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# THERMODYNAMIC ANALYSIS OF DENATURED LYSOZYME FOLDED ON MODERATELY HYDROPHOBIC SURFACE AT 298 K

*X. P. Geng*[1*], *H. Gao*[1], *B. H. Wang*[2], *A. L. Liu*[1] *and X. Y. Feng*[1]

[1]College of Environment and Chemical Engineering, Xi'an Polytechnic University, Xi'an 710048, China
[2]Institute of Physical Chemistry, Peking University, Beijing 100871, China

Both calorimetric determination of displacement adsorption enthalpies $\Delta H$ and measurement of adsorbed amounts of lysozyme (Lyz) denatured by 1.8 mol $L^{-1}$ guanidine hydrochloride (GuHCl) on a moderately hydrophobic packings at 298 K, pH 7.0 and various salt concentrations were carried out. Based on the thermodynamics of stoichiometric displacement theory (SDT) the fractions of thermodynamic functions, which related to four subprocesses of denatured protein refolding on the surface, were calculated and thermodynamic analysis that which one of the subprocesses plays major role for contribution to the thermodynamic fractions was made in detail. The moderately hydrophobic surface can provide denatured Lyz energy and make it gain more conformation with surface coverage or salt concentration increment. The displacement adsorptions of denatured Lyz onto PEG-600 surface are exothermic, more structure-ordered and enthalpy driven processes.

***Keywords:*** *adsorption, calorimetry, fractions of thermodynamic functions, hydrophobic surface, lysozyme, subprocesses of protein folding*

## Introduction

Study on folding of proteins adsorbed onto a moderately hydrophobic surface is of great significance in many research fields, such as protein renaturing, drug purification and biomaterials exploitation. In the last few decades, many researches have provided evidence for conformational changes induced by adsorption. Norde and Favier [1] reported that the amount of secondary structure of lysozyme (Lyz) and bovine serum albumin (BSA) decrease after adsorption by using circular dichroism (CD) to determine the content of α-helix. Larsericsdotter *et al.* [2] utilized mass spectrometry (HDX-MS) in combination with differential scanning calorimetry (DSC) to investigate the structure stability of lysozyme adsorbed to small colloidal silica particles and found that folding/unfolding of lysozyme occurs through a two-domain process. Giancola [3] described DSC, as a convenient tool, can be used to clarify the energetics of macromolecule transitions and to characterize the stability of proteins and nucleic acides. Norcross and Yeates [4] presented a mathematical framework for describing the notion of a topological folding barrier, which occurs when a protein chain must pass through a hole or opening, formed by other regions of the protein structure. Simultaneously, the research methods varied extensively, such as DSC, FTIR, NR, HDX-MS, fluorescence spectrum, CD and X-ray. According to the principle of reversibility between adsorption of denatured protein onto the stationary phase in hydrophobic interaction chromatography (HIC) at higher salt concentration and desorption of protein at lower salt concentration, Geng *et al.* [5, 6] investigated the mechanism of refolding and simultaneously purification of proteins in HIC as well as determination of free energy of several denatured proteins refolding on hydrophobic surface in terms of retention behavior. Recently, the microcalorimetric determination has been a reliable and sensitive method to investigate the adsorption subprocesses, which were elucidated by thermodynamic functions [7–12]. The analysis of displacement adsorption enthalpy ($\Delta H$) obtained directly from the microcalorimetric determination is a quite effective method to further explore protein adsorption mechanism on surface.

However, the protein adsorption was a complex process in which structural stability of a protein, ionic strength [13], pH of solution [14], and hydrophobicity of the adsorbent surface [7] were known to influence the affinity of a protein for a given interface. It is still difficult to fully understand the protein adsorption and conformational changes under various conditions at solid–liquid interfaces. In our previous study [12] on salt concentration dependence of 0.4 mg $mL^{-1}$ denatured Lyz adsorbed onto hydrophobic surface, the subprocesses occurred were generally analyzed just by obtained thermodynamics of total displacement adsorption ($\Delta H$, $\Delta S$ and $\Delta G$). In our recent study [15]

* Author for correspondence: xinpenggeng@163.com

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

on native Lyz adsorption, four subprocesses related to protein adsorption suggested that the possible two of them contribute to some thermodynamic fraction, making explanation of conformational loss of native Lyz. In this study, however, based on thermodynamics of stoichiometric displacement theory (SDT), the thermodynamic fractions of denatured Lyz adsorption in various coverage and salt concentrations are obtained to deeply investigate which one of the four subprocesses would take major role for contribution to a pointed thermodynamic fraction for denatured Lyz adsorption on the hydrophobic surface.

## Theoretical

Thermodynamic analysis of denatured protein folded on solid surface depends on the calculated fractions of thermodynamic functions and conformational changes associated with four subprocesses of displacement adsorption of protein. The calculating foundation of fractions of thermodynamic functions for protein adsorbed onto hydrophobic surface was expressed in recent study [15] as:

$$\Delta H=\Delta H_A+\Delta H_D=\Delta H_A+m\ln C \quad (1)$$

$$\Delta S=\Delta S_A+\Delta S_D \quad (2)$$

$$\Delta S_A=R\beta_a+\frac{\Delta H_A}{T} \quad (3)$$

and

$$\Delta S_D=\frac{\Delta H_D}{T}-\frac{q}{z}R\ln C \quad (4)$$

where $\Delta H_A$ and $\Delta S_A$ stands for (net) adsorption enthalpy and entropy of protein to the adsorbent, while $\Delta H_D$ and $\Delta S_D$ stands for (net) desorption enthalpy and entropy of solvent from the adsorbent for a general displacement adsorption in thermodynamics of stoichiometric displacement theory for adsorption (SDT-A) [16, 17]. $R$ is gas constant, $m$ a constant equal to $RT(q/z)$, $T$ absolute temperature, $C$ solution equilibrium concentration of protein, and $\beta_a$ and $q/z$ are constants and represent parameters for net adsorption of protein and net desorption of solvent in SDT-A, respectively, which can be obtained by linear plot of $\ln P_a$ *vs.* $\ln C$ in following linear equation:

$$\ln P_a=\beta_a-\frac{q}{z}\ln C \quad (5)$$

where $P_a$ represents partition coefficient of solute in the two phases, which can be calculated by adsorption isotherm [10]. The two fractions of enthalpy change, $\Delta H_A$ and $\Delta H_D$, can be obtained by linear plot of measured $\Delta H$ *vs.* $\ln C$. Further, $\Delta S_A$ and $\Delta S_D$ can also be calculated by inserting $\Delta H_A$, $\Delta H_D$, $\beta_a$ and $q/z$ to Eqs (3) and (4). Thus, (net) adsorption free energy, $\Delta G_A$, and (net) desorption free energy, $\Delta G_D$, can be written as

$$\Delta G_A=-RT\beta_a \quad (6)$$

and

$$\Delta G_D=RT\left(\frac{q}{z}\right)\ln C \quad (7)$$

The displacement adsorption of Lyz on a moderately hydrophobic surface includes four subprocesses, i.e. (*i*) Lyz affinity to surface; (*ii*) following molecular conformational change (loss for native or gain for denatured); (*iii*) dehydration between Lyz molecules and surface and (*iv*) dehydration (squeezing water for denatured Lyz) inside the hydrated protein molecules during formation of ordered structure or hydration (for native Lyz) of unfolded and exposed amino acid residues. For adsorption of denatured Lyz, the net adsorption thermodynamic fractions ($\Delta H_A$, $\Delta S_A$ and $\Delta G_A$) of Lyz are attributed to both (a) Lyz affinity to surface and (b) following conformational gain of protein, while the net desorption ones ($\Delta H_D$, $\Delta S_D$ and $\Delta G_D$) of Lyz are cooperative results of (c) dehydration between Lyz molecules and surface and of (d) dehydration (squeezing water) inside the hydrated protein molecules during formation of ordered structure. The schematic diagram of the subprocesses is shown in Fig. 1. These can be expressed as

$$\Delta H_A=\Delta H_a+\Delta H_{mo} \quad (8)$$

$$\Delta H_D=\Delta H_d+\Delta H_{md} \quad (9)$$

$$\Delta S_A=\Delta S_a+\Delta S_{mo} \quad (10)$$

$$\Delta S_D=\Delta S_d+\Delta S_{md} \quad (11)$$

$$\Delta G_A=\Delta G_a+\Delta G_{mo} \quad (12)$$

and

$$\Delta G_D=\Delta G_d+\Delta G_{md} \quad (13)$$

where the subscripts a, mo, d and md represent the subprocesses (a), (b), (c) and (d) of denatured protein, respectively. Based on Eqs (8)–(13) and conformational changes and adsorption isotherms of denatured protein, dominant subprocess of denatured protein refolding can be deduced.

## Experimental

*Materials*

Lysozyme (Lyz, chicken egg white) was purchased from Sigma Co. (St. Louis USA), PEG-600 made of a

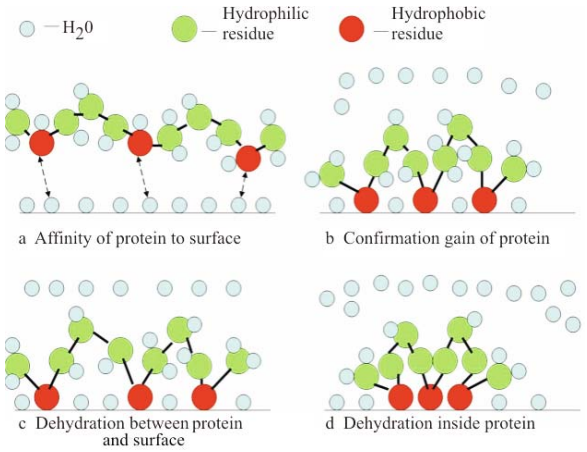


**Fig. 1** Schematic diagram of subprocesses of denatured Lyz folded at hydrophobic surface

silica base-HPHIC packings (particle size, 6.5 μm; pore diameter, 30 nm; the end-group of polyethylene glycol) were obtained from the Institute of Modern Separation Science, Northwest University, China, guanidine hydrochloride (GuHCl) bought from Shanghai State-medicine Group Chemical Reagent Ltd. Co., ammonium sulfate $(NH_4)_2SO_4$) from Tianjin Nankai Chemical Reagent Co., and potassium phosphate monobasic ($KH_2PO_4$) from Tianjin Dengfeng Chemical Reagent Co. Other chemicals are all analytic grade. The deionized water was produced by Milli-Q Academic (Millipore Co. Ltd., USA).

### *Methods*

#### Microcalorimetric procedure

The calorimetric measurements were carried out by a Micro DSC-III (Setaram, Calurie, France) [10]. Transfer 0.500 mL Lyz solution with a syringe into the lower chamber of 'measurement' mixing vessel and 'reference' mixing vessel, respectively. The solutions with 0.4, 0.7 and 1.0 mg $mL^{-1}$ Lyz, 0.05 mol $L^{-1}$ $KH_2PO_4$ (pH 7.0) and various concentrations of $(NH_4)_2SO_4$ (0, 0.3, 0.9, 1.5, 1.8, 2.1 mol $L^{-1}$) have been denatured by 1.8 mol $L^{-1}$ GuHCl for 24 h at 298 K. Put 20±0.01 mg PEG-600 packings in the upper chamber of the 'measurement' vessel while the corresponding 'reference' one being empty. The calorimetric operation is the same as that in the previous study [12]. The procedure to measure the blank heats, $Q_{blank}$, was the same except Lyz absent. The calorimetric data analysis is described in previous studies [12, 15].

#### Adsorbed amounts determination

Put the mixtures of PEG-600 and the Lyz solutions as same as that in the calorimetric mixing batch vessel into isothermal vibrator and keep them shaking for 5 h at 298 K so as to determine the adsorbed amounts of Lyz on the surface of PEG-600 packings corresponding to the calorimetric processes. After that use the UV-Vis spectrophotometer (Shimadzu UV-2450) to determine the absorbency (280 nm) of the supernatants obtained by centrifuging. The concentrations of Lyz in supernatants can be determined and adsorbed amounts can also be calculated.

## Results and discussion

### *Displacement adsorption enthalpies*

The heats directly observed during contacting the denatured Lyz solution sample with PEG-600 packings in a mixing batch vessel, $Q_{obs}$, include both displacement adsorption heat, $Q_i$, and corresponding blank sample (absent Lyz) heats, $Q_{blank}$. The individually calorimetric results in various concentration (0, 0.4, 0.7 and 1.0 mg $mL^{-1}$) of 0.5 mL denatured Lyz solutions (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) with mixing 20 mg PEG-600 packings at 298 K are listed in Tables 1–4, respectively. All relative average errors

**Table 1** $Q_{blank}$ at various concentrations of $(NH_4)_2SO_4$, 1.8 mol $L^{-1}$ GuHCl, 0.05 mol $L^{-1}$ $KH_2PO_4$, pH 7.0 at 298±0.001 K

| $C_{(NH_4)_2SO_4}$/ mol $L^{-1}$ | $Q_{blank}$/mJ | | | $Q_{mean}$/mJ |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| 0.0 | –184.3 | –182.9 | –185.0 | –182.2±1.9 |
| 0.3 | –176.7 | –175.4 | –170.1 | –174.1±2.7 |
| 0.9 | –160.1 | –154.7 | –157.2 | –157.1±1.8 |
| 1.5 | –141.3 | –142.1 | –138.5 | –140.4±1.5 |
| 1.8 | –131.3 | –134.5 | –130.2 | –132.0±1.7 |
| 2.1 | –124.1 | –125.3 | –121.4 | –123.6±1.5 |

**Table 2** $Q_{obs}$ of 0.4 mg $mL^{-1}$ denatured Lyz solutions (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) with mixing PEG-600 at 298±0.001 K

| $C_{(NH_4)_2SO_4}$/ mol $L^{-1}$ | $Q_{obs}$/mJ | | | $Q_{mean}$/mJ |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| 0.0 | –177.1 | –175.8 | –172.4 | –175.1±1.8 |
| 0.3 | –176.1 | –177.2 | –172.3 | –175.2±2.0 |
| 0.9 | –169.5 | –165.3 | –166.5 | –167.1±1.6 |
| 1.5 | –156.7 | –161.0 | –157.8 | –158.5±1.7 |
| 1.8 | –153.6 | –156.7 | –154.1 | –154.8±1.3 |
| 2.1 | –148.1 | –151.0 | –150.0 | –149.7±1.1 |

**Table 3** $Q_{obs}$ of 0.7 mg mL$^{-1}$ denatured Lyz solutions (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) with mixing PEG-600 at 298±0.001 K

| $C_{(NH_4)_2SO_4}$/ mol L$^{-1}$ | $Q_{obs}$/mJ | | | | $Q_{mean}$/mJ |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| 0.0 | –170.1 | –173.7 | –174.4 | –174.9 | –173.3±1.6 |
| 0.3 | –168.9 | –169.0 | –168.3 | –167.4 | –168.4±0.6 |
| 0.9 | –158.3 | –159.1 | –158.7 | –157.4 | –158.4±0.5 |
| 1.5 | –153.3 | –154.4 | –155.4 | –156.1 | –154.8±1.0 |
| 1.8 | –142.9 | –146.7 | –147.1 | –144.3 | –145.3±1.7 |
| 2.1 | –136.2 | –140.1 | –143.4 | –141.1 | –140.2±2.1 |

**Table 4** $Q_{obs}$ of 1.0 mg mL$^{-1}$ denatured Lyz solutions (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) with mixing PEG-600 at 298±0.001 K

| $C_{(NH_4)_2SO_4}$/ mol L$^{-1}$ | $Q_{obs}$/mJ | | | $Q_{mean}$/mJ |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| 0.0 | –168.0 | –164.7 | –165.3 | –166.0±1.3 |
| 0.3 | –162.7 | –161.2 | –161.7 | –162.0±0.6 |
| 0.9 | –153.8 | –151.6 | –151.2 | –152.2±1.1 |
| 1.5 | –148.4 | –147.7 | –145.3 | –147.1±1.2 |
| 1.8 | –135.3 | –134.1 | –134.7 | –134.7±0.4 |
| 2.1 | –122.0 | –127.2 | –125.6 | –124.9±2.0 |

**Table 5** Displacement adsorption enthalpies $\Delta H$ (kJ mol$^{-1}$) of denatured Lyz adsorbed onto PEG-600 from solutions (1.8 mol L$^{-1}$ GuHCl, *x* mg mL$^{-1}$ Lyz, 1.8 mol L$^{-1}$ $(NH_4)_2SO_4$, 0.05 mol L$^{-1}$ $KH_2PO_4$, pH 7.0) at 298±0.001 K

| $C_{(NH_4)_2SO_4}$/ mol L$^{-1}$ | $C_{Lyz}$/mg mL$^{-1}$ | | |
|---|---|---|---|
| | 0.4 | 0.7 | 1.0 |
| 0.0 | 511.2±266.4 | 366.2±144.0 | 466.6±92.2 |
| 0.3 | –79.2±338.4 | 234.5±135.8 | 384.5±104.9 |
| 0.9 | –720±244.8 | –535.5±947.4 | 141.1±83.5 |
| 1.5 | –1303±230.4 | –592.5±102.9 | –193.0±77.8 |
| 1.8 | –1642±216.1 | –547.2±139.9 | –77.8±60.5 |
| 2.1 | –1879±187.2 | –683.0±148.1 | –37.4±100.7 |

of these measured data are less than 2%, showing the calorimetric results to be very satisfactory.

The displacement adsorption enthalpies, $\Delta H$, corresponding to foregoing conditions can be obtained by $Q_i$ subtracted $Q_{blank}$ from $Q_{obs}$ and are listed in Table 5.

Notably, the data for 0.4 mg mL$^{-1}$ Lyz differ from that in previous study [12] because the hydrophobic packings used were separate batches and hydrophobicity of them was not identical. However, the results derived in this paper do not be affected, for instance, the trend that exothermic effects increase with salt concentration increment is as same for the two separate batches at 0.4 mg mL$^{-1}$ Lyz.

Table 5 shows that at a given salt concentration, the exothermic effects decrease with increasing of initial concentrations of Lyz (or surface coverage of Lyz increment), which accords with the fact that adsorption enthalpy (exothermic) in lower coverage is more than in higher coverage. At higher $(NH_4)_2SO_4$ concentrations (more than 0.9 mol L$^{-1}$), the $\Delta H$ values are all exothermic, predicting the denatured Lyz to refold more or less on the surface, because renaturation of denatured proteins is an opposite process of protein unfolding which is generally endothermic both on liquid-solid interface [15] and in solution [3, 18, 19]. Whereas, the changing tendency in $\Delta H$ values with salt concentration or protein coverage can not be elucidated simply because they are total results of enthalpy fractions of the four subprocesses during protein refolding on surface. This is why the fractions of thermodynamic functions are adopted to investigate adsorption and refolding of Lyz on surface.

### *Linear parameters* $\beta_a$ *and q/z in SDT-A*

The quantitative calculations of fractions of thermodynamic functions under the conditions of different salt concentrations and various protein concentrations employed depend on the precision of the parameters of linear net adsorption and net desorption, $\beta_a$ and $q/z$, which are attained by the plot of ln$P_a$ *vs.* ln$C$ in Eq. (5). In order to keep identical experimental conditions with calorimetric measurements, the adsorption isotherms of Lyz denatured by 1.8 mol L$^{-1}$ GuHCl at various concentrations of $(NH_4)_2SO_4$ and 298 K were performed. Because the adsorbed amounts are very little or even negative (in fact, apparent) at lower $(NH_4)_2SO_4$ concentrations, the ones at higher salt concentrations (1.5, 1.8 and 2.1 mol L$^{-1}$ $(NH_4)_2SO_4$) were chosen to guarantee reliability of calculation of the parameters. The corresponding partition coefficients $P_a$ can be calculated by method described in our previous study [12]. The plots of ln$P_a$ *vs.* ln$C$ are illustrated in Fig. 2 and the corresponding linear parame-

**Table 6** The linear parameters, $\beta_a$ and $q/z$, and correlation coefficients $r$

| $C_{(NH_4)_2SO_4}$/ mol $L^{-1}$ | $\ln P_a$ *vs.* $\ln C$ | | |
|---|---|---|---|
| | $r$ | $q/z$ | $\beta_a$ |
| 1.5 | 0.9831 | 0.5492 | 6.840 |
| 1.8 | 0.9792 | 0.5177 | 7.802 |
| 2.1 | 0.9964 | 0.6280 | 8.435 |

ters, $\beta_a$ and $q/z$, and linear correlation coefficients $r$ are listed in Table 6. The linear relationships of them are satisfactory, showing obtained $\beta_a$ and $q/z$ to be reliable. This makes a base to calculate the fractions of thermodynamic functions (see later).

*Thermodynamic fractions of subprocesses*

In order to make the thermodynamic fractions deduced be reliable, only the thermodynamic data at higher concentrations of $(NH_4)_2SO_4$ (1.5, 1.8 and 2.1 mol $L^{-1}$) were utilized, as were done in adsorption isotherms and linear adsorption parameters.

According to Eqs (1)–(4) and Eqs (6)–(7), the fractions of enthalpies, entropies and free energies calculated under the conditions researched are listed in Tables 7–9. The linear correlation coefficients, $r$, in Table 7 are all greater than 0.99, showing the net infinity enthalpies of protein, $\Delta H_A$, and net desorption enthalpies of solvent, $\Delta H_D$, obtained by plots of $\Delta H$ *vs.* $\ln C$ in Eq. (1) being very precise. Also, reliable values of $\Delta H_A$ and $\Delta H_D$ are vital to calculate the corresponding entropy fractions, $\Delta S_A$ and $\Delta S_D$, in Table 8, according to Eqs (3) and (4).

It is obviously shown in Tables 7–9 that the thermodynamic fractions of affinity for protein molecules, $\Delta H_A$, $\Delta S_A$ and $\Delta G_A$, are all negative, while that

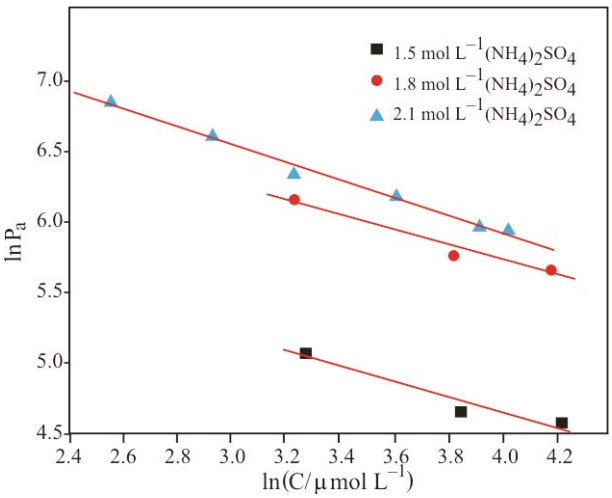


**Fig. 2** Plots of $\ln P_a$ *vs.* $\ln C$ of Lyz denatured by 1.8 mol $L^{-1}$ GuHCl and adsorbed from solutions ($x$ mol $L^{-1}$ $(NH_4)_2SO_4$, 0.05 mol $L^{-1}$ $KH_2PO_4$, pH 7.0) by PEG-600 at 298 K

of net desorption for solvent, $\Delta H_D$, $\Delta S_D$ and $\Delta G_D$, positive, and the general thermodynamic functions for total displacement adsorptions, $\Delta H$, $\Delta S$ and $\Delta G$, are all less negative than the corresponding $\Delta H_A$, $\Delta S_A$ and $\Delta G_A$ based on Eqs (1) and (2). The results reveal that the displacement adsorptions of denatured Lyz onto PEG-600 are exothermic, more structure-ordered and enthalpy driven processes. They differ from that of native Lyz, which are entropy driven processes contributed mainly by conformational loss of adsorbed Lyz [15]. The further mechanism exploration of denatured Lyz folded at hydrophobic surface can be obtained by analysis of the thermodynamic fractions associated with subprocesses of protein refolding.

Relative to less positive $\Delta H_D$, $\Delta S_D$ and $\Delta G_D$, the more negative $\Delta H_A$, $\Delta S_A$ and $\Delta G_A$ imply that contributions of subprocesses (a) Lyz affinity to

**Table 7** Enthalpy fractions of denatured Lyz adsorbed onto PEG-600 in various concentrations of $(NH_4)_2SO_4$ solutions at 298 K

| $C_0$*/mg $mL^{-1}$ | $\ln(C/\mu mol\ L^{-1})$ | $\Delta H$/kJ $mol^{-1}$ | $\Delta H_A$/kJ $mol^{-1}$ | $\Delta H_D$/kJ $mol^{-1}$ | $r$** |
|---|---|---|---|---|---|
| 1.5 mol $L^{-1}$ $(NH_4)_2SO_4$ | | | | | |
| 0.4 | 3.2663 | –1303±230 | –5171±230 | 3868±460 | |
| 0.7 | 3.8449 | –592±103 | –5171±103 | 4579±206 | 0.9996 |
| 1.0 | 4.2053 | –193±78 | –5171±78 | 4978±156 | |
| 1.8 mol $L^{-1}$ $(NH_4)_2SO_4$ | | | | | |
| 0.4 | 3.2272 | –1642±216 | –6977±217 | 5335±433 | |
| 0.7 | 3.8172 | –547±140 | –6977±141 | 6430±281 | 0.9960 |
| 1.0 | 4.1797 | –78±61 | –6977±61 | 6899±122 | |
| 2.1 mol $L^{-1}$ $(NH_4)_2SO_4$ | | | | | |
| 0.4 | 2.9267 | –1879±187 | –6823±187 | 4944±374 | |
| 0.7 | 3.6099 | –683±148 | –6823±148 | 6140±296 | 0.9996 |
| 1.0 | 4.0187 | –37±101 | –6823±101 | 6786±202 | |

*$C_0$: initial concentration of Lyz in solution; **$r$: linear correlation coefficient

**Table 8** Entropy fractions of denatured Lyz adsorbed onto PEG-600 in various concentrations of $(NH_4)_2SO_4$ solutions at 298 K

| $C_0$/mg mL$^{-1}$ | ln($C$/μmol L$^{-1}$) | $\Delta S$/kJ mol$^{-1}$ K$^{-1}$ | $\Delta S_A$/kJ mol$^{-1}$ K$^{-1}$ | $\Delta S_D$/kJ mol$^{-1}$ K$^{-1}$ |
|---|---|---|---|---|
| 1.5 mol L$^{-1}$ $(NH_4)_2SO_4$ | | | | |
| 0.4 | 3.2663 | –4.34±2.31 | –17.30±0.77 | 12.96±1.54 |
| 0.7 | 3.8449 | –1.96±1.02 | –17.30±0.34 | 15.34±0.68 |
| 1.0 | 4.2053 | –0.62±0.78 | –17.30±0.26 | 16.68±0.52 |
| 1.8 mol L$^{-1}$ $(NH_4)_2SO_4$ | | | | |
| 0.4 | 3.2272 | –5.46±2.18 | –23.35±0.73 | 17.89±1.45 |
| 0.7 | 3.8172 | –1.79±1.41 | –23.35±0.47 | 21.56±0.94 |
| 1.0 | 4.1797 | –0.22±0.61 | –23.35±0.20 | 23.13±0.41 |
| 2.1 mol L$^{-1}$ $(NH_4)_2SO_4$ | | | | |
| 0.4 | 2.9267 | –6.25±1.88 | –22.83±0.63 | 16.58±1.25 |
| 0.7 | 3.6099 | –2.24±1.49 | –22.83±0.50 | 20.59±0.99 |
| 1.0 | 4.0187 | –0.08±1.02 | –22.83±0.34 | 22.75±0.68 |

**Table 9** Free energy fractions of denatured Lyz adsorbed onto PEG-600 in various concentrations of $(NH_4)_2SO_4$ solutions at 298 K

| $C_0$/mg mL$^{-1}$ | ln($C$/μmol L$^{-1}$) | $\Delta G$/kJ mol$^{-1}$ | $\Delta G_A$/kJ mol$^{-1}$ | $\Delta G_D$/kJ mol$^{-1}$ |
|---|---|---|---|---|
| 1.5 mol L$^{-1}$ $(NH_4)_2SO_4$ | | | | |
| 0.4 | 3.2663 | –12.50±0.21 | –16.95±0.29 | 4.45±0.08 |
| 0.7 | 3.8449 | –11.72±0.20 | –16.95±0.29 | 5.23±0.09 |
| 1.0 | 4.2053 | –11.23±0.19 | –16.95±0.29 | 5.72±0.10 |
| 1.8 mol L$^{-1}$ $(NH_4)_2SO_4$ | | | | |
| 0.4 | 3.2272 | –15.19±0.31 | –19.33±0.40 | 4.14±0.09 |
| 0.7 | 3.8172 | –14.43±0.30 | –19.33±0.40 | 4.90±0.10 |
| 1.0 | 4.1797 | –13.97±0.30 | –19.33±0.40 | 5.36±0.11 |
| 2.1 mol L$^{-1}$ $(NH_4)_2SO_4$ | | | | |
| 0.4 | 2.9267 | –16.35±0.06 | –20.90±0.08 | 4.55±0.02 |
| 0.7 | 3.6099 | –15.28±0.06 | –20.90±0.08 | 5.62±0.02 |
| 1.0 | 4.0187 | –14.65±0.05 | –20.90±0.08 | 6.25±0.02 |

surface and (b) following conformational gain of protein dominate over the cooperative results of subprocesses (c) dehydration between Lyz molecules and surface and (d) dehydration (squeezing water) inside the hydrated protein molecules during formation of ordered structure. Whereas, for a given displacement adsorption system (at a constant concentration of $(NH_4)_2SO_4$), according to Eqs (1), (3) and (6), $\Delta H_A$, $\Delta S_A$ and $\Delta G_A$ are all constants with Lyz coverage increment, showing that subprocesses (a) and (b) should compensate each other based on Eqs (8), (10) and (12). Many reports [20, 21] including DSC, FTIR and LC [22] thought that adsorbed protein with higher surface coverage kept more secondary structure than that with lower coverage [1]. Thus, it can be deduced that with protein surface coverage increment, refolding of denatured Lyz or conformational gain (subprocess (b)) increases, while Lyz affinity to surface (subprocess (a)) decreases. Moreover, the increase for subprocess (b) is equal to the decrease for subprocess (a). It can be evaluated that the relative magnitude of negative thermodynamic fractions should be $\Delta H_{mo}>\Delta H_a$, $\Delta S_{mo}>\Delta S_a$ and $\Delta G_{mo}>\Delta G_a$ in Eqs (8), (10) and (12) with surface coverage of protein rise. We can image that energy of hydrophobic surface ($\Delta G$) is released by protein affinity to the surface ($\Delta G_A$) first and then a part of $\Delta G_A$ is delivered to protein molecular conformation ($\Delta G_{mo}$), remaining resulting protein affinity as $\Delta G_a$. Another part of $\Delta G$ ($\Delta G_D$) was expended in overcoming desorption of water molecules (dehydration) including subprocesses (c) and (d). The dehydration results in subprocesses (c) and (d) being endothermic ($\Delta H_D>0$), entropy gain ($\Delta S_D>0$) and partly counteractive to $\Delta G_A$, the original motive force. This mechanism is different from that in native Lyz adsorption onto PEG-600, where $\Delta G_D>0$ substantially results from entropy loss of hydration of polypeptide

amino acid residues in disordered structure ($\Delta S_{mh}<0$) [15]. In this presentation, however, it is subprocess (d) that dominates over subprocess (c) in dehydration to make $\Delta H_D$, $\Delta S_D$ and $\Delta G_D$ increase, i.e. $\Delta H_{md}>\Delta H_d$, $\Delta S_{md}>\Delta S_d$ and $\Delta G_{md}>\Delta G_d$ in Eqs (9), (11) and (13), with surface coverage of protein increment. The inference on subprocess (d) agrees with foregoing one on subprocess (b) because subprocess (d) occurs simultaneously with subprocess (b). These are deeply description in thermodynamics about denatured Lyz refolding on hydrophobic surface with surface coverage increment for the fact that lateral interaction and adsorbed amounts increase, and α-helical structural elements decrease while β-turn and β-sheets increase [2, 20].

At a constant initial concentration of Lyz, with salt concentration increment, the absolute values of fractions of thermodynamics in Tables 7–9 increase except for the fractions of enthalpy and entropy, $\Delta H_A$, $\Delta H_D$, $\Delta S_A$ and $\Delta S_D$, at 2.1 mol $L^{-1}$ $(NH_4)_2SO_4$. This indicates that the subprocesses enhance more or less and denatured protein folding becomes more effective. This is in accordance with the result obtained by DSC and FTIR measurements [21]. It can be elucidated as follows. Hydrophobic interactions (subprocess (a)) between the residues of protein and the ligands on hydrophobic surface strengthen, which made denatured protein molecules to refold (subprocess (b)), for instance, the lateral interactions of adsorbed protein enhance to form molecular β-sheet, and dehydration including subprocesses (c) and (d) is favored due to higher ionic strength. The exception to 2.1 mol $L^{-1}$ $(NH_4)_2SO_4$ mentioned above can be attributed to 'salt out effect' increment at very high salt concentration, which makes water molecules bounded by hydrogen bonding on protein and adsorbent surface decrease, leading to dehydration (subprocesses (c) and (d)) and conformational gain (subprocess (b)) less than at 1.8 mol $L^{-1}$ $(NH_4)_2SO_4$.

Also, under the same discussed conditions of a given Lyz initial concentration, $-\Delta G$ values increase with salt concentration increment, showing that $-\Delta G_A$ enhancement is more than $\Delta G_D$ rise due to $\Delta G=\Delta G_A+\Delta G_D$. In sight of contribution of the subprocesses, all the four subprocesses enhance but increase of processes (a) and (b) is greater than (c) and (d). Among processes (a) and (b), perhaps, subprocess (a) favors to (b) for contribution to $\Delta G_A$ especially at higher concentration (2.1 mol $L^{-1}$) of $(NH_4)_2SO_4$ because the adsorbed amounts of Lyz increase (equilibrium concentrations, $C$, due to identical initial Lyz concentrations decrease as shown in Tables 7–9) as a distinct remark of affinity of adsorbate to surface. Subprocess (c) which simultaneously occurred with subprocess (a) should be stronger than (d) for contribution to $\Delta G_D$.

## Conclusions

Denatured Lyz can obtain energy from the moderately hydrophobic surface to refold, which is different from some conformational loss for native Lyz adsorbed onto same surface in previous study [15] and makes refolding more perfect with salt concentration rise. Based on the thermodynamics of SDT the thermodynamic fractions which relate to four subprocesses of denatured Lyz refolding on the surface were calculated. The displacement adsorption of denatured Lyz onto PEG-600 are exothermic, more structure-ordered and enthalpy driven processes.

Thermodynamic analysis shows that with protein coverage increment, Lyz affinity to surface (subprocess (a)) and following conformational gain of protein (subprocess (b)) should compensate each other: the former decreases while the latter increases, more specifically, their negative fractions should be $\Delta H_{mo}>\Delta H_a$, $\Delta S_{mo}>\Delta S_a$ and $\Delta G_{mo}>\Delta G_a$; the dehydration inside protein molecules (squeezing water, subprocess (d)) dominates over the dehydration between protein molecules and surface (subprocess (c)): $\Delta H_{md}>\Delta H_d$, $\Delta S_{md}>\Delta S_d$ and $\Delta G_{md}>\Delta G_d$. With salt concentration increment, increase of subprocesses (a) and (b) is greater than that of (c) and (d), and subprocess (a) favors to (b) for contribution to $\Delta G_A$ especially at higher salt concentration, while subprocess (c) should be stronger than (d) for contribution to $\Delta G_D$.

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