

INTERACTION OF B-CYCLODEXTRIN WITH MOLTEN GLOBULE STATE OF HEN EGG-WHITE LYSOZYME

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ABSTRACT

The molten globule state of hen egg-white lysozyme (HEWL) is the major intermediate of a protein folding. Chaperones prevent aggregation and precipitation of proteins under conditions of stress. The present study aims to investigate the conformational state of the hen egg-white lysozyme when it in forming a high molecular weight complex with β -cyclodextrin (β -CyD) as a molecular chaperone using UV spectroscopy, fluorescence spectrophotometry, circular dichroism (CD) spectropolarimetry, Iso thermal titration (ITC) as well as the measurement of viscosity and Stokes radius of protein. The results showed that β -CyD reduces and delays the aggregation of lysozyme and increases the ANS (8-anilino-1-naphthalenesulfonic acid) fluorescence intensity of the spectra. Therefore, a hydrophobic interaction occurred between β -CyD and lysozyme. In the range of 204–250 nm CD spectra of lysozyme did not change at the presence of β -CyD in ellipticity relative to native lysozyme. Measuring Stokes radius of lysozyme in the presence and absence of β -CyD and comparing its value with denatured state showed that molten globule was an intermediate state in protein folding process.

Keywords: *Lysozyme, B-Cyclodextrin, Molten Globule, Circular Dichroism Spectropolarimetry, Fluorescence, Spectrophotometry, Iso Thermal Titration*

INTRODUCTION

Protein folding/unfolding process is a highly cooperative and a complex process of which only the completely folded/unfolded states have significantly been populated. However, under certain circumstances, several proteins exhibit a folding intermediate known as the molten globule (MG) (Kim and Baldwin, 1982; Ewbank and Creighton, 1991). The molten globule intermediates for different proteins have some common features, primarily a native-like secondary structure, high degree of compactness, and a disrupted tertiary structure (Kuwajima, 1989; Hildebrandt and Stockburger, 1989; Ptitsyn, 1987). The MG state has also been shown to exhibit a rotational correlation time and viscosity close to that of the native state indicating its highly compactness (Brazhikov *et al.*, 1981). It is important to elucidate the structure and the stabilizing mechanism of the MG state as an intermediate between the native and denatured states, in order to understand the principle of constructing a three-dimensional protein structure. X ray angle scattering studies have shown that MG states of various proteins have a wide range of structures from relatively disordered to highly ordered ones (Kataoka, 1993; Nishii *et al.*, 1994). The stability of the MG state is determined through a delicate balance of interaction, such as electrostatic repulsion between charged residues and opposing forces including hydrophobic interaction (Hamada, 1993; Goto and Nishikori, 1993). The MG state, first identified for cytochrome C (Chamani, 2003; Moosavi-Movahedi *et al.*, 2003) and α lactalbumin (Moosavi-Movahedi *et al.*, 2003; Moosavi-Movahedi *et al.*, 2011) however the MG state has now been recognized for various other proteins (Moosavi-Movahedi *et al.*, 2011; Rajabzadeh, *et al.*, 2011). Fluoroalcohols have been shown to exhibit remarkable structure stabilizing effects for peptides in aqueous solution (Terada and Kuwajima, 1999; Leavitt and Freire, 2001) and also hexafluoroacetone trihydrate is a far more potent structure inducer in peptides [23] and has been investigated in detail in the case of proteins (Kornblatt *et al.*, 2001). In the current study, we studied folding /unfolding of lysozyme in the presence and absence of β -CyD, as an chaperone molecule, and showed the effects of β -CyD on thermodynamic stabilization based on ITC, CD and ANS fluorescence spectrophotometry.

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MATERIALS AND METHODS

β -CyD, Hen-Egg White Lysozyme (HEWL), 8-anilino-1-naphthalenesulfonic acid (ANS), dithiotreitol (DTT), and other chemical reagents (salts, detergents) were obtained from Sigma-Aldrich. The buffer used for the preparation of stock solution of 1mM DTT and 10 mM ANS was 10 mM sodium phosphate (pH 7.0), 150 mM for preparation of 0.1% Lysozyme and 30 mM β Cyclodextrin.

Lysozyme Disaggregation

Lysozyme was aggregated as follows: solution containing lysozyme 0.1% in 150 mM sodium phosphate buffer (pH7.2) and 10 mM DTT was prepared. Its absorption was monitored at 37°C. at 360 nm by Jen Way spectrophotometer model 6505 for 50 min. The disaggregation of lysozyme was quantified as follows: solution containing lysozyme 0.1% in 150 mM sodium phosphate buffer (pH7.2) and different concentrations of β -cyclodextrin ranging from 0.3, 3 to 30 mM in presence of 10 mM DTT(40 μ l) were prepared. The absorbance was monitored at 360nm at 37°C using JenWay spectrophotometer model 6505

Fluorescence Spectrophotometry

To study The changes in tertiary structure of lysozyme on the basis of extrinsic fluorescence, the following solutions were prepared: a) lysozyme with concentration 0.177 mg/ml in 150 mM phosphate buffer (pH7.2), b) lysozyme with concentration 0.177mg/ml in the presence of 0.1M β -cyclodextrin and c) 0.1M β -cyclodextrin. All of the above were incubated at 37°C. for 50 min and carried to 0.4 ml cuvette then we measured their fluorescence in the presence of 10mM ANS(1-anilino-8-naphtalene sulfonate) by Cary Eclipse fluorospectrophotometer at $\lambda_{\text{excitation}}$ 365 nm at 37°C with a 10 nm bandwidth for every measurements and then all the results obtained were carried to sigma plot program for smoothing.

Circular Dichroism Spectropolarimetry

The secondary structure of lysozyme was investigated as follows: prepared samples of lysozyme 0.177mg/ml in 150 mM phosphate buffer(pH 7.2) in the presence /absence of 0.1M β -cyclodextrin were incubated for 20 and 50 min at 37°C. All samples were carried to cuvette 0.1 cm to study secondary structure by the circular dichroism spectropolarimeter AVIV model 215. The far UV spectra were recorded in the range of 190-260nm. The final results were obtained by Sigma plot program.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) measurements were made on a VP-ITC ultrasensitive microcalorimeter (MicroCal, Northampton, MA). All the solutions were degassed before titrations were performed. The procedure was carried out as follows: β -CyD (30 mM) and lysozyme (1.26 μ M) solution were prepared in buffer phosphate pH 7.2 at 37°C. During the titration, 8 μ L of β -CyD solution was injected with 5 min intervals into the calorimetric titration vessel, which contained lysozyme (1.26 μ M). The cell was stirred at 307 rpm. The titration was conducted at 37°C. The injection of β -CyD solution into the vessel was repeated 30 times, with 8 μ L per injection. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated by the "Thermometric Digitam 3" software program. The microcalorimeter was frequently calibrated electrically during the course of the study. The enthalpies of β -CyD-lysozyme interactions were calculated in kJ/mol and analyzed with GRB solvation model using a nonlinear least square method.

Measurement of Viscosity and Stokes radius

The viscosity was measured using a Haake D8 (W.Germany) microriscometer. The intrinsic viscosities, $[\eta]$, and stokes radii, R_s , of a lysozyme- β -cyclodextrin complexes were determined using the equation [25].

$$\eta_{sp}/c \approx [\eta] = \lim [(\eta/\eta_0 - 1)/c] = 2.5 N_A/M (4/3 \pi R_s^3)$$

Where η_{sp} is the specific viscosity, η_0 is the solvent viscosity, C is the protein concentration in grams per milliliter, N_A is Avogadro's number, M is the molecular weight of the protein and π is equal to 3.14.

RESULTS

Figure 1 shows the absorption of lysozyme at 360 nm vs. time and compares the effect of different concentrations of β -cyclodextrin on aggregation of lysozyme. It is observed that the aggregation of lysozyme is reduced and delayed in the presence of β -cyclodextrin.

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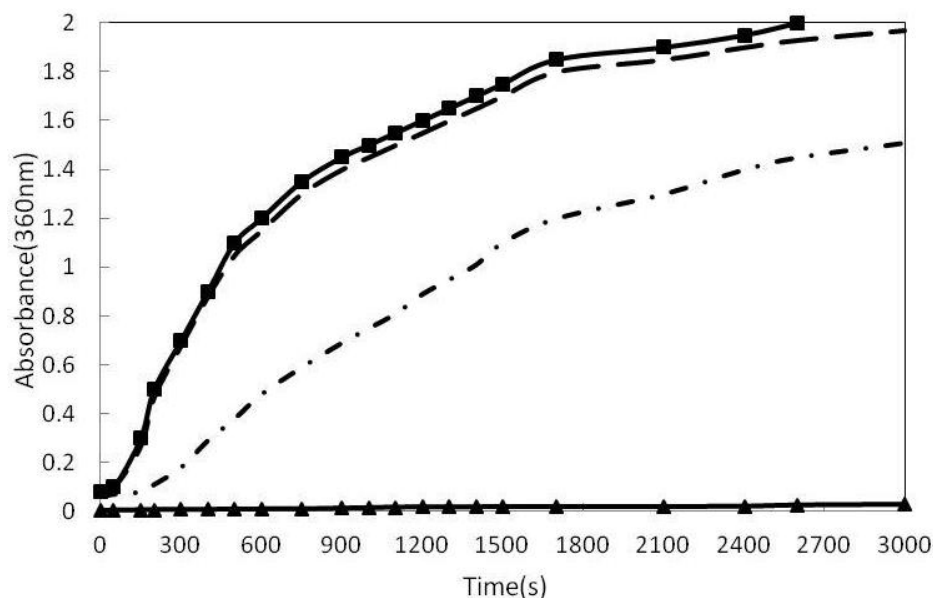


Figure 1: Chemical aggregation of lysozyme (0.1%) by DTT in the absence (■) and presence of different concentration of β -CyD, 0.3 mM (dash), 3 mM (dash dot), and 30 mM (▲) at 37 °C

In order to understand the changes in tertiary structure of lysozyme, we employed fluorescence substance for monitoring the effect of β -cyclodextrin on tertiary structure of lysozyme. The observations obtained from fluorescence spectrophotometry indicated the changes in conformation of tertiary structure of lysozyme Fig. 2. The results obtained from extrinsic fluorescence of lysozyme in figure 2 reveal that the intensity of spectra has increased in the presence of β -cyclodextrin. ANS (1-anilino-8-naphtalene sulfonate) is a kind of appropriate fluors has a weakness fluoresce in aqueous solution however in nonpolar environment the quantum yield increased and the spectrum shifts towards shorter wavelength. Hence, it is used to detect nonpolar reigen in proteins by binding to such a region and finally it leads to increasing fluorescence.

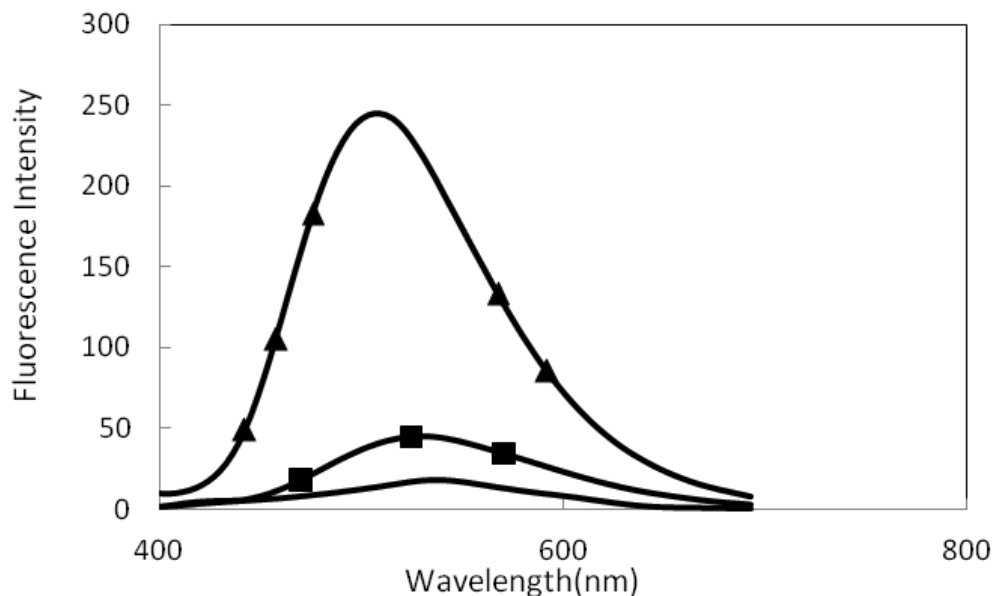


Figure 2: ANS fluorescence spectra of lysozyme in the absence (■), presence (▲) of β -CyD and ANS alone (-) incubated at 37 °C for 50 min.

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Circular dichroism spectropolarimetry was employed to study the changes in the secondary structure of lysozyme by conducting the reaction at 37 °C for 20 and 50 min in the presence and absence of β -cyclodextrin for lysozyme. The results obtained through circular dichroism spectra (Fig. 3) show that the secondary structure of lysozyme in the presence of β -cyclodextrin as compared to its native protein does not change.

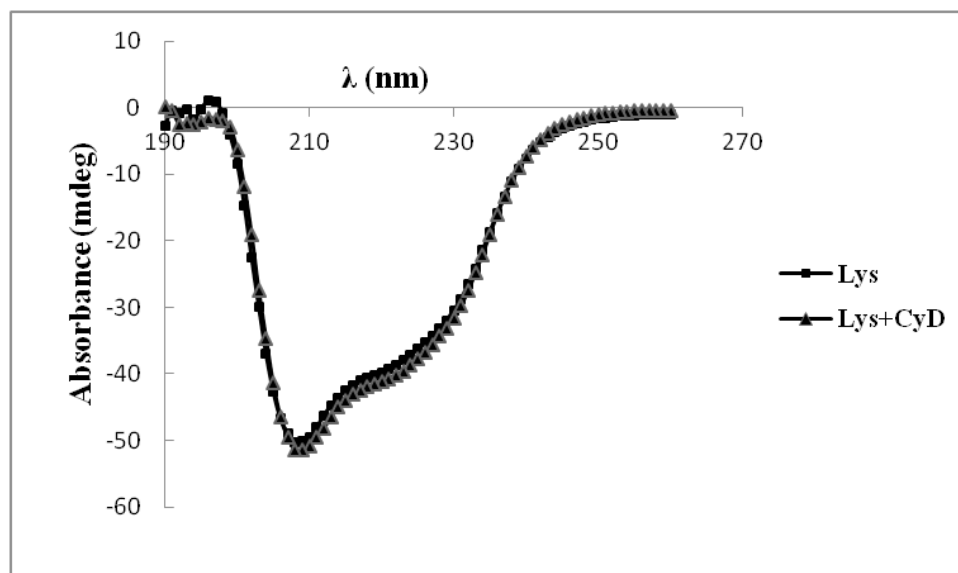


Figure 3: Molar ellipticity of lysozyme (■) and lysozyme with β -CyD (▲)

The results of ITC reveal that heat of complex formation between β -CyD and aromatic groups in the first binding sites and the second ones were as 112.84 and 225.75 kJ mol⁻¹, respectively (Table 1). The values of viscosity and Stokes radius of the lysozyme in presence and absence of β -cyclodextrin are given in table 2.

Table 1: Binding parameters for lysozyme+ β -CyD interaction recovered from Eqs. 1 and 2 at pH 7. $p=1$ indicates that the binding is non-cooperative in two sets of binding sites. The small negative values of δ_A^θ or δ_B^θ indicate that there are very little structural changes of lysozyme due to its interaction with cyclodextrin. The interaction is weak and just entropy driven.

Parameters	First binding sites	Second binding sites
P	1	1
g_i	1.86±0.01	4.22±0.01
K_d /mM	299.7	680.70
ΔH /kJ mol ⁻¹	112.84	225.75
ΔG /kJ mol ⁻¹	-1702.55	-543.46
$T\Delta S$ /kJ mol ⁻¹	1815.40	656.31
δ_A^θ	-0.04	
δ_B^θ		-0.04

Table 2: Intrinsic Viscosity and Stokes radius at 37°C. a) lysozyme (0.1M) b) lysozyme (0.1M) with β -CyD

Protein state	η (M ⁻¹)	R_s (Å)
protein ^a	0.265	11.9
protein ^b	0.272	13.4

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DISCUSSION

The DTT was used in this experiment to induce aggregation of lysozyme in the presence of different concentrations of β -CyD. The aggregation of lysozyme was reduced and delayed in the presence of β -CyD. Fig. 1 indicates the interaction between exposed hydrophobic patches of enzyme occurs upon interaction with hydrophobic patches of β -CyD which prevent hydrophobic-hydrophobic interactions (diminishing the aggregation) of the enzyme. The ANS fluorescence analysis indicated changes in the lysozyme conformation (Fig. 2). The ANS fluorescence intensity of the spectra increased in the presence of β -CyD, and the spectral maxima shifted towards shorter wavelength relative to the enzyme in the presence or absence of β -CyD. In the presence of DTT, the enzyme's disulfide bonds were broken, and therefore, the hydrophobic patches of lysozyme were exposed. Interactions among these hydrophobic patches resulted in protein aggregation. To test the hydrophobic effects under our study conditions, we monitored ANS fluorescence (Fig. 2). ANS exhibits weak fluorescence in aqueous solutions, however, in non-polar environments, its quantum yield increases (Moosavi-Movahedi, 1994; Stryer, 1968). Hence, it was used to detect the non-polar region in proteins by binding to such regions and increasing fluorescence. The results obtained from the extrinsic fluorescence of lysozyme revealed that there were interactions between the exposed hydrophobic patches of β -CyD and exposed hydrophobic patches of the protein (Fig. 2). Thus, β -CyD prevented the direct interactions between protein hydrophobic patches and reduced protein aggregation. The presence of β -CyD as a chaperone molecule prevented these hydrophobic-hydrophobic interactions in the enzyme. The exposed hydrophobic patches of the enzyme occurred upon interaction with hydrophobic patches of β -CyD which prevented hydrophobic-hydrophobic interactions diminishing the aggregation of the enzyme. CD spectra of lysozyme and lysozyme with β -CyD showed no change ellipticity relative to native lysozyme in the range of 204–250 nm (Fig. 3).

The positive values of transition enthalpy indicate the reaction between β -CyD and lysozyme is endothermic reaction (RezaeiBehbehani, *et al.*, 2009) (Table 1) $p=1$ indicates that the binding is non-cooperative in two sets of binding sites. The most common mechanism of protein aggregation is believed to involve protein denaturation, via hydrophobic interfaces. It is possible to introduce a correlation

between change in δ_A^θ and increase in the stability of proteins. The δ_A^θ value reflects the hydrophobic property of lysozyme, leading to the enhancement of water structure and protein stabilization [27]. δ_A^θ value (Table 1) for lysozyme + β -CyD interaction is -0.04 (Table 1), indicating that in the low concentration of β -CyD the lysozyme structure is destabilized. δ_B^θ value in high β -CyD concentration is -0.04, indicating that the lysozyme structure is destabilized by β -CyD in this region. The ITC results which revealed the native conformation of protein has slightly been destabilized, binding β -CyD to lysozyme reduces thermodynamic stability (table 1).

Table 2 compares the radii of lysozyme in native state and complex with β -CyD. The radius of lysozyme in the presence of β -CyD is 13.4 Å that reveals the native protein (lysozyme) is compactness than lysozyme in the presence of β -CyD. Comparing the radius denatured protein (21 Å) (Nicoli and Benedek, 1979) with lysozyme in the presence of β -CyD indicates lysozyme in the presence of β -CyD is more compact than the denatured protein. So the results obtained showed that in the presence of β -CyD; 1) secondary protein structure does not change 2) tertiary structure of protein changed in the presence of β -CyD 3) lysozyme in the presence of β -CyD is partially unfolded 4) lysozyme in the presence of β -CyD is more compact than the denatured protein. Therefore, there is an intermediate state between the native and denatured state for lysozyme in the presence of β -CyD that is called molten globule (MG).

Conclusion

The results of CD and fluorescence studies showed that the secondary structure of lysozyme in the presence of β -cyclodextrin does not change, but its tertiary structure changes in the presence of β -CyD. Also, the results of the Stokes radius of lysozyme in the presence of β -CyD are evident to exist MG state. The molten globule state induced by β -CyD is more compact than the lysozyme-unfolded state and lysozyme is unfolded partially in the presence of β -CyD.

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Conflict of interests

The authors declare that they have no competing interests.

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