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RESEARCH ARTICLE

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Denaturation study of bovine serum albumin induced by guanidine chloride or urea by microcalorimetry

Abstract The denaturation of bovine serum albumin (BSA) induced by guanidine chloride or urea at different pH values was studied by isothermal microcalorimetry measurements at 30°C. The simple bonding model, which was developed by Privalov, was employed to obtain the apparent bonding constant K, the apparent singular bonding Gibbs bonding energy ΔG and the total Gibbs energy $\Delta G(a)$ between the protein and denaturant, from analysis of the calorimetric data. Furthermore, linear extrapolation at the midpoint of transition was employed to determine the apparent denaturation enthalpy ΔH_d. The results showed that for guanidine chloride, the bonding between BSA and guanidine chloride could proceed more easily in an alkaline condition, and the apparent denaturation enthalpy ΔH_d of BSA due to guanidine chloride was 350 kJ·mol^{-1} at pH 6.97 and 7.05, while it was 275 kJ·mol^{-1} at pH 9.30, which indicated that BSA was more stabilized in a neutral condition. However, for urea, the bonding between BSA and urea could proceed more easily in an acidic condition, and the apparent denaturation enthalpy ΔH_d of BSA due to urea was 295 kJ·mol^{-1} at pH 6.97, while it was 230 kJ·mol^{-1} at pH 7.05 and 9.30. The results indicate that the degree of expansion of BSA in the two denaturants is different.

Keywords bovine serum albumin, isothermal microcalorimetry, guanidine chloride, urea, denaturation

The activity of proteins can only be demonstrated in the aqueous solution, therefore, key investigations of proteins focuses on the physical forces of the folding and denaturation mechanisms [1].

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Guanidine chloride and urea are two well-known denaturants, both having high solubility, and can interact with polar groups and non-polar groups. As early as the 1960 s, the study of denaturation mechanisms of protein induced by guanidine chloride and urea began [2], yet this is still a controversial issue until now. In general, there are three main points, as follows: 1) Guanidine chloride and urea molecules directly interact with the functional groups of proteins; 2) Changing the structure of water indirectly causes the structure of hydrogen bonding with water around the hydrophobic groups of the protein to be changed; 3) The abovementioned two factors work simultaneously. Watlaufer *et al*. [2] considered that the possibility of the first mechanism should be excluded through experiments, and Vanzi *et al*. [3] used a Random Network Model (RNM) to simulate the denaturation mechanism and calculate heat capacities of hydration, but thought that the second mechanism could not cause protein denaturation. Zou *et al*. [4] thought that it might be the third mechanism, by studying the protein denaturation induced by urea using microcalorimetry at 25°C. However, it has been clearly seen on the basis of experimental facts that, qualitatively speaking, the unfolding protein interacts with the denaturant more strongly than the folding one. Therefore, the main problem now focuses on developing a quantitative theory for forecasting and helping with understanding the factors affecting the stability of proteins [5]. An understanding of the denaturation process and mechanism in proteins is still not clear mainly because of the lack of direct information about proteins with denaturants. Privalov *et al*. [6] indicated that calorimetry and spectrometry are two analytic methods for estimating the stability of protein structures. Using microcalorimetric technology to study complex systems such as proteins has a unique advantage because the thermal effect only associates with the primary state and the final state of the system.

Bovine serum albumin (BSA) is a relatively large myosin, and can be found in cattle blood and milk [7]. It contains 607 residues (there are also reports of 582 residues [8]), 17 disulfide bonds, one free cysteine group, with a large spiral structure, and is remarkably stable at pH

7.00 [9]. It has relatively high water-solubility and is rich in ionizable amino acids. In this paper, microcalorimetry was used to study the denaturation process of bovine serum albumin induced by guanidine chloride and urea, and then we calculated the relevant thermodynamic parameters.

1 Experiments

1.1 Materials and instruments

Bovine serum albumin (BSA), was purchased from Sigma, purity > 98% (M_W= 68000). Guanidine hydrochloride ($CH_5N_3 \cdot HCl$), was purchased from Pharmaceutical Group, Chemical Reagents Corporation of Shanghai, China, analytical reagent grade (AR). Urea (CH_4N_2O) was purchased from the KeMiOu Chemical Reagents and Development Centers, Tianjin, China, analytical reagent grade. Other materials were also analytical reagent grade. The main instruments were: microcalorimeter (C-80 type, made by the SETARAM Company, France), acidometer (PHS-2C type, made by DaPu Instrument Co. Ltd., Shanghai).

1.2 Solution preparation

1) Twelve concentrations of guanidine hydrochloride solution(0.5, 1.0, 1.5, …, 6.0 $mol \cdot L^{-1}$) and 8 concentrations of urea solution(1.0, 2.0, 3.0, …, 8.0 $mol \cdot L^{-1}$) were prepared respectively in buffers, and then these solutions were adjusted to pH 4.80, pH 7.05 and pH 9.30, respectively, with hydrochloric acid (HCL) or sodium hydroxide (NaOH) (0.1 $mol \cdot L^{-1}$).

2) Bovine serum albumin solution with a certain molality was prepared in buffer and then adjusted to pH 6.97, pH 7.05 and pH 9.30, respectively, with hydrochloric acid or sodium hydroxide (0.1 $mol \cdot L^{-1}$). The molality m could be converted into molarity c by determining the density of the bovine serum albumin at 30°C with a pycnometer [10] (cubage is 5 mL). This protein solution should be prepared and used immediately because bovine serum albumin solution is easily denatured. Each time the concentrations of preparation were as close as possible to 3.3×10^{-3} $mol \cdot L^{-1}$, the error was $(\pm 0.01 \times 10^{-3})$ $mol \cdot L^{-1}$.

2 Methods for treatment of microcalorimetric data

In accordance with the simple model of bonding, that is, with the bonding position at random, one position only bonded with one denaturant molecule, and there was freedom from the influence of others. The bonding reaction can be expressed as

$$p + s = ps \tag{1}$$

where p is the bonding position, s is the denaturant molecule, and ps refers to the bonding positions that have bonded with denaturant molecules. According to the law of mass action, the bonding equilibrium constant is expressed as

$$K = \frac{[ps]}{[p][s]} \tag{2}$$

where [ps] and [p] mean the numbers of positions that have bonded the denaturant molecules or not, respectively, so-called the concept of concentration, and it is free from the influence of other positions. And because it is simple bonding, it also can therefore be seen as activity. [s] is the concentration of the denaturant molecules. There are many different kinds of bonding positions in protein molecules, therefore, many different equilibrium constants K_i exist, and the thermal effects for various bonding position are different, thus, the thermal effect $Q(a)$ from the protein bonding with the denaturant is expressed as [11]

$$Q(a) = \sum \Delta H_i n_i \times K_i \frac{a}{1 + K_i a} \tag{3}$$

where n_i means the number of bonding positions of i-Type, K_i means the bonding equilibrium constant of i-Type, a means the activity of the denaturant, and ΔH_i means the bonding enthalpy between the single bonding position and the denaturant. Because it is difficult to differentiate the bonding enthalpy from different bonding positions, Privalov assumed that all bonding positions were equivalent, and the above formula can be changed as

$$Q(a) = n\Delta H \times K \frac{a}{1 + Ka} \tag{4}$$

where n is the effective number of equivalent bonding positions and K is the effective bonding constant, which can also be seen as the apparent bonding constant. Then the above formula can be rearranged as

$$\frac{Q(a)}{a} = (n\Delta H)K - KQ(a) \tag{5}$$

Plotting $\frac{Q(a)}{a}$ *vs.* $Q(a)$, a straight line can be obtained. K can be obtained from the slope, and $n\Delta H$ can be calculated by the intercept. According to thermodynamic relations, the apparent singular bonding Gibbs bonding energy is expressed by the equation:

$$\Delta G = -RT\ln K \tag{6}$$

Because of the existence of a denaturant, the total Gibbs energy is expressed as

$$\Delta G(a) = -RT\ln(1 + Ka) \tag{7}$$

Compared to the apparent singular bonding Gibbs bonding energy, Eq. (6) has positive values but Eq. (7) has negative values, and the absolute values increase rapidly along with increases in denaturant activity [1].

3 Results and discussion

In the study of protein denaturation, we focused on the thermal effect $Q(a)$ from the bonding between protein and denaturant. The data are shown in Tables 1 and 2.

When the activity of the denaturant is lower, the thermal effect of bonding between protein and denaturant only contains the bonding enthalpy. When the activity of the denaturant increases, the protein structures begin stretching (begin denaturation); at this time, the thermal effects include not only the apparent bonding enthalpy but also the denaturation enthalpy. If we only deal with the thermal effects at lower activity, that is, the so-called apparent bonding enthalpy, a straight line can be obtained according to Eq. (5); from the slope and intercept of the straight line, the bonding constant K and the total bonding enthalpy $n\Delta H$ for the reaction of bonding between bovine serum albumin and guanidine chloride or urea can be obtained, respectively. The apparent singular bonding Gibbs bonding energy ΔG can be obtained from Eq. (6). All results are shown in Tables 3 and 4.

From Table 3, it can be found that the values of $n\Delta H$ are basically the same at different pH; this result suggests that the pH had little influence on the total bonding enthalpy $n\Delta H$ between bovine serum albumin and guanidine chloride. The apparent bonding constant K increased, but ΔG decreased with the increase in pH, thus it can be shown that the bonding between bovine serum albumin and guanidine chloride could proceed more easily in an alkaline condition; the positive value of ΔG shows that the entropy decreased distinctly.

From Table 4, it can be found that with increasing pH, the absolute value of $n\Delta H$ also increases. It can be seen that pH influenced the total bonding enthalpy $n\Delta H$ between bovine serum albumin and urea. From the data on K and ΔG, the entropy decreased more distinctly in this bonding reaction. With the increase in pH, K decreased while ΔG increased; thus, it can be seen that the bonding between bovine serum albumin and urea would proceed more easily in an acidic condition. This was opposite to the bonding reaction of guanidine chloride.

The relationship of the total Gibbs free energy $\Delta G(a)$ and the activity of guanidine hydrochloride or urea could be obtained from Eq. (7), as shown in Figs. 1 and 2.

It was found that the absolute values of the total Gibbs free energy $\Delta G(a)$ increased with the increase in denaturant activity in solution, and it was seen that the higher the activity of the denaturant, the easier the denaturation of bovine serum albumin.

The relationships between $-Q(a)$ (the thermal effect that results from bovine serum albumin interacting with guanidine chloride or urea) vs. a (the activity of guanidine chloride or urea) are shown in Figs. 3 and 4. From the figures, it can be found that the slope of the straight line showing the unfolding bovine serum albumin was greater than that of the folding line, thus showing that the

Table 1 The thermal effect of bonding $Q(a)$ ($kJ \cdot mol^{-1}$) between BSA and guanidine chloride

a/($mol \cdot L^{-1}$)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
$Q(a)$ (pH = 4.80)	–91	–140	–182	–226	–238	–241	–267	–409	–503	–543	–630	–698
$Q(a)$ (pH = 7.05)	–89	–150	–199	–229	–230	–233	–271	–419	–478	–538	–646	–670
$Q(a)$ (pH = 9.30)	–90	–150	–200	–217	–232	–241	–351	–401	–436	–522	–601	–657

Table 2 The thermal effect of bonding $Q(a)$ ($kJ \cdot mol^{-1}$) between BSA and urea

a/($mol \cdot L^{-1}$)	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0
$Q(a)$ (pH = 4.80)	–230	–357	–389	–401	–431	–433	–609	–739
$Q(a)$ (pH = 7.05)	–78	–155	–198	–248	–261	–271	–352	–444
$Q(a)$ (pH = 9.30)	–81	–144	–207	–258	–264	–273	–320	–401

Table 3 The value of $n\Delta H$, K and ΔG for the reaction of bonding between BSA and guanidine chloride

	$n\Delta H$/($kJ \cdot mol^{-1}$)	K	ΔG/($kJ \cdot mol^{-1}$)
Guanidine chloride pH = 4.80, BSA pH = 6.97	–399	0.573	1.40
Guanidine chloride pH = 7.05, BSA pH = 7.05	–396	0.597	1.30
Guanidine chloride pH = 9.30, BSA pH = 9.30	–387	0.626	1.18

Table 4 The value of $n\ \Delta H$, K and ΔG for the reaction of bonding between BSA and urea

	$n\Delta H$/($kJ \cdot mol^{-1}$)	K	ΔG/($kJ \cdot mol^{-1}$)
Urea pH = 4.80, BSA pH = 6.97	–563	0.720	0.83
Urea pH = 7.05, BSA pH = 7.05	–693	0.133	5.09
Urea pH = 9.30, BSA pH = 9.30	–748	0.122	5.31

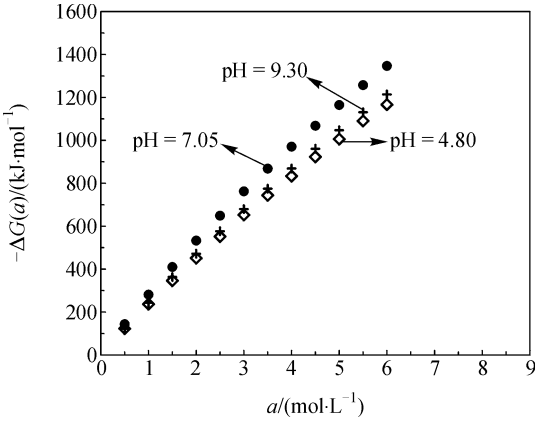

Fig. 1 Effect of guanidine chloride activity on $-\Delta G(a)$ for the interaction of BSA and guanidine chloride

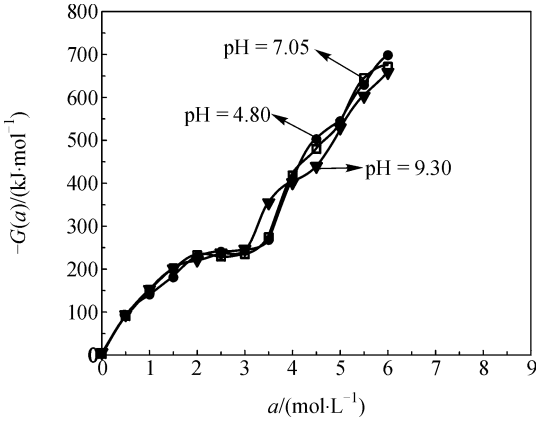

Fig. 3 Effect of guanidine chloride activity on $-G(a)$ for the interaction of BSA and guanidine chloride

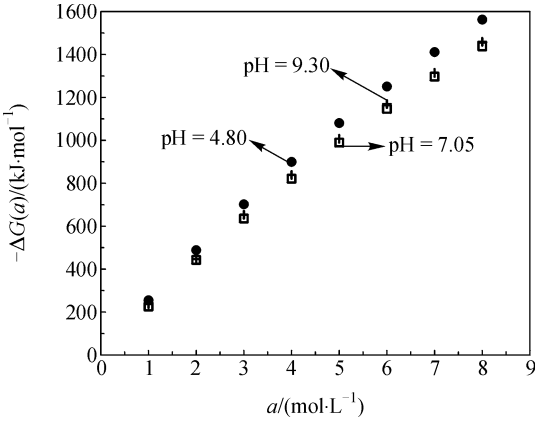

Fig. 2 Effect of urea activity on $-\Delta G(a)$ for the interaction of BSA and urea

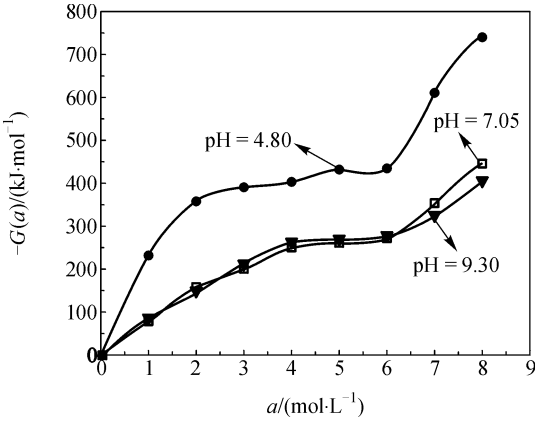

Fig. 4 Effect of urea activity on $-G(a)$ for the interaction of BSA and urea

folding bovine serum albumin could bond more denaturant molecules than the natural state. From Fig. 3, according to the linear extrapolation at the midpoint of transition ($2.5\ \mathrm{mol\cdot L^{-1}}$), the apparent denaturation enthalpy ΔH_d of bovine serum albumin due to guanidine chloride was $350\ \mathrm{kJ\cdot mol^{-1}}$ at pH 6.97 and 7.05, while it was $275\ \mathrm{kJ\cdot mol^{-1}}$ at pH 9.30, which indicated that bovine serum albumin was more stabilized in a neutral condition.

From Fig. 4, according to the linear extrapolation at the midpoint of transition ($4.5\ \mathrm{mol\cdot L^{-1}}$), the apparent denaturation enthalpy ΔH_d of bovine serum albumin due to urea was $295\ \mathrm{kJ\cdot mol^{-1}}$ at pH 6.97, while it was $230\ \mathrm{kJ\cdot mol^{-1}}$ at pH 7.05 and 9.30. The results indicated that the expanding degrees of bovine serum albumin in the two denaturants were different.

4 Conclusion

The apparent bonding constant K, the apparent singular bonding Gibbs bonding energy ΔG and the total Gibbs energy $\Delta G(a)$ between the protein and denaturant were obtained from analyzing the calorimetric data. The results showed that bovine serum albumin was relatively stable in neutral pH. The value of ΔH_d is close to that in literature, (i.e., $297\ \mathrm{kJ\cdot mol^{-1}}$ at pH 5.00 and $301\ \mathrm{kJ\cdot mol^{-1}}$ at pH 6.50–7.00 [1]). It was found that bovine serum albumin was a two-domain protein by studying its thermal denaturation using differential scanning calorimetry (DSC) [12]; one domain was unstable and could denature at a lower temperature, and its denaturation enthalpy was $328\ \mathrm{kJ\cdot mol^{-1}}$. There would appear a gel phenomenon or aggregation at higher concentrations of protein and denaturant, and these exothermic processes would interfere with the endothermic process of denaturation, therefore, the denaturation enthalpy would be decreased. Furthermore, the values of enthalpy by mid-point linear extrapolation are apparent values, and they only exist under the priority condition that the interaction be carried out with denaturalized protein and a denaturant [1].

The chemical denaturation of protein is because of the bonding between protein and the denaturant. The calorimetry experiment showed that the influences of pH in GuHCl-induced and urea -induced denaturation of bovine

serum albumin are opposite - for guanidine chloride, the bonding between bovine serum albumin and guanidine chloride can proceed more easily in an alkaline condition, but for urea, the bonding between bovine serum albumin and urea can proceed more easily in an acidic condition.

The denaturation enthalpy of bovine serum albumin induced by guanidine chloride is significantly higher than that where it is urea-induced, showing that the denaturation induced by guanidine chloride is significantly higher than that which is urea-induced. Guanidine chloride is an electrolyte, however, and urea is a non-ionic denaturant, so, the electrostatic interaction that maintains the tertiary structure of proteins can be weakened by the guanidinium-ion, but not by urea. Therefore, the mechanisms of protein denaturation induced by the two denaturants have great differences [13].

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