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# Refolding of Lysozyme Upon Interaction with β-Cyclodextrin

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## Abstract

Effects of β-cyclodextrin, βCD, on refolding of lysozyme was investigated at pH 12 employing isothermal titration calorimetry (ITC) at 300K in 30mM Tris buffer solution. βCD was employed as an anti-aggregation agent and the heats obtained for lysozyme+βCD interactions are reported and analyzed in terms of the extended solvation model. It was indicated that there are two sets of identical and non-cooperative sites for βCD. Enthalpic force in the first binding sites is more important than entropic one, indicating that electrostatic interaction plays an important role in the interaction of lysozyme with βCD. The interaction in the second binding sites is stronger and both enthalpy and entropy driven but hydrophobic interaction has more important than electrostatic force. These results suggest that the effects of βCD on lysozyme refolding are attributed to its ability to suppress aggregation of the protein.

**Keywords:** Lysozyme; Isothermal titration calorimetry; β-Cyclodextrin; Binding parameters

## Introduction

Cyclodextrins (CDs) have been reported to suppress aggregate formation during the refolding of a wide range of proteins. Their potency is often ascribed to their affinity for aromatic amino acids, whose surface exposure would otherwise lead to protein association. However, no detailed structural studies are available. CDs, consisting of six, seven, or eight D-glucopyranose units, which are referred to as α-, β-, and γ-cyclodextrins, respectively. CDs inhibited the chemically induced aggregation and its inhibition was generally in the order of γ-CDs < α-CDs < β-CDs. Hydrophilic CDs reduced the thermally induced unfolding of lysozyme, suggesting that CDs destabilize native lysozyme or stabilize the unfolded state of lysozyme [1-4]. Electrophoresis data indicate that CDs, which promoted lysozyme refolding, arrested

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aggregation at the stage of smaller soluble aggregates [3]. The presence of both anionic and cationic substituents on the same CD molecule was found to partially restore its renaturation ability. The reports show that Beta-cyclodextrin improved thermal stability and biological activity of lysozyme [4-10]. Previously published results suggest that the effects of CDs on protein refolding are attributed to their ability to suppress aggregation of proteins. CDs may show properties similar to chaotropic agents, which may help explain their anti-aggregation and protein refolding ability. Biological assay exhibited that lysozyme and lyophilized protein/cyclodextrin better maintained protein biological activity compared to lyophilized lysozyme in absence of cyclodextrin [1-14]. CDs have hydrophobic cavities that prevent direct interactions on the hydrophobic surfaces of proteins and in this way suppress protein aggregation [12-14].

The tendency of lysozyme to aggregate is most distinct at pH 12. Thus, exposure to alkaline pH of 12 serves as a convenient approach to initiate the aggregation of lysozyme. In these conditions we can follow the anti-aggregation effect of βCD clearly.

## Materials and Methods

Hen egg-white lysozyme was obtained from Sigma. The isothermal titration calorimetric experiments were carried out on a VP-ITC ultra sensitive titration calorimeter. The microcalorimeter consists of a reference cell and a sample cell of 1.8mL in volume, with both cells insulated by an adiabatic shield. All solutions were thoroughly degassed before use by stirring under vacuum. The sample cell was loaded with lysozyme solution (1.26 mM) and the reference cell contained buffer solution. The solution in the cell was stirred at 307 rpm by the syringe (equipped with micro propeller) filled with βCD solution (30mM) to ensure rapid mixing. Injections were started after baseline stability had been achieved. The titration of lysozyme with βCD were carried out on a VP-ITC ultra sensitive titration calorimeter (MicroCal, LLC, Northampton, MA). The measurements were performed at a constant temperature of 27.0±0.02 °C and the temperature was controlled using a Poly-Science water bath. The titration of lysozyme with βCD solution involved 30 injections, the first injection was 5 μL and the remaining ones were 10 μL. To correct the thermal effects due to βCD dilution, control experiments were done in which identical aliquots were injected into the buffer solution with the exception of lysozyme. In the ITC experiments, the heat changes associated with processes occurring at a constant temperature are measured.

## Results and Discussion

The determined heats for lysozyme+βCD interaction were shown graphically in Figure 1.

We have shown previously [15-27] that the heats of the macromolecules+ligands interactions in the aqueous solvent systems can be reproduced by the following equation:

$$q = q_{\max} x'_{\mathrm{B}} - \delta^{\theta}_{\mathrm{A}}(x'_{\mathrm{A}} L_{\mathrm{A}} + x'_{\mathrm{B}} L_{\mathrm{B}}) - (\delta^{\theta}_{\mathrm{B}} - \delta^{\theta}_{\mathrm{A}})(x'_{\mathrm{A}} L_{\mathrm{A}} + x'_{\mathrm{B}} L_{\mathrm{B}}) x'_{\mathrm{B}} \quad (1)$$

The parameters $\delta^{\theta}_{\mathrm{A}}$ and $\delta^{\theta}_{\mathrm{B}}$ reflect to the net effect of βCD on the lysozyme stability in the low and high dextrin concentrations respectively. The positive values for $\delta^{\theta}_{\mathrm{A}}$ or $\delta^{\theta}_{\mathrm{B}}$ indicate that βCD stabilizes the lysozyme structure and vice versa. $x'_{\mathrm{B}}$ can be expressed as follows:

$$x'_{\mathrm{B}} = \frac{p x_{\mathrm{B}}}{x_{\mathrm{A}} + p x_{\mathrm{B}}} \quad (2)$$

$p > 1$ or $p < 1$ indicate positive or negative cooperativity of macromolecule for binding with ligand respectively; $p$=1 indicates that the binding is non-cooperative. $x'_{\mathrm{B}}$ is the fraction of bound βCD and $x'_{\mathrm{A}} = 1 - x'_{\mathrm{B}}$ is the fraction of unbound βCD. We can express $x_{\mathrm{B}}$ as follows:

$$x_{\mathrm{B}} = \frac{[CD]}{[CD]_{\max}} \qquad x_{\mathrm{A}} = 1 - x_{\mathrm{B}} \quad (3)$$

[$\beta CD$] is the concentration of βCD after every injection and [CD] is the maximum consternation of βCD upon saturation of all lysozyme molecule. $L_{\mathrm{A}}$ and $L_{\mathrm{B}}$ can be calculated from heats of dilution of CD in water, $q_{\mathrm{dilut}}$, as follows:

$$L_{\mathrm{A}} = q_{\mathrm{dilut}} + x_{\mathrm{B}}\left(\frac{\partial q_{\mathrm{dilut}}}{\partial x_{\mathrm{B}}}\right), L_{\mathrm{B}} = q_{\mathrm{dilut}} - x_{\mathrm{A}}\left(\frac{\partial q_{\mathrm{dilut}}}{\partial x_{\mathrm{B}}}\right) \quad (4)$$

The heats of lysozyme+βCD interactions were fitted to Eq. 1 over the entire βCD concentrations. In the fitting procedure, the only adjustable parameter ($p$) was changed until the best agreement between the experimental and calculated data was approached.

There are two distinct sets of binding sites on lysozyme, which are clear in Figure 1. The dissociation equilibrium constant ($K_d$) and the number of binding sites "$g$" can be determined by the following equation [15-27]:

$$\frac{\Delta q}{q_{\max}} M_0 = (\frac{\Delta q}{q}) L_0 \frac{1}{g} - \frac{K_d}{g} \quad (5)$$

Where $\Delta q = q_{\max} - q$ and $q$ represents the heat value at a certain βCD ($L_0$) and lysozyme ($M_0$) concentrations and $q_{max}$ represents the heat value upon saturation of all lysozyme molecule. Therefore, the plot of $(\frac{\Delta q}{q_{\max}})M_0$ *vs.* $(\frac{\Delta q}{q})L_0$ should be a linear plot with slope of *"1/g"* and the vertical-intercept of $\frac{K_d}{g}$, which "*g*" and $K_d$ can be obtained (Table 1). If $q$ and $q_{max}$ are calculated per mole of lysozyme, then the standard molar enthalpy of binding for each binding site, $\Delta H^0$, will be $\Delta H^0 = \frac{q_{\max}}{g}$. The change in the standard Gibbs free energy, $\Delta G^0$, and change in standard entropy of binding, $\Delta S^0$, could be calculated by using association equilibrium constant, $K_a = 1/K_d$, and $\Delta H^0$ value in equations 6 and 7, respectively.

$$\Delta G^0 = -RT \ln K_a \quad (6)$$

$$\Delta S^0 = \frac{\Delta H^0 - \Delta G^0}{T} \quad (7)$$

The binding parameters in Table 1 suggest that the effects of βCD on lysozyme refolding are attributed to its ability to suppress aggregation of lysozyme. βCD reduced the unfolding of lysozyme as evidenced by large values of association equilibrium constants ($K_a$=49967.89 and 130462 $M^{-1}$ at the first and second set of binding sites respectively), suggesting that βCD stabilize native or unfolded state of lysozyme. The binding process for inhibition of lysozyme aggregation at the first set of binding sites was both enthalpy and entropy driven (Table 1), but electrostatic interaction plays an important role in the binding processes. The interaction in the second set of binding sites is stronger and both enthalpy and entropy driven but hydrophobic interaction is more important than electrostatic force for the inhibition of lysozyme aggregation (Table 1). βCD has a stronger affinity for lysozyme at the second set of binding sites, as evidenced by larger association equilibrium constant. A negative $\delta_A^\theta$ value ($\delta_A^\theta = -0.34$) for the interaction is a characteristic of the electrostatic interactions underlying many non-specific ligand-protein interactions, indicating that βCD destabilizes lysozyme structure. Destabilization of lysozyme by βCD indicates that βCD binds preferentially to the unfolded lysozyme or to a partially folded intermediate form of lysozyme. Such effects are characteristic of nonspecific interactions, in that the nonspecific ligand binds weakly to many different groups at the protein/water interface. Therefore, the calorimetric results suggest that inhibition of lysozyme aggregation is the result of nonspecific interactions at the first set of binding sites. In the other words, the negative $\delta_A^\theta$ values followed by positive value of $\delta_B^\theta$ indicates that firstly, the non-specific binding of βCD to exposed side-chains on unfolded lysozyme will destabilize the native folded form of lysozyme. Alternatively, interactions with

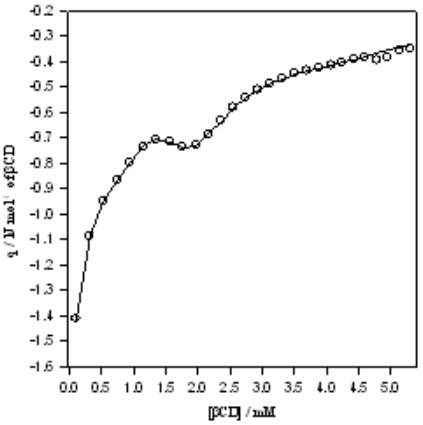


**Figure 1.** Comparison between the experimental heats, *q*, (O), for lysozyme+βCD interaction and calculated data (lines) via Eq. 1. [βCD] are concentrations of βCD solutions in mM at pH 12.

**Table 1.** Binding parameters for lysozyme+βCD interaction at pH 12. *p*=1 indicates that the binding is non-cooperative in two sets of binding sites. Enthalpic force in the first binding sites is more important than entropic one, indicating that electrostatic interaction plays an important role in the interaction of lysozyme with βCD. The interaction in the second binding sites is stronger and both enthalpy and entropy driven but hydrophobic interaction has more important than electrostatic force

| Parameters | First binding sites | Second binding sites |
|---|---|---|
| $p$ | 1 | 1 |
| $g_i$ | 2.00±0.03 | 5.11±0.08 |
| $K_a$/M | 49967.89±21.15 | 130462.30±13.06 |
| ΔH/kJ $mol^{-1}site^{-1}$ | -2.05±0.05 | -0.08±0.01 |
| ΔG/kJ $mol^{-1}$ $site^{-1}$ | -26.80±0.15 | -29.18±0.25 |
| ΔS/kJ $mol^{-1}$ $site^{-1}$ | 0.09±0.01 | 0.10±0.01 |
| $\delta_A^\theta$ | -0.36±0.03 | |
| $\delta_B^\theta$ | | 0.09±0.02 |

groups on oligomeric folded proteins can lead to dissociation of these protein aggregates. Finally, cyclodextrin interaction with unfolded proteins may enhance the solubility of partially denatured lysozyme by masking the exposed hydrophobic residues, thereby assisting the refolding of lysozyme molecule.

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