A direct calorimetric determination of denaturation enthalpy for lysozyme in sodium dodecyl sulfate

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Received 7 August 2007; received in revised form 9 August 2007; accepted 15 August 2007
Available online 21 August 2007

Abstract
Thermodynamics of the interaction between sodium dodecyl sulfate (SDS) with lysozyme were investigated at pH 7.0 and 27 °C in phosphate buffer by isothermal titration calorimetry. A new method to follow protein denaturation, and the effect of surfactants on the stability of proteins was introduced. The new solvation model was used to reproduce the enthalpies of lysozyme–SDS interaction over the whole range of SDS concentrations. The solvation parameters recovered from the new equation, attributed to the structural change of lysozyme and its biological activity. At low concentrations of SDS, the binding is mainly electrostatic, with some simultaneous interaction of the hydrophobic tail with nearby hydrophobic patches on the lysozyme. These initial interactions presumably cause some protein unfolding and expose additional hydrophobic sites. The enthalpy of denaturation is 160.81 ± 0.02 kJ mol−1 for SDS.

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Keywords: Lysozyme; Sodium dodecyl sulfate; Proteins; Isothermal titration; Folding; Unfolding

1. Introduction
Protein denaturation is a key method in thermodynamics and binding site analysis and can be used to enhance our understanding of the protein structure-function relationship. Interaction of a protein with a surfactant (e.g., at a cell membrane) plays an important role in many biological processes. Many proteins possess specific binding sites for the surfactants [1–12]. Protein–surfactant interaction is an important area of research, which has many practical applications including washing detergents and stabilizing emulsions. Interaction of ionic detergents with oppositely charged proteins has been investigated for a long time, and there are numerous reports. The interaction of sodium n-dodecyl sulfate (SDS) as an anionic surfactant with lysozyme and other proteins has been widely investigated from viewpoints of binding isotherms, energetics and phase behavior [13]. It has been shown that, at the beginning of the interaction and at low concentration of SDS, the trend is specific and electrostatic in which negative SDS binds to positive residues, neutralizes the protein surface charges and leads to precipitation and turbidity of the solution. Reducing the net charge of the protein sets the stage for penetration of hydrophobic tail of SDS and leads to conformational changes of the protein at higher concentration. Meanwhile, it has been reported that lysozyme keeps its compactness at low SDS concentrations despite secondary structure changes [1].

The ionic surfactants consist of polar and non-polar portions on the same molecule. The dual nature of an anionic surfactant is typified by sodium n-dodecyl sulfate (SDS), CH₃–(CH₂)₁₀–CH₂–OSO₃⁻Na⁺, which has found wide application in biology [1]. The widespread application of surfactants, such as SDS, in the field of biochemistry has given impetus to fundamental studies of the nature of the interaction between protein and surface-active agents in biological phenomena such as biological membranes and protein solubilization. It has also been suggested that surfactant–protein systems can be used as a model for biological membranes. Since phospholipid membranes help to stabilize the three-dimensional structure of membrane proteins, the use of detergents for the reconstitution of the proteins seems plausible. The mechanism of the surfactant-induced unfolding of protein will improve our understanding of protein folding. One of the important

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applications of surfactants is the breakdown of protein structure (denaturation). Ionic surfactants, such as SDS, are unique in the way that they denature proteins at millimolar concentrations in marked contrast to other denaturants, such as guanidinium chloride or urea, which are effective only at molar concentrations [7–10]. Denaturation studies are capable of yielding information about the native state of a protein in terms of its thermodynamic stability, cooperativity, and the nature of the forces required to maintain its tertiary structure [7–10]. However, no direct calorimetric studies have been reported for unfolding reactions of lysozyme associated with denaturing agents at constant temperature. In this paper we report a unique procedure for direct calorimetric determination of denaturation enthalpy for proteins in the presence of denaturing agents.

2. Materials and methods

2.1. Materials

Hen egg-white lysozyme was obtained from Sigma and sodium n-dodecyl sulfate (SDS) was purchased from Merck. Protein concentrations were determined from absorbance measurements at 277 nm in 1 cm quartz cuvettes. The molar extinction coefficient of lysozyme was 7690 M⁻¹ cm⁻¹. All other materials and reagents were of analytical grade, and solutions were made in 50 mM buffer phosphate using double-distilled water.

2.2. Methods

The isothermal titration calorimetric experiments were performed with the four channel commercial calorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. The titration vessel was made from stainless steel. A solution of SDS (30 mM) was injected by use of a Hamilton syringe into the calorimetric titration vessel, which contained 1.8 mL lysozyme (68 µM). Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of SDS solution into the perfusion vessel was repeated 30 times, with 40 µL per injection. The heat of each injection was calculated by the “Thermometric Digitam 3” software. The heat of dilution of the SDS solution was measured as described above except lysozyme was excluded. The calorimeter was frequently calibrated electrically during the course of the study. The molecular weight of lysozyme was taken to be 14.7 kDa.

3. Results and discussion

Protein folding is a process in which an extended polypeptide chain acquires a maximally compact structure through formation of specific secondary and tertiary architecture. Large numbers of hydrophobic residues are buried inside the molecule upon protein folding, resulting in decreased heat capacity. However, a significant and opposite contribution by the burial of polar groups is also evident [1,7,8].

It has been suggested that solvation of a solute in binary solvent mixtures is analogous to complexation, with the better solvent taking the role of the ligand. The model used to analyze the enthalpies of transfer of a solute from a pure solvent into a mixed solvent system has been presented in detail previously [14–20]. Briefly this takes account of preferential solvation by the components of a mixed solvent, the extent to which the solute disrupts or enhances solvent–solvent bonding and the interaction of the solute with the surrounding solvent molecules. This treatment leads to:

\[ \Delta H^\circ = \Delta H^\circ (A \rightarrow B) = \Delta H^\circ A(x'_A - x'_B)(x'_A L_A + x'_B L_B) \]  

\[ \Delta A H^\circ (A \rightarrow B) \]

is the enthalpy of transfer from pure solvent A to pure solvent B. \( x'_A \) and \( x'_B \) are the local mole fractions of the components A and B in the solvation sphere, where the solvent molecules are the nearest neighbours of the solute, which can be expressed as follow:

\[ x'_A = \frac{n_B}{n}, \quad x'_B = 1 - x'_B \]

\[ \Delta H^\circ (A \rightarrow B) \]

is the enthalpy of transfer of the solutes from solvent A to the mixture of solvent A and B. \( x'_A \) and \( x'_B \) represent the bulk mole fractions of the components A and B in the binary mixtures. \( L_A \) and \( L_B \) are the relative partial molar enthalpies of A and B in the mixed solvent. The parameter \((an + \beta N)\) reflects the net effect of the solute on the solvent–solvent bonding, with \(cn\) resulting from the formation of a cavity wherein n solvent molecules become the nearest neighbours of the solute and \(\beta N\) reflecting the enthalpy change from strengthening or weakening of solvent–solvent bonds of N solvent molecules \((N \geq n)\) around the cavity \((\beta < 0\) indicates a net strengthening of solvent–solvent bonds). The constants \(\alpha\) and \(\beta\) represent the fraction of the enthalpy of solvent–solvent bonding associated with the cavity formation or restructuring, respectively. The degree \((\delta)\) in all cases refers to the quantities in infinite dilution of the solute. \(p < 1\) or \(p > 1\) indicate a preference for solvent A or B, respectively; \(p = 1\) indicates random solvation. As the parameters, \(\beta, n, N\) and \((an + \beta N)\) are not constant during the solvent compositions; thereby the net effect of the solute on solvent–solvent bonds in mixture \((an + \beta N)^{mix} = \delta^{mix}\) is changed over the solvent compositions and we can express this parameter as follow:

\[ \delta^{mix} = \delta_A x_A + \delta_B x_B = \delta_A + (\delta_B - \delta_A) x_B \]

\[ x'_A \] and \( x'_B \) are the local mole fractions of the components A and B in the vicinity of the solute or solvation sphere. \(\delta_A\) and \(\delta_B\) are the net effects of the solute on solvent–solvent bonds in water-rich domain and co-solvent-rich region, respectively. Therefore, Eq. (1) changes to:

\[ \Delta H^\circ = \Delta A H^\circ (A \rightarrow B) \]

Substituting \(\delta^{mix}\) from Eq. (3) into Eq. (4), leads to:

\[ \Delta H^\circ = \Delta A H^\circ (A \rightarrow B) \]

\[ \Delta \delta_A(x'_A L_A + x'_B L_B) \]

\[ -\delta_B(x'_A L_A + x'_B L_B)x'_B \]
With simple modification of Eq. (5), it is possible to use this equation to reproduce the enthalpies of surfactant–protein interaction as follow:

\[
Q = Q_{\text{max}}x_A' - \delta_A^a(x_A'L_A + x_B'L_B) - (\delta_B^a - \delta_A^a)(x_A'L_A + x_B'L_B)x_B' + f_D\Delta H_D
\]  

(6)

where \( Q \) is heat of lysozyme–surfactant interaction at certain ligand concentrations and \( Q_{\text{max}} = \Delta\Delta H_{12} + \delta_B^a\Delta H_{SDS}^S - \delta_B^a\Delta H_w^o \) represents the heat value upon saturation of all lysozyme. \( \Delta\Delta H_{12} \) is the difference between the enthalpies of water–lysozyme and SDS–lysozyme interactions. \( \Delta\Delta H_{12} > 0 \) indicates that the interaction of the lysozyme with SDS is weaker than with water. \( \Delta H_w^o \) is the enthalpy of condensation of pure water (−44.7 kJ mol\(^{-1}\)) and \( \Delta H_{SDS}^S \) is the enthalpy of solution of SDS in water (0.47 kJ mol\(^{-1}\)). \( x_A \) and \( x_B \) are the local mole fractions of the water and SDS in the vicinity of the lysozyme or solvation sphere. \( \delta_A^a \) and \( \delta_B^a \) are the net effects of the solute on solvent–solvent bonds in water-rich domain and SDS-rich region, respectively. \( L_A \) and \( L_B \) are the relative partial molar enthalpies, \( f_D \) is the fraction of lysozyme molecules undergoing denaturation and we can express this parameter as follow:

\[
f_D = \frac{Q_N - Q}{Q_N - Q_D}
\]

(7)

\( Q_N \) and \( Q_D \) is the heat of lysozyme–SDS interactions in the native and denatured state, respectively. \( x_A \) and \( x_B \) are bulk mole fractions in solvation model theory and we can express them in lysozyme–SDS interaction as the total ligand concentrations divided by the maximum concentration of SDS as follow:

\[
x_B = \frac{[SDS]_f}{[SDS]_{\text{max}}}x_A = 1 - x_B
\]

(8)

\( [SDS]_f \) is the total concentration of SDS and \( [SDS]_{\text{max}} \) is the maximum concentration of SDS upon saturation of all lysozyme. \( L_A \) and \( L_B \) are the relative partial molar enthalpies and can be calculated from heats of dilution of SDS in water, \( Q_{\text{dilut}} \), follows (Table 1):

\[
L_A = Q_{\text{dilut}} + x_B \left( \frac{\partial Q_{\text{dilut}}}{\partial x_B} \right),
\]

\[
L_B = Q_{\text{dilut}} - x_A \left( \frac{\partial Q_{\text{dilut}}}{\partial x_B} \right)
\]

(9)

\( Q \) values were fitted to Eq. (6) over the whole SDS compositions. In the procedure the only adjustable parameter (\( p \)) was changed until the best agreement between the experimental and calculated data was approached over the whole range of solvent composition. \( \delta_A^a \) and \( \delta_B^a \) are the net effects of lysozyme on solvent–solvent bonds in water-rich region and SDS-rich region, respectively, which are recovered from the coefficients of the second and third terms of Eq. (6). \( p < 1 \) or \( p > 1 \) indicate a preferential solvation of lysozyme by or SDS, respectively; \( p = 1 \) indicates random solvation. Solvation parameters for lysozyme–SDS interaction recovered by Eq. (6) are listed in Table 2. The agreement between the calculated and experimental results (Figs. 1 and 2) is striking, and gives considerable support to the use of Eq. (6).

A non-polar residue dissolved in water induces a solvation shell in which water molecules are highly ordered. When two non-polar groups come together on the folding of a polypeptide chain, the surface area exposed to the solvent is reduced and part of the highly ordered water in the solvation shell is released to bulk solvent. Therefore, non-polar moieties come together in aqueous solvent, resulting in formation of multimers and, in extreme cases, aggregation and precipitation. The most common mechanism of protein aggregation is believed to involve protein denaturation, via hydrophobic interfaces and often results in loss of biological activity. It is possible to introduce a correlation between change in \( \delta_A^a \) and increase in the stability of proteins. The \( \delta_A^a \) value reflects the hydrophobic property of lysozyme, leading to the enhancement of water structure. The greater the extent of this enhancement, the greater the stabilization of the lysozyme structure and the greater the value of \( \delta_A^a \). As it is shown in Fig. 2, at low concentrations of SDS (up to 8 \( \mu \)M), the binding is mainly electrostatic, with some simultaneous interaction of the hydrophobic tail with nearby hydrophobic patches on the lysozyme. These initial interactions presumably cause some protein unfolding and expose additional hydrophobic sites. \( \delta_A^a \) value (Table 2) for lysozyme–SDS interaction is negative and small (−0.43) indicating that small amounts of SDS destabilizes

<table>
<thead>
<tr>
<th>[Lysozyme] (( \mu )M)</th>
<th>[SDS] (( \mu )M)</th>
<th>( Q )</th>
<th>( Q_{\text{dilut}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>65.106</td>
<td>1.277</td>
<td>−0.433</td>
<td>−0.473</td>
</tr>
<tr>
<td>62.45</td>
<td>2.449</td>
<td>13.407</td>
<td>−0.403</td>
</tr>
<tr>
<td>60</td>
<td>3.529</td>
<td>50.809</td>
<td>−0.347</td>
</tr>
<tr>
<td>57.73</td>
<td>4.528</td>
<td>98.122</td>
<td>−0.304</td>
</tr>
<tr>
<td>55.64</td>
<td>5.454</td>
<td>125.164</td>
<td>−0.267</td>
</tr>
<tr>
<td>53.84</td>
<td>6.316</td>
<td>139.323</td>
<td>−0.239</td>
</tr>
<tr>
<td>51.86</td>
<td>7.119</td>
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<td>−0.215</td>
</tr>
<tr>
<td>50.16</td>
<td>7.869</td>
<td>153.931</td>
<td>−0.195</td>
</tr>
<tr>
<td>48.57</td>
<td>8.571</td>
<td>159.985</td>
<td>−0.178</td>
</tr>
<tr>
<td>47.08</td>
<td>9.231</td>
<td>165.05</td>
<td>−0.164</td>
</tr>
<tr>
<td>45.67</td>
<td>9.851</td>
<td>169.054</td>
<td>−0.152</td>
</tr>
<tr>
<td>44.35</td>
<td>10.435</td>
<td>172.518</td>
<td>−0.141</td>
</tr>
<tr>
<td>43.1</td>
<td>10.986</td>
<td>175.549</td>
<td>−0.132</td>
</tr>
<tr>
<td>41.92</td>
<td>11.507</td>
<td>178.163</td>
<td>−0.124</td>
</tr>
<tr>
<td>40.8</td>
<td>12</td>
<td>180.402</td>
<td>−0.116</td>
</tr>
</tbody>
</table>

\( Q_{\text{dilut}} \) is the enthalpies of dilution of SDS with water. Precision is ±0.005 kJ or better.

\( \Delta\Delta H_{12} > 0 \) indicates that the interaction of the lysozyme with SDS is weaker than with water.
the lysozyme structure, leading to a mild denaturation. At high SDS concentrations, presumably two different complexes coexist: one is similar to that obtained at low SDS concentrations and has a compact structure, and the other is larger complex in which lysozyme probably has a more open, expanded structure, presumably caused by the binding of a greater amount of SDS. In the SDS-rich region $\delta_A^\circ$ value is high and negative ($-57.58$), indicates that the hydrophobic sites of lysozyme have been saturated by the hydrophobic tail of SDS in this region and hydrophobic property of lysozyme is rapidly decreased by the addition of SDS. We can attribute decreasing $\delta_B^\circ$ value to the loss of hydrophobic property of lysozyme in SDS-rich region and destabilizing of its native conformation, resulting in the great denaturation of lysozyme. The electrostatic interaction, which is accompanying by preliminary hydrophobic interaction is occurred initially (in agreement with $\delta_A^\circ$ value), followed by a more extensive pure hydrophobic interaction (Fig. 2) which is in agreement with $\delta_B^\circ$ value in SDS-rich region. A procedure is described for direct calorimetric determination of denaturation enthalpies of proteins in the presence of denaturing agents. The method has been applied to the reversible transition of lysozyme between native and unfolded conformations in SDS at 27 $^\circ$C. The heat of unfolding has been measured at various concentrations of SDS using Eq. (6) and found to be 160.81 ± 0.02 kJ mol$^{-1}$ under conditions of complete transition. The corresponding SDS induced van’t Hoff enthalpy of denaturation was determined from difference spectral measurements at 301 nm and found to be 148 ± 15 kJ mol$^{-1}$ [21]. Agreement between the calorimetric and van’t Hoff enthalpies of denaturation is a good support for Eq. (6). The calorimetric and the heat induced van’t Hoff enthalpy of denaturation (245 ± 10 kJ mol$^{-1}$) [21] are different, indicating the presence of appreciably populated intermediate species between native and denatured forms of lysozyme which is again in agreement with our conclusion from $\delta_A^\circ$ and $\delta_B^\circ$ values. Lysozyme is preferentially bonded by SDS as the $p$-value is more than one ($p = 4.17$).

Acknowledgements

Financial support from the Universities of Imam Khomeini (Qazvin), Tehran and the Iranian National Science Foundation (INSF) are gratefully acknowledged.

References