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MICROCALORIMETRIC DETERMINATION OF DISPLACEMENT ADSORPTION ENTHALPIES OF PROTEIN REFOLDING ON A MODERATELY HYDROPHOBIC SURFACE AT 308 K

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Both microcalorimetric determination of displacement adsorption enthalpies Δ*H* and measurement of adsorbed amounts of guanidine – denatured lysozyme (Lys) refolding on the surface of hydrophobic interaction chromatography (HIC) packings at 308 K were carried out and compared with that at 298 K. Study shows that both temperature and concentration of guanidine hydrochloride (GuHCl) affect the molecular mechanism of hydrophobic interaction of protein with adsorbent based on the analysis of dividing Δ*H* values into three kinds of enthalpy fractions. The adsorption in higher concentrations of GuHCl (>1.3 mol L^{-1}) at 308 K is an enthalpy-driving process, and the adsorption under other GuHCl concentrations is an entropy-driving process. The fact that the Lys denatured by 1.8 mol L^{-1} GuHCl forms a relatively stable intermediate state under the studied conditions will not be changed by temperature.

Keywords: *displacement adsorption enthalpy, hydrophobic interaction mechanism, liquid/solid system, lysozyme, microcalorimetry, protein folding*

Introduction

The behavior of protein adsorption with refolding on a moderately hydrophobic surface is very important in many research fields, such as protein renaturing and purification, process of metabolism, pathological changes and life process, etc. In the last few decades, many reports concerned focused on the effects of protein adsorption in hydrophobic interaction chromatography (HIC), specifically, the characterization of the retention behavior of protein in HIC has been intensively studied [1–5]. Recently the investigation of mechanism of simultaneously refolding and purification of protein in HIC [6] has both theoretical and practical significance. However, all these studies were still carried out in terms of retention behavior. Microcalorimetric determination of adsorption enthalpies of protein folding at liquid/solid interface is the most direct method to explore energetically the properties and rule of protein folding. Up to now, the directly measured enthalpy of protein adsorption with refolding onto a moderately hydrophobic surface has seldom performed because the analysis of the enthalpies is complicated and rather difficult for an adsorption process which is substantially a displacement adsorption process including both adsorption affinity of solute onto adsorbent and desorption of solvent from the adsorbent and the hydrated solute [7]. Chen and his coworkers [8] measured the adsorption enthalpies of two proteins with sepharose by isothermal titration calorimetry (ITC) at 298 and 310 K, respectively. They considered mainly the dehydration process of hydrophobic ligands of adsorbents and the hydrophobic interaction between proteins and hydrophobic ligands for the analysis of the adsorption enthalpies and obtained the mechanism of hydrophobic interaction affected by temperature with combining the adsorption isotherm assay. However, the investigation of directly measured adsorption enthalpy of denatured protein during adsorption with simultaneously refolding on the liquid/solid interface was just carried out recently. With a biomicrocalorimeter, we directly determined the displacement adsorption enthalpies (Δ*H*) of guanidine-denatured lysozyme (Lys) during the protein adsorption with simultaneously refolding on the surface of HIC packings at 298 K [1]. Furthermore, this viewpoint can be proposed that the measured Δ*H* may be divided into three enthalpy fractions induced by the corresponding subprocesses: (a) dehydration enthalpy ΔH_d (endothermic) induced by dehydration process occurred both at the contacted region between the hydrated denatured protein molecules and the hydrated hydrophobic ligands of surface as the molecules approach-

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ing the surface and between the hydrated protein molecules during the formation of the microdomains or intermediates of protein [9]; (b) adsorption affinity enthalpy ΔH_a (exothermic) led by the adsorption affinity process of protein molecules onto a moderately hydrophobic surface; (c) molecular conformation enthalpy ΔH_m (exothermic) produced by forming orderly domain of polypeptide amino acid residues. In order to discuss conveniently the effect of the enthalpy fractions, the endothermic effect induced by dehydration between the residues, i.e. squeezing water, as the unfolded protein molecules refolding from its denatured state to native or intermediate state can be included in ΔH_d. In this paper, the microcalorimetric measurement at 308 K imitating the one at 298 K [1] is performed, and the thermodynamic analysis with combining equilibrium adsorption amounts and the further exploration on the enthalpy fractions changing with denaturing extent are made. Also, the effect of temperature on the mechanism of protein adsorption with simultaneously refolding of denatured Lys on the hydrophobic PEG-600 surface is discussed.

Experimental

Materials

PEG-600 made of a silica base-HPHIC packings (particle size, 6.5 μm; 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 analytical grade. The deionized water was prepared with Milli-Q Academic (Millipore Co. Ltd, USA).

Preparations of denatured and calorimetric Lys solutions

A set of partially denatured Lys solutions with 1.0 mg mL^{-1} Lys with 0.05 mol L^{-1} KH_2PO_4 (pH 7.0) were prepared with different concentrations of GuHCl and standing for 24 h at 298 K. The Lys concentration in the reaction solution was 0.40 mg mL^{-1}. The C_{GuHCl} was 0.0, 0.40, 0.80, 1.30, 1.80, 2.20, and 2.60 mol L^{-1}, respectively. The other composites were 2.1 mol L^{-1} ammonium sulfate and 0.05 mol L^{-1} potassium phosphate monobasic (pH 7.0).

Method

The concentrations of Lys solution were measured with a UV-Vis spectrophotometer (Model 8453, Agilent Co., USA). 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 on the PEG-600 packing surface were all made by Guohua Electromachine Co., Changzhou, China.

Microcalorimetric measurement

A Micro DSC-III calorimeter (Setaram, Calurie, France) available for measurement of microcalorimetric data occurred in liquid-solid system [1] was used.

Transfer 0.500 mL 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 procedure of Lys solution sample at 308±0.001 K is as same as that at 298±0.001 K [1]. The detection limit and calorimetric resolution of signal were 0.2 μW and 40 nW, respectively.

The procedures to measure the two disturbing heats were as same as that of Lys solution sample except PEG-600 packings absent in the upper chamber for the rubbing heat of putting down operation rod and except Lys absent in the liquid sample for the mixing heat of PEG-600 packings with blank solution sample, respectively.

Adsorbed amounts

The mixtures of PEG-600 packings and Lys in various C_{GuHCl} solutions with the same ratios (W/V) as that in the mixing batch vessel employed were taken to shake for 4 h at 308±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 8453 UV-VIS Spectrophotometer to determine the concentrations of Lys. Based on the differences of equilibrium concentration 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 curves

To obtain the displacement adsorption enthalpy (Q_i or ΔH_i) of Lys on a moderately hydrophobic surface of PEG-600, the Q_{mix} denoting the mixing (wetting) heat

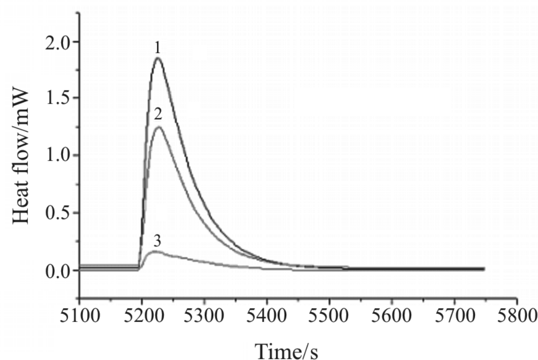

Fig. 1 Typical calorimetric curves of the involving fractions during mixing Lys solution with PEG-600 at 308±0.001 K; 1 – Q_{obs} (at 1.8 mol L^{-1} GuHCl), 2 – Q_{mix} (with Lys blank solution), 3 – Q_{rub}

of PEG-600 packings with solution with absent Lys (blank sample) and the rubbing heat Q_{rub} of pushing the rod should be subtracted from the directly measured heat effect Q_{obs} produced by mixing the denatured Lys solution sample with the packings in calorimetric mixing batch vessel. Then, the following expression exists:

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

Figure 1 shows the above three typical curves at 308 K, 1.8 mol L^{-1} GuHCl, 2.1 mol L^{-1} $(NH_4)_2SO_4$, 0.05 mol L^{-1} KH_2PO_4, pH 7.0.

Each area under the three 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 rubbing heats and mixing (wetting) heats at 308 K are listed in Table 1.

Table 1 Rubbing heats and mixing heats at 308±0.001 K

No.	Q_{rub}/mJ	Q_{mix}/mJ
1	–12.4	–113.4
2	–15.5	–105.2
3	–14.5	–98.0
4	–14.6	–110.5
5	–15.6	–110.2
6	–15.0	
mean	–14.6±0.4	–107.5±2.4

The mean values of measured Q_{rub} and Q_{mix} are –14.6±0.4 and –107.5±2.4 mJ, respectively, and the corresponding relative average errors of them are 2.74 and 2.23% respectively, indicating the measuring results to be quite satisfactory.

Equilibrium adsorption amounts

The equilibrium adsorption amounts of Lys molecules which are denatured by different concentrations of GuHCl and adsorbed from the solutions (2.1 mol L^{-1} $(NH_4)_2SO_4$, 0.05 mol L^{-1} KH_2PO_4, 0.4 mg mL^{-1} Lys, pH 7.0) by PEG-600 at 308 K are shown in Fig. 2. Also, that at 298 K in the previous study [1] are given for convenience of comparison.

With decreasing GuHCl concentrations (imitating gradual removal of denaturing agent in HIC), the adsorbed amounts firstly increase to the maximum value (0.5830 mmol g^{-1}) corresponding to 0.8 mol L^{-1} GuHCl and then decrease at 308 K, as the same changing tendency at 298 K. This is because during the process of adsorption with simultaneously refolding of Lys in variously denatured states on PEG-600 packing surface, the molecular conformations of the adsorbed Lys are different from each other due to the presence of GuHCl with various concentrations (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}, but Lys molecules were not all denatured completely, and even at 2.6 mol L^{-1} GuHCl, some secondary and tertiary structures remained [10]) and steric-hindered effect of the three-dimensional structure affecting adsorption [1]. However, as temperature increases, the relative magnitude of the adsorbed amounts is different in various GuHCl concentrations. Above 1.3 mol L^{-1} C_{GuHCl}, the values of adsorbed amounts of Lys at 308 K are greater than that at 298 K while below 1.3 mol L^{-1} C_{GuHCl} that at 308 K

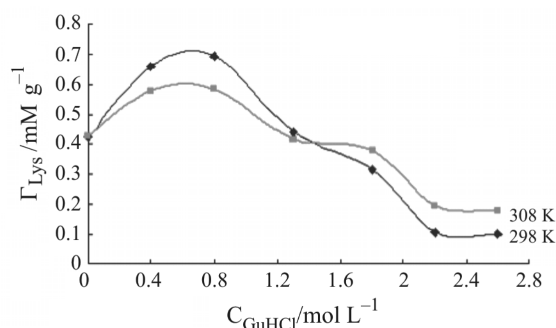

Fig. 2 The adsorption curves of 0.4 mg mL^{-1} Lys denatured by different concentrations of GuHCl on PEG-600 surface; the data at 298 K are derived from the previous study [1]

are lower than that at 298 K. This perhaps reveals different molecular mechanism of hydrophobic interaction for partly denatured Lys binding with a moderately hydrophobic ligands of PEG-600 surface as temperature increasing and the thermodynamic analysis on this will be seen later.

The values of equilibrium adsorption amounts at higher salt concentrations are the net results of the effect of both temperature and C_{GuHCl} and imply the important information on reverse thermodynamics of protein adsorption with protein refolding on a moderately hydrophobic surface. Herein we follow our previous studies [7, 11–14] on the thermodynamics in liquid-solid system. Under the investigation of stoichiometric displacement theory (SDT) [15] for partition coefficient between solid and liquid phases, P_a, of adsorbate, which was derived from a true surface concentration based on the correct volume of adsorbed layer [11] ($P_a = n^s/(CN_0V_0n^s_{max})$, 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: $\Delta G = -RT\ln P_a$ (R is the universal gas constant, T stands for Kelvin temperature) [7, 12–14], P_a and ΔG can be obtained according to equilibrium adsorption data. Then, combining the calorimetric enthalpies measured directly, ΔH, the entropies can be calculated. The calculated results are separately shown in Tables 2 and 3. The relative average error of Γ in Table 2 is about 1.94%, and that of P_a is about 9.05%. The detailed implication about the thermodynamic data will be discussed in the next section.

Displacement adsorption enthalpies

Since the displacement adsorption enthalpy ΔH can be divided into three fractions [1], as mentioned above, the discussion of ΔH for studying the effects of GuHCl concentration and temperature can also be done similarly. It is necessary to elucidate that there is little effect of GuHCl binding to Lys on measured enthalpy. Because the GuHCl binding to Lys occurred before the enthalpy was measured, which mainly depends on the three subprocesses mentioned above. The extent of departure of GuHCl from binded Lys was little when the denatured Lys in solution with a fixed GuHCl concentration contacts with hydrophobic packings in a calorimetric cell. Thus, the effect of GuHCl binding to Lys on enthalpy can be ignored, especially for the lower GuHCl concentrations. Table 4

Table 2 Partition coefficients of partly denatured Lys (0.4 mg mL^{-1}) adsorbed onto PEG-600

C_{GuHCl}/mol L^{-1}	C_{Lys}/mg mL^{-1}		Γ_{Lys}/mg		$\ln P_a$	
	298 K	308 K	298 K	308 K	298 K	308 K
0.0	0.1562	0.1542	0.1219	0.1229	7.7464	7.9398
0.4	0.0192	0.0676	0.1904	0.1662	10.2896	9.0665
0.8	0.0012	0.0642	0.1994	0.1679	13.1082	9.1282
1.3	0.1456	0.1590	0.1272	0.1205	7.8581	7.8894
1.8	0.2190	0.1824	0.0905	0.1088	7.1077	7.6499
2.2	0.3380	0.2874	0.0310	0.0563	5.6048	6.5361
2.6	0.3420	0.2980	0.0290	0.0510	5.5265	6.4012

The adsorbed amounts Γ_{Lys} at 298 K were derived from literature [1]

Table 3 Thermodynamic functions of Lys (0.4 mg mL^{-1}) adsorbed onto PEG-600

C_{GuHCl}/mol L^{-1}	ΔG/kJ mol^{-1}		ΔH/kJ mol^{-1}		$T\Delta S$/kJ mol^{-1}	
	298 K	308 K	298 K	308 K	298 K	308 K
0.0	–19.19	–20.33	6566.4	1072.8	6585.6	1093.1
0.4	–25.49	–23.22	5976.0	813.6	6001.5	836.8
0.8	–32.48	–23.37	1828.8	417.6	1861.3	441.0
1.3	–19.47	–20.20	1591.2	–244.8	1610.7	–224.6
1.8	–17.61	–19.59	–669.6	–2073.6	–652.0	–2054.0
2.2	–13.89	–16.74	1807.2	–993.6	1821.1	–976.9
2.6	–13.69	–16.39	6559.2	–223.2	6572.9	–206.8

The ΔH values at 298 K were based on the experimentally measured heats Q in literature [1] and derived by calculating via the initial concentration of Lys (0.4 mg mL^{-1})

Table 4 The ΔH of the Lys at interface of PEG-600/solution (2.1 mol $L^{-1}(NH_4)_2SO_4$ x mol L^{-1} GuHCl, 0.05 mol L^{-1} KH_2PO_4, pH 7.0) at 308±0.001 K

C_{GuHCl}/ mol L^{-1}	Γ/ mg	Q_{obs}/ mJ	Q_i/ mJ	ΔH/ kJ mol^{-1}
0.0	0.1229	–107.2	14.9	1072.8
0.4	0.1662	–110.8	11.3	813.6
0.8	0.1679	–116.3	5.8	417.6
1.3	0.1205	–125.5	–3.4	–244.8
1.8	0.1088	–150.9	–28.8	–2073.6
2.2	0.0563	–135.9	–13.8	–993.6
2.6	0.0510	–125.2	–3.1	–223.2

Γ – adsorbed amount of Lys on PEG-600; Q_{obs} – observed value; Q_i – total heat effect of displacement adsorption of Lys (offsetting the mixture heat of blank sample with the HPHIC packings, –107.5 mJ and the heat effect of putting down operation rod, –14.6 mJ)

shows the directly measured displacement adsorption heat Q_i or the corresponding adsorption enthalpy ΔH_i of denatured Lys on the interface between PEG-600 packings and solutions (2.1 mol L^{-1} $(NH_4)_2SO_4$, x mol L^{-1} GuHCl, and 0.05 mol L^{-1} KH_2PO_4, pH 7.0). The condition of calorimetric test is as same as that of determination of adsorbed amounts above. It is noted that in the quantification of the ΔH, 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. Moreover, this method of quantification of displacement adsorption enthalpy coincides with that in the literature [8] .

It was shown that with the decreasing of C_{GuHCl}, the heat effects of both Q_i and ΔH_i exhibit exothermic firstly and then endothermic, and their values decrease until the minimum values (exothermic) corresponding to 1.8 mol L^{-1} GuHCl, and lastly they tend to increase. This changing tendency is similar to the previous study reported at 298 K [1]. Figure 3 gives the comparison of ΔH change with the C_{GuHCl} at 298 and 308 K.

In fact, the relative values of the above three enthalpy fractions of ΔH are affected by all the C_{GuHCl}, $C_{(NH_4)_2SO_4}$ and temperature. When the temperature (at 298 or 308 K) and the concentration of salt are fixed, the changing trend of ΔH with decreasing GuHCl concentrations (imitating continuous removal of denaturing agent in HIC mobile phase) can be depicted as follows: (1) ΔH_a (exothermic) gets more negative because hydrophobic interaction between the protein and adsorbent increases gradually. The number of protein molecules in partly unfolded state, which cover the binding sites of adsorbent, reduces and the effective hydrophobic interaction occurred at the binding sites increases. This also can be seen from the changing trend of adsorbed amounts with decreasing C_{GuHCl} in Table 4; (2) ΔH_d (endothermic) increases because with the decrease in C_{GuHCl}, the molecular conformations of Lys are getting closer and closer to form to microdomain or intermediate and lastly to its native state, resulting in the increment in dehydration of the surface of those amino acid residues originally exposed to solution phase and then placed inside of the refolded molecules [5]; (3) ΔH_m (exothermic) induced by orderly orientation rises in algebraic value or the exothermic effect reduces because the range of conformational change narrows down with decreasing C_{GuHCl}. Therefore, the total displacement adsorption enthalpies ΔH at 308 and 298 K shown in Fig. 3 and Table 4 are just right the algebraic sum of the three kinds of enthalpy fractions analyzed above and corresponding to various C_{GuHCl}. Although the changing trend of ΔH with C_{GuHCl} at 308 and 298 K can not be examined directly by the enthalpy fractions yet because of the difficulty to evaluate them individually, the significance is that the result exhibits the relative extent of effect of the subprocesses leading to enthalpy fractions on the mechanism of the partly denatured protein refolding on the adsorbent. The ΔH values at 308 K and C_{GuHCl} ranged from 1.3 to 2.6 mol L^{-1} are all negative (exothermic), implying that both adsorption affinity or hydrophobic interaction (marked (1) above) and molecular conformation induced by orderly orientation (marked (3) above) dominate over the dehydration (marked (2) above), while the ΔH values between 0~1.3 mol L^{-1} GuHCl at 308 K behave positive (endothermic), indicating an oppositely predominant relation with that above between the subprocesses. It is worthy of taking notice that no matter whether at 298 or at 308 K the ΔH value at 1.8 mol L^{-1} GuHCl is the lowest (valley point). This

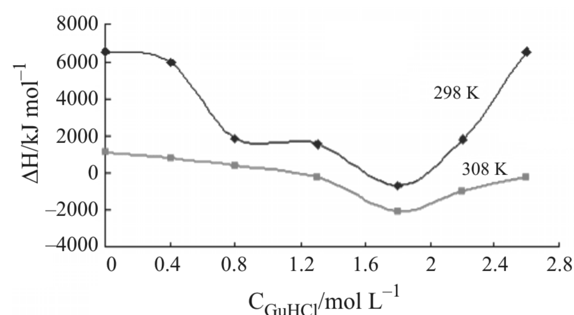

Fig. 3 The displacement adsorption enthalpy of Lys with C_{GuHCl}; the ΔH values at 298 K here were based on the measured heats Q in literature [1] and derived by calculating via the initial concentrations of Lys

phenomenon of concave point also makes us easily understand the changing trend of ΔH. The previous study [1] specially pointed out the possibility of existence of an intermediate state with relatively lower energy or called ‘energy well’. The partly denatured Lys molecules easily form a relatively stable three-dimensional structure, which is the result of cooperative effect of the analyzed three kinds of fractions above, and the ΔH become more negative. In other words, a state which closes nearer the lowest point has a stronger adsorption affinity and molecular conformation induced by orderly orientation than a state which departs away from the valley point. A conclusion can be made: it is crucial that the C_{GuHCl} which determines the denaturing degree of protein can affect distinctly the change of ΔH values. This can be demonstrated by the same ΔH changing trend with C_{GuHCl} at 298 and 308 K.

Additionally, the ΔH ranged from 2.6 to 1.3 mol L^{-1} C_{GuHCl} at 308 K, different from that at 298 K, are all exothermic, and all the enthalpies in the whole GuHCl concentrations investigated at 308 K are lower than that at 298 K. This is because, on the one hand, when temperature rises, the thermal motion of protein molecules speeds up in the adsorption system and the opportunity of binding of the hydrophobic residues in denatured protein molecules with the hydrophobic ligands of surface enlarges, resulting in favoring the protein molecules to obtain energy from the solid surface and to form microdomain and then to grow into their native state [15]. Herein, both the adsorption affinity or hydrophobic interaction and the molecular conformation enhance and facilitate ΔH to be more exothermic with temperature increasing; on the other hand, the value of heat required for the dehydration process of hydrophobic ligands of adsorbent also decreases with increasing temperature [8], resulting in less endothermic effect. That is to say that rise of temperature facilitates probably Lys refolding and renaturing, exhibiting the ΔH values at 308 K to be less than that at 298 K. As denaturing degree of Lys gets stronger, for example from 1.3 to 2.6 mol L^{-1} GuHCl, the phenomenon that ΔH values at 308 K show more exothermic than that at 298 K is more distinct. The changes of both increase of the exothermic effect induced by hydrophobic interaction and molecular conformation and decrease of endothermic effect led by dehydration with temperature increase can be elucidated further by thermodynamics as following.

Table 3 shows that not only the enthalpies measured by microcalorimetry at 308 K are all less than that at 298 K, but also the entropies evaluated from the equilibrium adsorption amounts at 308 K are also lower than that at 298 K under the same other conditions. This can be explained as following. Dehydration (marked (2) above) both between hydrated protein and hydrated ligands of surface and between the hydrated residues of protein contributes to entropy gain, while both hydrophobic interaction between hydrophobic residues of protein and hydrophobic ligands of surface (marked (1) above) and molecular conformation (marked (3) above) make entropy and enthalpy reduce. When temperature increases from 298 to 308 K, the latter (marked (1) and (3) above) gets stronger than the former (marked (2) above), as analyzed above, therefore, the entropies at 308 K are less than that at 298 K. The analysis of effect of temperature from 298 to 308 K on entropies is in accordance with that on enthalpies discussed above, confirming the reasonability of dividing ΔH into three fractions. It is found that, compared with the enthalpies and entropies under the various concentrations of GuHCl at 298 and 308 K, respectively, listed in Table 3, the adsorption with protein refolding of partly denatured Lys on PEG-600 surface at 298 K is an entropy-driving process (exclude that at 1.8 mol L^{-1} GuHCl), as pointed out in our previous study [1], while that at 308 K depends on GuHCl concentrations. The adsorption with protein refolding at lower GuHCl concentrations (less than 1.3 mol L^{-1}) at 308 K belongs to an entropy-driving process induced by dehydration, i.e. enthalpy enhancement with decrement of GuHCl concentrations is compensated by the greater entropy gain, while that at higher GuHCl concentrations (from 1.3 to 2.6 mol L^{-1}) corresponds to an enthalpy-driving process led by both the hydrophobic interaction and the molecular conformation, i.e. the entropy loss with decrement of GuHCl concentrations is compensated by the more exothermic enthalpies.

Refolding enthalpies of protein

Since refolding enthalpy of protein $\Delta\Delta H$ means the difference between the ΔH at native state (C_{GuHCl}=0) and that at variously denatured state under the same HIC condition, i.e. a complementary enthalpy needed to make a denatured protein corresponding to a given C_{GuHCl} and ΔH entirely refold into the native state [1], both the magnitude and physical meaning of $\Delta\Delta H$ relate closely to that of ΔH due to the denatured state of protein. The plottings of $\Delta\Delta H$ *vs*. C_{GuHCl} under the same conditions as that in Fig. 3 are illustrated in Fig. 4. Figure 4 shows that the $\Delta\Delta H$ values at 308 and 298 K (the $\Delta\Delta H$ values at 298 K are derived by calculating via the initial concentration of Lys instead of that in the previous study [1]) are all positive and higher than that of folding in solution, indicating that more dehydration (marked (2) above) is needed for the Lys molecules in variously denatured states to

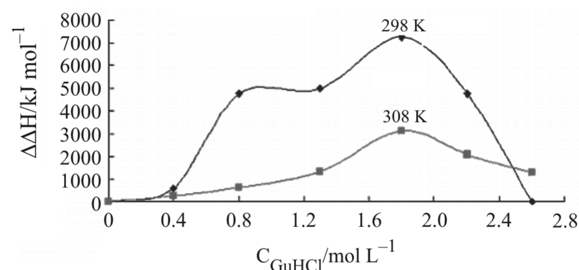

Fig. 4 The folding enthalpy $\Delta\Delta H$ of Lys with C_{GuHCl}; the $\Delta\Delta H$ values at 298 K were based on the experimentally measured heats Q in literature [1] and derived by calculating via the initial concentration of Lys (0.4 mg mL^{-1})

refold into their native states and they must obtain enough energy provided by a moderately hydrophobic surface of adsorbent at higher salt concentrations. This is why the efficiency of denatured protein refolding at liquid-solid interface is higher than that in solution. The fact that $\Delta\Delta H$ values at 308 K are almost lower than that at 298 K at the fixed C_{GuHCl} shows that the heat required for the denatured protein refolding into its native state at 308 K are less than that at 298 K. This coincides with the analysis in last section that rise in temperature from 298 to 308 K facilitates Lys refolding and renaturing.

The maximum refolding enthalpy of Lys due to 1.8 mol L^{-1} GuHCl at 308 K implies the existence of an intermediate state with relatively stable (lower) energy, as be called 'energy well' above, which is in accordance with that at 298 K for the same studied system [1]. The intermediate state of Lys refolding occurs at same concentration of GuHCl (1.8 mol L^{-1}) and at both 298 and 308 K, indicating that it is independent of temperature but is affected by C_{GuHCl}. This study demonstrates again that during the process of denatured protein refolding and renaturing (continuously removing the denaturing agent from sample solution) in the HIC the intermediate state in lower energy exists in the protein folding pathway.

Conclusions

The displacement adsorption enthalpies ΔH and equilibrium adsorption amounts of guanidine-denatured Lys refolding on a moderately hydrophobic surface at 308 K are respectively measured and compared with that at 298 K. The discussion shows how temperature and C_{GuHCl} affect the enthalpy and entropy behavior of protein adsorption in liquid/solid system.

The adsorption thermodynamics calculated by the adsorbed amounts combining with the measured enthalpy shows that adsorption with refolding of partly denatured Lys on PEG-600 surface at 298 K is an entropy-driving process (exclude that at 1.8 mol L^{-1} GuHCl), while that at 308 K depends on GuHCl concentrations. The protein adsorption with refolding at lower GuHCl concentrations (less than 1.3 mol L^{-1}) at 308 K belongs to an entropy-driving process induced by dehydration, while that at higher GuHCl concentrations (from 1.3 to 2.6 mol L^{-1}) corresponds to an enthalpy-driving process led by both the hydrophobic interaction and the molecular conformation.

No matter whether at 298 or 308 K, both the lowest ΔH value (valley point) and the maximum refolding enthalpy of Lys $\Delta\Delta H$ corresponding to 1.8 mol L^{-1} GuHCl imply the existence of an intermediate state with relatively stable (lower) energy, revealing the partly denatured Lys molecules easily form a relatively stable three-dimensional structure, which is the results of cooperative effect of the analyzed three kinds of fractions. The C_{GuHCl} which determines the denaturing degree of protein can affect distinctly the change of ΔH value. The intermediate state of Lys refolding is independent of temperature but is affected by C_{GuHCl}. This study demonstrates again that during the process of denatured protein refolding and renaturing in HIC the intermediate state in lower energy exists in the protein folding pathway.

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