relatively predominant over ΔH_d led by dehydration at 1.8 mol L^{-1} GuHCl, compared with that at other GuHCl concentrations, as pointed out in the previous study [10], in order to interpret the salt concentration dependence of molecular mechanism of hydrophobic interaction in terms of adsorption enthalpies, it is necessary to analyze firstly the change of molecular conformation enthalpy with salt concentrations, especially with higher ones. The changing trend of ΔH values with the concentrations of $(NH_4)_2SO_4$ is as same as that of equilibrium adsorption amounts, as be seen in Fig. 3 where the plot of ΔH values *vs*. Γ, the equilibrium adsorption amounts of Lys, is almost a linear line (the linear correlation coefficient is 0.9883). It seems that during the process of adsorption with simultaneously refolding of Lys partly denatured by 1.8 mol L^{-1} GuHCl on PEG-600 packing surface with various salt concentrations, the molecular conformations of the adsorbed Lys have probably no distinct change. Moreover, this inference can also be demonstrated by following investigation. According to Perkins *et al*. [4], the contact area of interaction between protein molecule and ligands of PEG-600 surface, which was

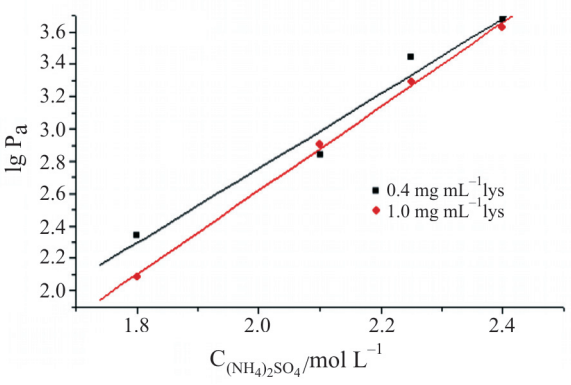

Fig. 4 Plot of lgP_a *vs*. $C_{(NH_4)_2SO_4}$

derived from the slope of the linear plotting of the logarithm of protein capacity factors *vs*. salt concentrations (shown in Fig. 4) based on the solvophobic theory [1, 2], is constant under the same experimental conditions as that in calorimetric measurement.

Based on the solvophobic theory [1, 2] , we have a linear equation:

$$lg\ P_a = J + SC \quad (4)$$

where J is the intercept, S denotes the linear slope and a constant related to protein-ligand contact area, and C stands for the salt concentration. P_a represents the capacity factor calculated from $P_a=C_S/C_i$, where C_S is the real concentration of protein on the hydrophobic surface, and C_i is the equilibrium concentration of protein. The linear plots of lg P_a *vs*. C for the initial concentrations of protein being 0.4 mg mL^{-1} and 1.0 mg mL^{-1}, respectively, are illustrated in Fig. 4. The linear regression coefficients corresponding to the initial protein concentration of 0.4 and 1.0 mg mL^{-1} are 0.9829 and 0.9991, respectively, which exhibited good linear correlation, indicating that the Lys molecules denatured by 1.8 mol L^{-1} GuHCl have a constant hydrophobic area contacting with the ligands of hydrophobic adsorbent PEG-600, which is independent of the concentration of $(NH_4)_2SO_4$. Meanwhile, the fact that the slope values corresponding to the initial protein concentration of 0.4 and 1.0 mg mL^{-1} are 2.31 and 2.59, respectively, and close to each other suggests that the contact area of interaction has little correlation with the protein concentration.

It should be emphasized that the inference that molecular conformations of the adsorbed Lys partly denatured by 1.8 mol L^{-1} GuHCl with various salt concentrations may be little change relates closely with the fact that the secondary and tertiary structures of Lys in solution under the same conditions were not destroyed completely [19]. The investigation of differential ultraviolet spectrum and circular dichroism (CD) showed that the denatured degree of Lys in solutions increased with the GuHCl concentrations from 0.4 to 2.6 mol L^{-1}, and the secondary and tertiary structures lost gradually, but Lys molecules were not all denatured completely, and even at 2.6 mol L^{-1} GuHCl, some secondary and tertiary structures remained [19]. In order to avoid too many denatured states during a process of refolding of completely denatured protein, the choice of moderately denatured Lys in this study is suitable for beginning exploration of salt concentration dependence of molecular mechanism of hydrophobic interaction of protein folding with solid in terms of adsorption thermodynamics.

Although the ΔH_m values at various salt concentrations seem to be unchanged, the exothermic enthalpies exhibited at 1.8 mol L^{-1} GuHCl because of more orderly structure induced by quite effective protein refolding contribute indeed to the measured ΔH values. Similarly, according to forgoing same reason, we can imply that among the entropies calculated by equilibrium adsorption amounts at 1.8 mol L^{-1} GuHCl and various salt concentrations, the fractions of entropy decrement for molecular conformation may be also identical with each other and take a part of the individual entropies. This means that in the following discussion, we can no longer consider the effects of molecular conformation on the changes of ΔH and ΔS with changing salt concentrations.

The result in Table 2 indicates that all the ΔH values of Lys adsorption with refolding onto PEG-600 surface decrease with increasing concentrations of ammonium sulfate. This is because $(NH_4)_2SO_4$ is a lyotropic salt and has significant solvation and a strong surface tension enhancement of an aqueous solution. The increment of surface tension of the aqueous solution results in a more hydrophobic aqueous environment for both protein and adsorbent. Therefore, a higher concentration of aqueous $(NH_4)_2SO_4$ solution provides a highly hydrophobic environment favoring the hydrophobic interaction between protein and adsorbent. Moreover, the water molecules existed in terms of the hydrated adsorbent and hydrated protein residue surface may reduce with increasing salt concentration. Therefore, on the one hand, the adsorption affinity enthalpy ΔH_a (exothermic) induced by hydrophobic interaction of Lys molecules with ligands of PEG-600 surface (substantially by van der Waals forces) becomes more negative with $(NH_4)_2SO_4$ concentrations; on the other hand, dehydration enthalpy ΔH_d (endothermic), i.e. the heat required for dehydration of hydrated protein molecules and hydrated ligands of adsorbent surface decreases with salt concentration increasing [9]. Therefore the total ΔH values of Lys adsorption with refolding onto PEG-600 surface decrease with increasing concentrations of ammonium sulfate.

It is shown in Table 2 that the ΔH values of denatured Lys with PEG-600 surface behave negative value until 0.9 mol L^{-1} $(NH_4)_2SO_4$, demonstrating an entropy-driving process for Lys adsorption at lower salt concentrations. It's dehydration, especially the squeezing water molecules from amino acid residues in protein interior that predominates over hydrophobic interaction (including the orderly orientation of molecular conformation) and promotes mainly the orderly water molecules in protein interior to be released to solution, indicating the entropy gain plays a major role at lower $(NH_4)_2SO_4$ concentrations. This may be in accordance with the fact that the adsorbed amounts of Lys corresponding to less than 1.0 mg mL^{-1} initial concentration of Lys solutions

onto PEG-600 surface at lower salt concentrations are almost undetected because of relatively weaker hydrophobic interaction. At higher salt concentrations, the ΔH values are all negative, indicating that the adsorption affinity or hydrophobic interaction (also including the orderly orientation of molecular conformation, as be shown above) induced by van der Waals forces predominates over dehydration. The gradually increasing exothermic effect behaves enhancement of heat released by only the hydrophobic interaction as salt concentrations increase because the contributions of conformation are regarded to be invariable. Moreover, it can be seen in Table 3 that the exothermic enthalpies at higher salt concentrations become major driving force of partly denatured Lys adsorption and refolding on the PEG-600 surface because the enthalpy values are less than that of corresponding entropy terms ($T\Delta S$) evaluated by equilibrium adsorption amounts. That is to say that at higher salt concentrations adsorption with simultaneously refolding of Lys partly denatured at 1.8 mol L^{-1} GuHCl at PEG-600 packing surface is an enthalpy-driving process induced by both hydrophobic interaction between the hydrophobic peptide chain of protein and a moderately hydrophobic surface and orderly orientation of molecular conformation. Also, Table 3 shows that the entropy values at higher salt concentrations are negative and decrease with salt concentration increment, revealing that the cooperation of hydrophobic interaction and molecular conformation making adsorbed protein molecules orderly orientation predominates over dehydration making released water molecules free. And, the steadily decreased entropy may also be mainly attributed to the strengthening of orderly state induced by hydrophobic interaction as salt concentrations increase. Obviously, at higher salt concentrations, the analysis of entropies evaluated by equilibrium adsorption amounts coincides with that of enthalpies measured by micro-calorimetry for the study of molecular mechanism of hydrophobic interaction. In sight of thermodynamic concept on enthalpy-entropy compensation, the decrease of entropies with salt concentration increment is compensated by the more exothermic enthalpies.

Notably, in Table 3 the ΔH and ΔG values at 2.4 mol L^{-1} $(NH_4)_2SO_4$ (–1375.2 and –20.96 kJ mol^{-1}, respectively) are all negative and lower in algebraic value than that at 2.1 mol L^{-1} $(NH_4)_2SO_4$ (–669.6 and –16.2 kJ mol^{-1}, respectively), which corresponded to the minimum enthalpy and was called as 'energy well' in the previous study [10]. This means that 2.4 mol L^{-1} $(NH_4)_2SO_4$ is more favorable to make Lys molecules denatured by 1.8 mol L^{-1} GuHCl refold to their more stable intermediate state than 2.1 mol L^{-1} $(NH_4)_2SO_4$. This concentration (2.4 mol L^{-1} $(NH_4)_2SO_4$) is just right the upper limit of range from 1.8~2.4 mol L^{-1} $(NH_4)_2SO_4$, adopted in HIC [20] for the same studied system imitated in this paper. From the trend of variation of both ΔG and ΔH values with salt concentrations, above 2.4 mol L^{-1} $(NH_4)_2SO_4$ concentrations seem more effective than those listed in Table 3, but, in fact, are impracticable because of the limit of solubility of the protein. One of the significances in this study is that it can provide theoretical basis of choosing the suitable salt concentration for retention of protein in HIC.

Conclusions

According to the dividing ΔH into three enthalpy fractions, the net consequence of ΔH affected by salt concentrations are obtained by analyzing the effects of salt concentrations on the three fractions of enthalpy. That is, with increasing $(NH_4)_2SO_4$ concentrations, the molecular conformation enthalpy of the adsorbed Lys has probably no distinct change at 1.8 mol L^{-1} GuHCl; the adsorption affinity enthalpy (exothermic) becomes more negative and the dehydration enthalpy (endothermic) decreases.

At lower salt concentrations, it is dehydration, especially squeezing water molecules of molecular conformation that predominates over hydrophobic interaction (including the orderly orientation of molecular conformation), indicating the entropy gain plays a major role. At higher salt concentrations, the adsorption affinity or hydrophobic interaction (also including the orderly orientation of molecular conformation) predominates over dehydration, and the adsorption with simultaneous refolding of Lys is an enthalpy-driving process. In sight of thermodynamic concept on enthalpy-entropy compensation, the decrease of entropies with salt concentration increment is compensated by the more exothermic enthalpies. The measured ΔH and calculated ΔG values show that 2.4 mol L^{-1} $(NH_4)_2SO_4$ is more favorable to make Lys molecules denatured by 1.8 mol L^{-1} GuHCl refold to their more stable intermediate state than 2.1 mol L^{-1} $(NH_4)_2SO_4$.

Acknowledgements

We thank the Natural Sci. Found. of Shaanxi Province, China (2004B25), Sci. & Tech. Found. of Xi'an City, China (GG200270), and both opening and visiting scholar Founds. of Shaanxi Key Lab. of Modern Separ. Sci. of Northwest University, China for their sponsoring the project.

References

1 W. R. Melander and Cs. Horvath, Arch. Biochem. Biophys., 183 (1977) 200.
2 W. R. Melander, D. Corradini and Cs. Horvath, J. Chromatogr., 317 (1984) 67.
3 Z. E. Rassi and Cs. Horvath, J. Liq. Chromatogr., 9 (1986) 3245.
4 T. W. Perkins, D. S. Mak, T. W. Root and E. N. Lightfoot, J. Chromatogr., A, 766 (1997) 1.
5 M. Lu and F. Tjerneld, J. Chromatogr., A, 766 (1997) 99.
6 L. Szepesy and Cs. Horvath, Chromatographia, 26 (1998) 13.
7 X. D. Geng, Q. Bai, Y. J. Zhang, X. Li and Dan Wu, J. Biotechnol.,113 (2004) 137.
8 X. D. Geng and Q. Bai, Sci. China (Ser.B), 32 (2002) 460.
9 Y. S. Tsai, F. Y. Lin, W. Y. Chen and C. C. Lin, Colloids Surf., A: Physicochem. Eng. Aspects, 197 (2002) 111.
10 X. P. Geng, H. F. Zhang, B. H. Wang, X. D. Geng and J. W. Xing., J. Therm. Anal. Cal., 82 (2005) 193.
11 X. P. Geng, H. F. Zhang, B. H. Wang, Y. N. Wu, X. D. Geng and J. W. Xing, 4th International and 6th Japan-China Joint Symp. Cal. Therm. Anal., (CATS-2005), Oct. 6–8, 2005 Fukuoka, Japan, p. 90.
12 K. S. Kim, S. Kim, H. J.Yang and D. Y. Kwon, Int. J. Food. Sci. Tech., 39 (2004) 385.
13 X. P. Geng, Y. N. Guo, X. Y. Wang and X. D. Geng, Proceedings of the 3rd China Int. Wool Textile Conf., 116, Sept. 26~28, 2002 Xi'an, China.
14 Y. Y. Chen and X. D. Geng, Chem. J. Chin. Univ., 14 (1993) 1432.
15 X. P. Geng, T. S. Han and C. Cao, J. Therm. Anal., 45 (1995) 157.
16 X. P. Geng, Thermochim. Acta, 308 (1998) 131.
17 X. P. Geng, F. X. Xie, H. F. Zhang, X. Y. Wang and J. W. Xing, Colloids Surf., A: Physicochem. Eng. Aspects, 257 (2005) 89.
18 X. D. Geng, Stoichiometric Displacement Theory and its application, Beijing: Sci. press, 2004, p. 82.
19 H. F. Zhang, Master thesis, Xi'an University of Engineering Science & Technology, China, 2005.
20 J. Zhang, Master Thesis, Northwest University, China, 1999.

DOI: 10.1007/s10973-006-7644-9