DSC study of cold and heat denaturation processes of \(\beta\)-lactoglobulin A with guanidine hydrochloride*

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Abstract The cold and heat denaturations of bovine \(\beta\)-lactoglobulin A (\(\beta\)-lg A) has been studied in solutions of guanidine hydrochloride (GuHCl) by differential scanning calorimetry (DSC). The experimental results are presented and discussed. It is shown that the number of protons bound by the monomeric molecules of \(\beta\)-lg A was unchanged before and after its heat denaturation below pH 3, and that the activation energy of the heat denaturation was depressed owing to the presence of GuHCl. In the solutions with 2.50 and 3.06 mol/L of GuHCl, both the cold and heat denaturations of \(\beta\)-lg A were observed. In comparison with the heat denaturation, the activation energy of cold denaturation was far lower and the number of GuHCl molecules bound by the unfolded polypeptide chains after cold denaturation increased a lot. The absolute value of the enthalpy of cold denaturation was larger than that of heat denaturation. It was found by the analysis that the contribution to the total denaturational enthalpy of conformational change itself of the monomeric molecules of \(\beta\)-lg A was the lowest among the globulins, according to the average of the number of heavy atoms.

Keywords: cold denaturation, heat denaturation, \(\beta\)-lactoglobulin A, guanidine hydrochloride, differential scanning calorimetry.

Protein molecules in an aqueous environment exist in their native state at ordinary temperature. The change of temperature may induce two kinds of denaturation. One is the commonest, the so-called heat denaturation induced by increasing temperature. Its progress is accompanied by heat absorption and an increase in enthalpy and entropy. The other is the cold denaturation predicted over thirty years ago by Brandts\(^{[1]}\), who used the spectroscopic data and made a long extrapolation. That is, with decreasing temperature, the protein molecules with a compact ordered structure in native state N are induced into those with a disordered structure in denatured state D. The progress of cold denaturation is accompanied by heat release and a decrease in enthalpy and entropy. In contrast to the spectroscopies such as NMR, CD and UV, application of calorimetry allows ones to determine directly the enthalpy of a denaturation process, having no need of supposing the existence of a two-state equilibrium N\(\leftrightarrow\)D during the denaturation. Therefore it has been a long-cherished strong desire that the exothermic cold denaturation could be observed directly by calorimetry. However, since 1986 when the first report appeared, this long-cherished desire has been realized only for a few proteins\(^{[2\text{-}5]}\). Consequently, the knowledge about cold denaturation is too little. Furthermore, the concentrations of the proteins involved in the foreign reports were lower than 1%, the lowest temperature reached by cooling was only about \(-8^\circ\text{C}\), and in some cases the recorded peaks of cold denaturation were incomplete. In order to know more completely the phenomenon of cold denaturation, this work was undertaken with the aim at studying

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directly the behavior of β-lactoglobulin A (β-lg A) during cold and heat denaturations in small quantity of aqueous solutions by differential scanning calorimetry (DSC).

1 Materials and methods

1.1 Materials

The bovine β-lg A (L7880, Lot 13H7020) used was purchased from the Sigma Chemical Co. In checking its purity by electrophoresis, a single band was displayed essentially. Therefore the reagent was used without further purification. After baking for 4 h at (105 ± 1)°C, the two samples of the protein were immediately put into a desiccator with Mg(ClO$_4$)$_2$. By weighing after 24 h, the water content of the protein was determined to be 6.3%, from which the dry weight of the protein was calculated. The reagents used, NaH$_2$PO$_4$ and guanidine hydrochloride (GuHCl), were Chinese commercial ones of the CP grade. The former was baked in 100—110°C, the latter was dried long enough in a desiccator with silica gel, and then the required solutions were prepared by weighing and using the suitable measuring flasks. The pH values of the solutions were adjusted to the desired values using Chinese commercial reagent H$_3$PO$_4$ of AR grade, and then the solutions were used as solvents of β-lg A.

1.2 Instrumental

A Perkin-Elmer Model DSC-2C differential scanning calorimeter equipped with a Model 3500 Data Station and a Chinese Model PHS-2 pH-meter were used in this work. Their calibration methods and accuracy were the same as those in the previous work$^5$.

1.3 Methods

The lyophilized powder of β-lg A, with its weight being 7.05 mg ± 0.03 mg, was mixed with 40 μL of the required solution in a stainless steel vessel, which was then sealed carefully with a Viton O-ring and placed overnight, and then used for DSC determination. For the DSC determinations related to low temperature, absolute alcohol was used as reference; otherwise, distilled water was used.

2 Results and discussion

The lyophilized powder of β-lg A was soaked with 40 μL of the solution. Therefore the water content of the samples was larger than 4.5 g H$_2$O per g dry protein. According to the investigation by Ruegg et al.$^6$, the β-lg A in this work should be hydrated completely. That is to say, in the samples there are freezable secondary hydration water and normal bulk water in addition to the unfreezable primary hydration water. The results of DSC determinations of the completely hydrated β-lg A are summarized in table 1. The typical DSC curves are presented in figure 1.

In table 1, the rates with negative values represent cooling rates, and the rates with positive values, heating rates; $T_i$ is the initial temperature of a peak, $T_p$ is the peak temperature, and $T_f$ is the final temperature of a peak; ΔH is the specific enthalpy obtained from the total area of a peak, its positive value represents the heat absorbed, and its negative value the heat released. All the ΔH values are those recalculated by using the dry weight which is calculated based on the
Table 1  DSC determination results of β-lg A in various solutions

<table>
<thead>
<tr>
<th>Protein weight/mg</th>
<th>Solvent composition</th>
<th>Solvent pH</th>
<th>Rate/ K·min⁻¹</th>
<th>Td/K</th>
<th>Tp/K</th>
<th>Tc/K</th>
<th>ΔH / J·g⁻¹</th>
<th>ΔHcal/ΔHcal</th>
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<td>7.04</td>
<td>A</td>
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<td>5.00</td>
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<td>353.69</td>
<td>362.23</td>
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<td>5.00</td>
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<td>358.43</td>
<td>365.80</td>
<td>13.3</td>
<td>1.8</td>
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<tr>
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<td>2.50</td>
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<td>361.91</td>
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<td>-2.50</td>
<td>267.38</td>
<td>256.54</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>A + 2.50 mol/L GuHCl</td>
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<td>-2.50</td>
<td>276.82</td>
<td>258.45</td>
<td>254.38</td>
<td>-13.0</td>
<td>1.3</td>
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<td>-2.50</td>
<td>280.43</td>
<td>295.46</td>
<td>308.53</td>
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<td>5.00</td>
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<td>355.11</td>
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<tr>
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<td>320.71</td>
<td>355.59</td>
<td>344.60</td>
<td>3.48</td>
<td>2.2</td>
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</table>

A = 0.02 mol/L NaH₂PO₄; - , not determined because of an incomplete peak of cold denaturation caused by freezing of freezable water at 253.42 K.

Fig. 1. Typical DSC curves. Sample: 7.07 mg of lyophilized powder of β-lg A + 40 μL of solution (0.02 mol/L NaH₂PO₄ + 3.06 mol/L GuHCl, pH 2.04); reference: absolute alcohol. Scanning rate: curve 1, -2.50 K/min; curve 2, 5.00 K/min.

As for all the renaturation peaks, the values of ratio ΔH_exh/ΔH_cal are not computed because the signal-to-noise ratio (ratio of the maximal peak height to the amplitude of noise) was too small. In fig. 1, the cooling and heating curves of β-lg A in the solution with 3.06 mol/L GuHCl are given. In this figure the initial point, top and final points of a peak have been marked by three perpendicular short bars, and the dashed lines are the interpolating baselines used for calculating the peak area. It can be seen from fig. 1 and table 1 that only the peaks of heat denaturation could be observed in the solutions without GuHCl, while the exothermic peaks of cold denaturation could be observed in both solutions with GuHCl. If the protein undergone cold denaturation is heated, the molecules of the protein will renature first into the native state, and then undergo heat denaturation, as shown by curve 2 in fig. 1. The experiments demonstrated that the processes of cold denaturation and its renaturation were reproducible. In the solution with 2.50 mol/L GuHCl, when the samples were reheated after heat denaturation and being kept at room temperature for several hours or three days, the protein weight and its water content. The values of ratio ΔH_exh/ΔH_cal are given in the rightest column of table 1. Here ΔH_exh was calculated from formula ΔH_exh = 2R²Tp(MΔC_p)²/²; ΔH_cal is equal to the molecular weight M of the β-lg A monomer multiplied by the specific enthalpy ΔH mentioned above. During the calculation, the molecular weight M of β-lg A monomer was computed at 18 366 u by means of the primary structure given by Grosclaude et al. [8], and then the molecular weight computed to calculate the molar quantities, the van’t Hoff enthalpy ΔH_exh and the calorimetric enthalpy ΔH_cal. As for all the renaturation peaks, the values of ratio were not computed because the signal-to-noise ratio (ratio of the maximal peak height to the amplitude of noise) was too small. In fig. 1, the cooling and heating curves of β-lg A in the solution with 3.06 mol/L GuHCl are given. In this figure the initial point, top and final points of a peak have been marked by three perpendicular short bars, and the dashed lines are the interpolating baselines used for calculating the peak area. It can be seen from fig. 1 and table 1 that only the peaks of heat denaturation could be observed in the solutions without GuHCl, while the exothermic peaks of cold denaturation could be observed in both solutions with GuHCl. If the protein undergone cold denaturation is heated, the molecules of the protein will renature first into the native state, and then undergo heat denaturation, as shown by curve 2 in fig. 1. The experiments demonstrated that the processes of cold denaturation and its renaturation were reproducible. In the solution with 2.50 mol/L GuHCl, when the samples were reheated after heat denaturation and being kept at room temperature for several hours or three days, the
peaks of heat denaturation of β-lg A reappeared, but the peak areas decreased by over 50%. The process of heat denaturation as a whole was not reproducible. In the solution with 3.40 mol/L GuHCl, only a DSC curve without any peak was recorded in both cooling and heating cases. This indicates that the molecules of β-lg A in this solution have been in denatured state at room temperature.

Armstrong et al. [9] indicated that the adjustment of pH to 2 was a condition under which the dimer of β-lg tended to dissociate into its two single chains. Therefore, in all the solutions at pH 2, β-lg A exists in the monomer form at room temperature. At pH 3, the dimers should have dissociated into its monomers before heat denaturation, because the heat denaturation occurs above 55°C [10]. Consequently it may be approved that the β-lg A undergoes heat denaturation in monomer form in this work. Moreover, it should be pointed out that the portion of DSC curve around 55°C was smooth, and there was no peak of dimer dissociation.

Just like the case for the calculation of van’t Hoff enthalpy, it may also be supposed that the denaturational process has been completed to a half extent at the peak temperature \( T_p \). Then equation

\[
\Delta n = n_D - n_N = (\Delta H_{cal}/2 \cdot 303 R T_p^2) \cdot \partial T_p/\partial pH
\]

(1)
can be obtained. Using eq. (1), one may inspect the change, which is caused by heat denaturation, of number \( n \) of protons bound with one monomeric molecule of β-lg A. Substituting the data for pH 2 and 3 in table 1 into eq. (1), and replacing the partial derivative by \( \Delta T_p/\Delta pH \), \( \Delta n \) can be calculated. The absolute value of \( \Delta n \) calculated is far less than unit. From this it may be concluded that the number of protons bound with one monomeric molecule of β-lg A is unchanged below pH 3 during heat denaturation. This shows that all the ionizable groups have exposed to the aqueous solutions before denaturation. Therefore one need not consider the contribution of the ionization enthalpies of the protein and buffer to the denaturational enthalpy obtained by calorimetry. Starting from the fact that the ionization enthalpies of the protein and buffer are usually small and possess the signs opposite to each other, Privalov[3] also came to the conclusion that the contribution of the ionization enthalpies need not be considered. It can be seen from table 1 that the values of ratio \( \Delta H_{vH}/\Delta H_{cal} \) for the processes of heat denaturation are between 1.5 and 2.2; according to Sturtevant's [11] view, this indicates that the cooperation among the monomeric molecules has occurred during the heat denaturation and an average “cooperation unit” comprises at least 1.5—2.2 monomeric molecules of β-lg A. Starting with this, we suggest a possible thermodynamic model \( N_c \rightarrow D \rightarrow F \) for the heat denaturation of β-lg A. Here N represents the native state, the subscript c implies that \( N_c \) is a “cooperation unit” in native state; F represents the final state. The single-directional step from the unfolded state D to F causes the process of heat denaturation as a whole to be irreversible. Azuaga and Lapanje stated the facts that the peak of heat denaturation at low pH values may reproduce [4] or reproduce partly [10]. In view of these facts, we are of opinion that the SH and SS exchanges may be involved in this single-directional step, which causes the occurrence of disulfide bond mismatch. Because the total number of chemical bonds is not changed, the mismatch of disulfide bonds does not influence the value of denaturation enthalpy. Disappearance of the mismatch of disulfide bonds during cooling or staying at room temperature results in the reappearance of the peak of heat denaturation.
The results of the DSC determinations at different rates have been given in table 1. On the basis of these data, following Ozawa's approach\textsuperscript{[12]}, the activation energy $E_a$ of denaturation can be estimated by rate $\beta$ and peak temperature $T_p$. Calculated with formula $\Delta \ln \beta / \Delta (1 / T_p) = \pm E_a / R$ (plus sign is suitable for cold denaturation, minus sign for heat denaturation), the activation energies are: 173.1 kJ/mol for the cold denaturation in the solution with 2.50 mol/L GuHCl; 253.5 kJ/mol for the heat denaturation in the same solution; and 309.0 kJ/mol for the heat denaturation in the solution only with 0.02 mol/L of NaH$_2$PO$_4$. From these it could be said that the presence of GuHCl depresses the activation energy of heat denaturation of $\beta$-lg A; and that the difference between the cold and heat denaturations is that the activation energy of cold denaturation is far lower than that of heat denaturation in addition to the opposite signs of denaturational enthalpies. By use of the activation energy 309.0 kJ/mol, the peak temperature of heat denaturation of $\beta$-lg A at the rate of 1.00 K/min in the solution only with 0.02 mol/L NaH$_2$PO$_4$ at pH 2 may be estimated to be 352.95 K. From the straight line in Privalov's fig. 3\textsuperscript{[11]}, the 353.4 K peak temperature of heat denaturation of $\beta$-lg A in the solution with 0.02 mol/L NaH$_2$PO$_4$ at pH 2 at the rate of 1.00 K/min can be obtained by interpolating. Considering the $\pm 0.2^\circ$ error of measuring temperature, the coincidence of both peak temperatures mentioned above is satisfactory.

It can be seen from the data of table 1 that the cooperation among monomeric molecules in cold denaturation is very weak and the reproducibility of cold denaturation implies that the unfolded monomeric molecules may refold. The values of $\Delta H_{da}/\Delta H_{cal}$ for the cold denaturation are very close to one. Therefore it may be described thermodynamically by the two-state model N$\rightleftharpoons$D. For the cold denaturation progressing in the low temperature range, the slower cooling rate must be used for obtaining the longer observation time and for recording the complete peak of cold denaturation before the freezing of freezable water in the system. But in order to obtain a satisfactory peak of heat denaturation, the higher heating rate is usually used to increase the signal-to-noise ratio. This is another difference between the cold and heat denaturations.

Replacing the pH in eq. (1) by the concentration of hydrogen ions $C$, the following equation

$$\Delta n = n_D - n_N = (- \Delta H_{cal} / RT_p^C) \cdot \partial T_p / \partial \ln C$$

(2)
can be obtained. When $C$ in eq. (2) is the molar concentration of GuHCl, this equation can be used for inspecting the change of the number $n$ of GuHCl molecules bound with one molecule of $\beta$-lg A before and after denaturation. When the average value of both denaturational enthalpies corresponding to two close concentrations of GuHCl and the monomeric molecular weight $M$ are used to calculate $\Delta H_{cal}$, and $\Delta T_p / \Delta \ln C$ is used to replace the partial derivative, $\Delta n = 6.1$ will be obtained for heat denaturation (using the data at 5.00 K/min in table 1); and $\Delta n = 13.0$ will be obtained for cold denaturation (using the data at $-2.50$ K/min). This estimation indicates qualitatively that the unfolded polypeptide chains of $\beta$-lg A monomers can bind with more GuHCl molecules after both the cold and heat denaturations, and that the $\Delta n$ corresponding to the cold denaturation is larger. If the binding of $\beta$-lg A with GuHCl is exothermic, that is, the sign of the binding enthalpy is the same as that of the cold denaturation enthalpy and is opposite to that of the heat denaturation enthalpy, then the absolute value of the cold denaturation enthalpy obtained by calorimetry would be larger than that of the heat denaturation enthalpy in the same system. The relevant data in table 1 show that such is the case. Indeed the results from Lapanje\textsuperscript{[13]} show that the binding of $\beta$-lg with GuHCl is exothermic. The conclusion that the absolute value of the cold
denaturation enthalpy in the presence of urea is larger has also been obtained by Privalov\textsuperscript{[31]} . The residues with the stronger hydrophobicity expose to the aqueous solution after the folded polypeptide chains are unfolded. Therefore, \( n_D > n_N \), this shows that the binding force between the GuHCl molecules and a \( \beta\)-lg A monomeric chain is not electrostatic; it may be of hydrogen bonding. The similar conclusion that the binding force of GuHCl with a protein is of hydrogen bonding has been obtained by Privalov\textsuperscript{[14]} in the investigation of the binding of GuHCl with RNase, lysozyme and cytochrome C.

On account of water being the major component in the solutions, we inspect the influence of hydration on the heat denaturation of bovine \( \beta\)-lg A in the solution only with 0.02 mol/L NaH\(_2\)PO\(_4\) at pH 3.02. Although there is a cooperation among the monomeric molecules, in order that the molar quantities corresponding to a polypeptide chain can be calculated by the monomeric molecular weight, the following cycle is used to analyse the heat denaturation:

\[
\begin{align*}
\text{N (nonhydrated)} & \xrightarrow{\text{Water}} \text{D (nonhydrated)} + \text{Water} \\
\text{N (hydrated)} & \xrightarrow{\Delta H_d} \text{D (hydrated)} \\
\Delta H_d & = \Delta H^\text{N}_d + (\Delta H^\text{D}_0 - \Delta H^\text{N}_0) = \Delta H^\text{N}_0 + \Delta H^\text{D}_0,
\end{align*}
\]

where \( \Delta H_d \) is the denaturational enthalpy obtained by calorimetry; \( \Delta H^\text{N}_0 \) is the contribution of the conformational change of the protein to the denaturational enthalpy \( \Delta H_d \); \( \Delta H^\text{D}_0 \) is the difference \((\Delta H^\text{D}_0 - \Delta H^\text{N}_0)\) between the hydration enthalpies of the protein in the native and denatured states, it is the contribution of the hydration of the protein during heat denaturation. Because the atomic coordinates of \( \beta\)-lg A were not available, we used the primary structure of bovine \( \beta\)-lg A given by Grosclaude\textsuperscript{[8]} and the enthalpies and heat capacities of hydration of amino acid residues in Ooi\’s table \textsuperscript{[15]}, then followed the method of residue additivity and made a deduction for the N-terminal residue Ala and the C-terminal residue Ala, and finally obtained the result \( \Delta H^\text{D}_0 = -557 \times 4.184 \) kJ/mol of monomer at 353.69 K for the \( \beta\)-lg A monomer having 1285 heavy atoms. Combined with the values of denaturational enthalpy in table 1 and eq. (3), the results \( \Delta H_d = 63.2 \times 4.184 \) kJ/mol of monomer and \( \Delta H^\text{N}_0 = 620 \times 4.184 \) kJ/mol of monomer at 353.69 K were obtained further for the \( \beta\)-lg A. Compared with the corresponding estimated values of the 14 globulins respectively at their denaturational temperatures in Ooi\’s table \textsuperscript{[15]}, it is found that the molar denaturational enthalpy \( \Delta H_d \) of \( \beta\)-lg A monomer is the lowest among those of the globulins. According to Privalov\’s argument\textsuperscript{[2]}, that the denaturational enthalpy is very low is a condition favorable for easily observing the cold denaturation peak. According to Ooi\’s approach, the engendering of the thermodynamic quantities of hydration is relevant to the change of the solvent accessible area of the heavy atoms before and after the denaturation. In comparison, to evade the inconvenience due to the difference of the numbers of heavy atoms in the molecules of proteins, the thermodynamic quantities are to be normalized by averaging the numbers of heavy atoms. That is, the values of \( |\Delta H^\text{D}_0| \) and \( \Delta H^\text{N}_0 \) of \( \beta\)-lg A are divided by its number of heavy atoms, and then the quotients are compared with the corresponding values of the 14 globulins. Consequently, it is found that the value of \( |\Delta H^\text{D}_0|/N_A \) of \( \beta\)-lg A is larger only than that of protein 4PTI, and close to that of protein 2SSI, and lower than those of the remaining 12 globulins; the value of
$\Delta H_d^0/N_A$ of $\beta$-lg A is the lowest among those of all the globulins. This shows that the denaturational enthalpy of hydration of $\beta$-lg A caused by the change of the solvent accessible area of heavy atoms is relatively low in view of the averages of the numbers of heavy atoms; and the summation of all the interactions (hydrogen bonding, hydrophobic bond, electrostatic interaction and disulfide bond), which result in a stable native three-dimensional structure of $\beta$-lg A, is the lowest among the globulins, in view of the averages of the numbers of heavy atoms.

References

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