Asian Journal of Chemistry Vol. 18, No. 3 (2006), 1963–1972

Thermodynamic Stability of the Non-Native α-Helical Intermediates of β-Lactoglobulin by Conjugation with Three Different Kinds of Carboxymethyl Cyclodextrins

J. CHAMANI
Department of Biology, Faculty of Science
Islamic Azad University, Mashhad Branch, Mashhad, Iran
Fax: (98)(511)8435050; Tel: (98)(511)8435050; E-mail: chamani@ibb.ut.ac.ir

β-Lactoglobulin-carboxymethyl cyclodextrin conjugates were prepared by using water soluble carbodiimide. Three kinds of carboxymethyl cyclodextrins (CM-CyDs) differing in molecular mass were used to investigate the effects of different CM-CyD contents and hydrophobicity on the structural changes in β-lactoglobulin (β-LG). The effect of CM-CyDs on the structure of β-LG was utilized to investigate the contribution of hydrophobic interactions to the stability of the protein. It is generally assumed that folding intermediates contain partially formed native-like secondary structures. However, on considering the fact that the conformational stability of the intermediate state is simpler than that of the native state, it would be expected that the secondary structures in a folding intermediate would not necessarily be similar to those of the native state. β-LG is a predominantly β-sheet protein, although it has a markedly high intrinsic preference for α-helical structure. The formation of non-native α-helical intermediate of β-LG was conjugated by CM-CyDs including CM-α-CyD, CM-β-CyD and CM-γ-CyD as special condition. The m values of the intermediate state of β-LG by CM-CyDs showed substantial variation. The enhancement of m values as the stability criterion of non-native α-helical intermediate state corresponded with increasing glucose units of the cited CM-CyDs. The present results suggest that the folding reaction of β-LG follows a non-hierarchical mechanism and hydrophobic interactions play important roles in stabilizing the non-native α-helical intermediate state.

Key Words: β-Lactoglobulin, α-Helical Intermediate, Cyclodextrin, Stability, Hydrophobic force.

INTRODUCTION

β-Lactoglobulin (β-LG), a major whey protein, is a globular protein of m.w. 18400 with two disulfide bridges and a free cysteine, containing plenty of essential amino acids[1]. The results of X-ray crystallography[2,3] and protein sequencing[4] have shown that there is remarkable similarity between β-LG and plasma retinol binding protein. β-LG has a calyx fold and it is categorized as a member of the lipocalin superfamily[5]. The function of β-LG is tentatively considered to be the binding and transportation of small hydrophobic ligands such as retinol, fatty acids and so on[6]. It is also well known that β-LG has various functional properties such as gelling[7,8], foaming and emulsifying properties[9,10]. Although β-LG is considered to be a valuable protein in terms of its nutritional feature, but also is known to be a potent allergen responsible for milk allergy; about 82% of milk allergy patients are sensitive to β-LG[11]. The fact that β-LG is stable at low pH and resistant to

proteolysis is considered to be one of the reasons β-LG is allergenic[12]. Hence, it is desirable to develop new means of decreasing the allergenicity and enhancing the functional properties of β-LG. To achieve this, Hattori *et al.*[13] studied neoglycoconjugates of β-LG. Many studies on neoglyco conjugates of proteins have been performed during the past 20 years and various improvements in the functional properties of proteins have been achieved. As far as β-LG is concerned, several studies[14–21] on conjugates of β-LG have been reported, focusing on improvement of its solubility, heat stability, foaming properties and emulsifying properties. Conjugation of a protein with a polymer is thought to change the protein structure more effectively than conjugation with low molecular weight components. In particular, multiple changes to protein structure can be expected by conjugation with a charged polymer due to the difference in molecular weight or charge of the chemical species conjugated. To achieve low allergenicity, conjugation of a protein with polysaccharides is thought to be more effective than conjugation of a protein with low molecular weight molecules because it can be expected that polysaccharides will cover the epitopes of the allergens more effectively than low molecular weight molecules[22]. Hattori *et al.*[23-28] have reported that conjugates of β-LG and acidic saccharides such as carboxymethyl dextran and alginic acid oligosaccharides showed enhanced heat stability, improved emulsifying properties and reduced immunogenicity. Previous studies[29, 30] have shown that the alcohol-induced denaturation of proteins can be interpreted in terms of two processes: disruption of the native state and induction of an α-helical conformation. Alkanols (alkyl alcohols) with bulky alkyl groups are more effective than those with small alkyl groups in denaturing proteins, suggesting that hydrophobic interactions are involved in the denaturation process. Hamada *et al.*[31] suggested that the folding reaction of β-LG follows a non-hierarchical mechanism, in which non-native α-helical structures play important roles.

In the present study, carboxymethyl α,β,γ-cyclodextrins as useful saccharides for conjugation with β-LG to study the non-native α-helical structural properties of a protein have been chosen. Cyclodextrins (CyDs) are cyclic carbohydrates, which form inclusion complexes with various hydrophobic molecules and they are widely used in food and pharmaceutical applications[20, 32–34]. Cyclodextrins are used in order to stabilize unstable materials and to solubilize insoluble or poorly soluble materials. Three CMCyDs are readily available: CM-α-CyD, CM-β-CyD and CM-γ-CyD having six, seven and eight glucose units, respectively. CyDs with fewer than six glucose residues are too strained to exist, whereas those with more than eight residues are very soluble, difficult to isolate and hardly studied to date. α-CyD, β-CyD and γ-CyD are commonly referred to as the native CyDs. Many very covalently modified CyDs have been prepared from native forms. β-LG-CMCyDs conjugates were prepared using water-soluble carbodiimide. In this study, the conformation of non-native α-helical intermediates of β-LG was characterized by conjugate ion with CMCyDs. The results indicate that non-native α-helical structures may play important roles in the folding of β-LG.

EXPERIMENTAL

Bovine β-LG and three kinds of CyDs (α β, γ, cyclodextrins) were obtained from Sigma. The other substances of reagent grade were purchased from Merck. Other

chemicals were of reagent grade. The molar mass for β-LG is 18400 g mol^{-1} (Dalton).

Carboxymethylation of CyDs

CyDs were carboxymethylated by a method previously used for carboxymethylation of dextran[35], with some modifications. In brief, monochloro acetic acid (9 g) was dissolved in 48 mL of methanol and 10.5 mL of NaCl solution (1 g mL^{-1}) was added with gentle stirring. After adding methanol (12 mL), CMCyD (3 g) was added to the solution and the mixture was incubated at 45°C for 48 h. After washing with 70% methanol, filtration and lyophilization, CMCyD was obtained.

Preparation of the β-LG-CMCyD conjugates

The β-LG-CMCyD conjugates were prepared by the method of Hattori *et al.*[24] (method 1) and by referring to the method of Kitabatake *et al.*[17] (method 2).

Method 1: Each of CMCyDs (1 g, α, β, γ) was dissolved in 100 mL of distilled water and 100 mL of an EDC solution (10 mg/mL) was added separately. After the pH was adjusted to 5.5 with 1 N HCl, 100 mL of a β-LG solution (10 mg/mL) was added. The reaction mixture was incubated at 4°C for 6 h while gently stirring. The reaction was stopped by adding 18 mL of acetic acid and the solution was dialyzed against distilled water to form a separated oily phase of a coacervate. Crude β-LG-CMCyDs conjugates were obtained after lyophilization of the coacervate.

Method 2: β-LG and CMCyDs were dissolved in 30 mL of distilled water and adjusted to pH 4.75 with 1 N HCl and EDC solution (413 mg/mL of distilled water) was added gradually during 30 min while maintaining the pH at 4.75 with 1 N HCl. The reaction mixture was incubated at 25°C for 3 h. The reaction was stopped by adding 2 mL of a 2 M sodium acetate buffer at pH 5.5. After dialysis against distilled water and lyophilization, crude β-LG-CMCyDs conjugates were obtained.

Free CMCyDs were removed by salting out. The crude conjugates were dissolved in a 0.067 M phosphate buffer at pH 7.0 at a concentration of 5 mg/mL and the proteinaceous component was salted out with ammonium sulphate to a final concentration of 5 M. The precipitate, which was recovered by centrifuging at 18000 rpm for 15 min at 25 °C and washing with the 0.067 M phosphate buffer at pH 7.0, containing 5 M ammonium sulphate, was next dissolved in the 0.067 M phosphate buffer at pH 7.0. After dialysis against distilled water and lyophilization, the β-LG-CMCyDs conjugates without free CMCyDs were obtained.

Free β-LG containing polymerized β-LG was removed by ion-exchange chromatography. A DEAE-Toyopearl 650 S column (Tosoh, 2.2 i.d. × 20 cm) was equilibrated with the 0.067 M phosphate buffer at pH 7.0 and at a flow rate of 4.0 mL/min. Each β-LG-CMCyD conjugate after salting out (30 mg/3 mL) was applied to the column and eluted with a linear gradient from 0 to 1 M NaCl concentration. To detect the protein and CMCyD, the absorbance was monitored at 280 and 490 nm after colouring according to the phenol-sulfuric acid method[36], respectively.

Circular dichroism (CD) measurements

All measurements in this work were carried out at 20°C with thermostatically controlled cell holders. CD spectra were measured with a Jasco J-715 spectropolarimeter (Japan) equipped with an interface and a personal computer. The instruments were calibrated with ammonium D-10-camphorsulfonic acid[37]. The results were

expressed as the mean residue ellipticity [θ], which is defined as $[\theta] = 100 \times \theta_{obsd}/(lc)$, where θ_{obsd} is the observed ellipticity in degrees, c is the concentration in residue mol/L, and l is the length of the light path in cm. The helical content of the β-LG and three kinds of β-LG-CMCyDs conjugates were calculated by the method of Chen *et al.*[38] The CD spectra were measured in a 1 mm path length cell from 250 to 190 nm. The rotatory contributions of a protein can be determined by $X = f_H X_H + f_\beta X_\beta + f_R X_R$ where X can be either the ellipticity or the rotation at any wavelength. f is the fraction of the helix (f_H), beta form (f_β) and unordered form (f_R); the sum of f is equal to unity and each f is greater than or equal to zero. With the f values of five proteins obtained by X-ray diffraction studies, the X of the protein at any wavelength is filled by a least-squares method, which defines the X_H, X_β and X_R. The CD for the helix, beta and random forms determined thus can be conversely used to estimate the secondary structure of any protein with X at several wavelengths for the same equation. The α-helical content (f_H) was estimated from the ellipticity value at 222 nm ($[\theta]_{222}$) as described bellow[39, 40]:

$$f_H = -([\theta]_{222} + 2340/30300)$$

Fluorescence measurements

Fluorescence measurements were made on JASCO SP-6200 spectrofluorometer at an excitation wavelength of 283 nm. Trp fluorescence emission was followed at 339 nm[13]. The temperature of the cell compartments was kept constant at 20°C by water circulation.

Differential scanning calorimetry (DSC) measurements

DSC experiments were performed on a Scal differential scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia) with cell volumes of 0.48 mL at a scanning rate of 1 K/min (was kept constant in all experiments), interfaced with a personal computer (IBM compatible). Before the measurements, samples were degassed by stirring in an evacuated chamber at room temperature and then immediately loaded into the calorimeter cell; the final dialysis buffer (also degassed) was loaded into the reference cell. A pressure of 152 kPa (1.5 atm) of dry nitrogen was always kept over the liquids in the cells throughout the scans to prevent any degassing during heating. The reversibility of the thermal transitions was checked by a second heating of the cool sample immediately following the first scan. The calorimetric traces were corrected for the instrumental background by subtracting a scan with buffer in both cells. The reactive errors of the values of molar enthalpy changes are in the range of 3% and the absolute errors of given transition temperatures T_m are 0.3°C. The thermogram analysis and fitting were done based on Privalov and Potekhin theory[40] which was installed as DOS program in software package (named Scal-2) and supplied by Scal (Russia). Scal-2 program, which is installed in DSC instrument, enables to determine the native and denatured lines based on fitting error. The best fitting error is selected as a best thermogram.

RESULTS AND DISCUSSION

The conjugation of β-LG and three different kinds of CM-CyDs were confirmed by the coincidence of the protein and saccharides strained bands by SDS-PAGE and by the shift of the isoelectric point (pI) of β-LG to the acidic side (data not shown).

The far-UV CD spectra of β-LG and CM-α-CyD conjugate is shown in Fig. 1. Native β-LG had a negative maximum at 216 nm, since β-LG was rich in β-sheet. In the case of the β-LG-CM-α-CyD conjugate, the broad negative maximum and the blue shift show that the β-sheet regions had been changed by conjugation with CM-α-CyD. The CD spectra at different concentrations of CM-α-CyD show an isodichronic point at 200 nm for β-LG. This suggests that the conjugated process is a two-state transition[35]. The α-helix content of native β-LG estimated according to the method of Chen *et al.*[38] was 9.3% corresponding to the results of X-ray crystallography[2]. However, the α-helix contents of β-LG-CM-α-CyD, β-LG-CM-β-CyD and β-LG-CM-γ-CyD conjugates were 14.4, 19.6 and 23.1, respectively, on the basis of the ellipticity values at 222 nm as calculated by the method of Chen *et al.*[38] The maximum ellipticities observed for various CM-CyDs are similar, indicating that the maximum helical content, estimated to be about 80% by the method of Chen *et al.*[38], is independent of CM-CyD species. It is evident that CM-γ-CyD is much more effective in inducing the transition than CM-β-CyD and CM-α-CyD. The order of effectiveness of various CM-CyDs is CM-γ-CyD, CM-β-CyD, CM-α-CyD.

The inset of Fig. 1 shows the dependence on CM-γ-CyD concentration of the ellipticity at 222 nm. Transition curves induced by CM-β-CyD and CM-α-CyD are also shown. The midpoint concentration (C_m) values of the transitions indicate that the addition of one glucose unite enhances the denaturant activity of CM-CyDs (Table-1). The fluorescence emission spectra of the native β-LG and β-LG-CM-α-

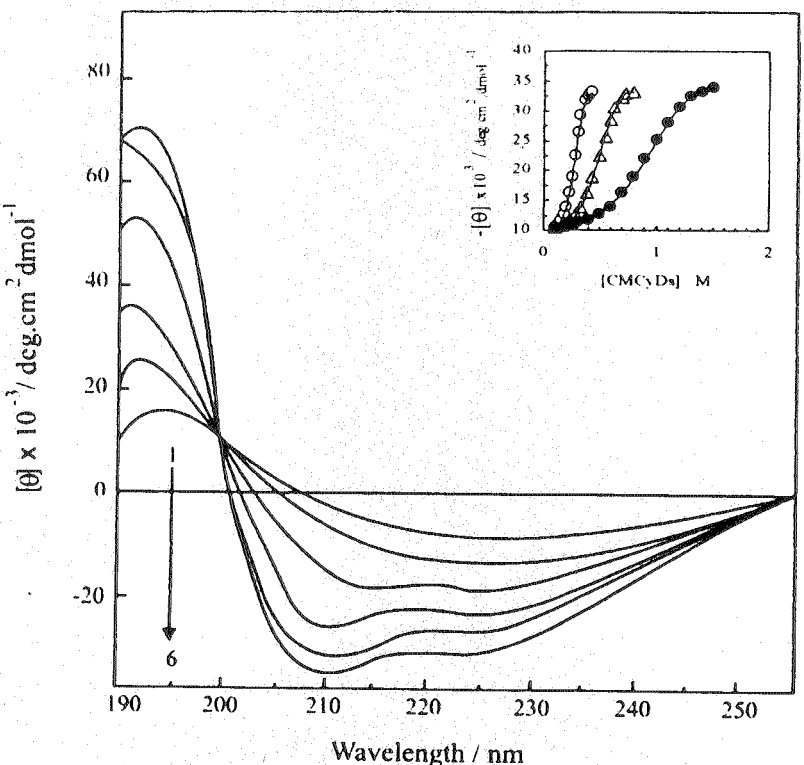

Fig. 1. Far-UV CD spectra of β-LG (ellipticity, [θ]) conjugated with CM-α-CyD. Inset: Plots of molar ellipticity ($[\theta]_{222}$) *vs.* concentrations of CM-CyDs conjugates. CM-α-CyD conjugate (closed circle), CM-β-CyD conjugate (open triangle) and CM-γ-CyD conjugate (open circle). Arrow indicates the direction of the change.

CyD conjugate at different concentrations of CM-α-CyD are shown in Fig. 2. When excited at 283 nm, native β-LG exhibited a fluorescence emission maximum at 336 nm. It has previously been clarified that the fluorescence intensity increases with red shift of the wavelength for maximum emission as the conformation of β-LG changes[23,41,42]. In this experiment, each conjugate also exhibited the same emission maximum wavelength. Hence, the conformation around Trp residues (19 Trp and 61 Trp) of the conjugates seems to have been the same as that of native β-LG. However, the fluorescence intensity of CM-CyDs conjugates at different concentrations was higher than that of native β-LG. This increase is thought to have been due to the denaturing effect by the polysaccharide chain bound to β-LG in each conjugate. The inset of Fig. 2 shows the dependence on various kinds of CM-CyDs concentration of the fluorescence intensity. The midpoint concentration (C_m) values of the transitions indicate that the addition of one glucose unit in CM-CyDs enhances the denaturant activity of them (Table-1).

TABLE-1
ΔG° (H_2O), m VALUES AND INFLECTION POINTS FOR THE αI STATE OF β-LG AS CONJUGATED WITH CM-CYDS

	ΔG° (H_2O)[a] (kJ mol^{-1})	m[b] (kJ mol^{-1} M^{-1})	C_m^c(M)
β-LG-CM-α-CyD Conjugate	24.5 ± 1.2	28.3 ± 0.4	0.90
β-LG-CM-β-CyD Conjugate	43.1 ± 1.5	87.4 ± 0.7	0.49
β-LG-CM-γ-CyD Conjugate	56.8 ± 1.6	203.1 ± 1.5	0.28

[a]ΔG° (H_2O) was calculated by the least-squares method from Eqn. (3).
[b]A parameter reflecting the hydrophobicity of the transition state.
[c]The midpoint concentration of transition.

The insets of Fig. 1 and 2 show the sigmoidal curves (drawn by a numerical analysis method, called cubic-spline in the MATLAB program, version 6.1 for the native (N) to the α-helical intermediate (αI) of β-LG upon the conjugation with CM-CyDs. β-LG is a small protein with a single subunit. Therefore, a two-state analysis based on the Pace theory was performed[43]. It is now possible to obtain equilibrium constant (K) for the N-αI states and to calculate the corresponding Gibb's free energy changes, ΔG°as follows:

$$\Delta G^\circ = -RT \ln (A_{obs} - A_N)/(A_{\alpha I} - A_{obs}) \quad (2)$$

where A_N, $A_{\alpha I}$ and A_{obs} are the physical parameters of molar ellipticity and fluorescence intensity of N, αI and any observed states with conjugation of CM-CyDs, respectively.

Fig. 3 shows the plot of ΔG° against CM-CyDs concentrations. The free enegies of αI formation in the absence of CM-CyDs, ΔG° (H_2O), were calculated by the least-squares method from the following equation[43]:

$$\Delta G^\circ = \Delta G^\circ(H_2O) - m[CM - CyDs] \quad (3)$$

where m is the slope of linear curve reflecting the cooperativity and also hydrophobicity of the transition state. The m value correlates very strongly with the amount of protein surface exposed to the solvent upon unfolding[43–45]. A similar dependence on accessible surface area has been found for the heat capacity change (ΔC_p), which was confirmed for a set of proteins[45,46]. The m values and

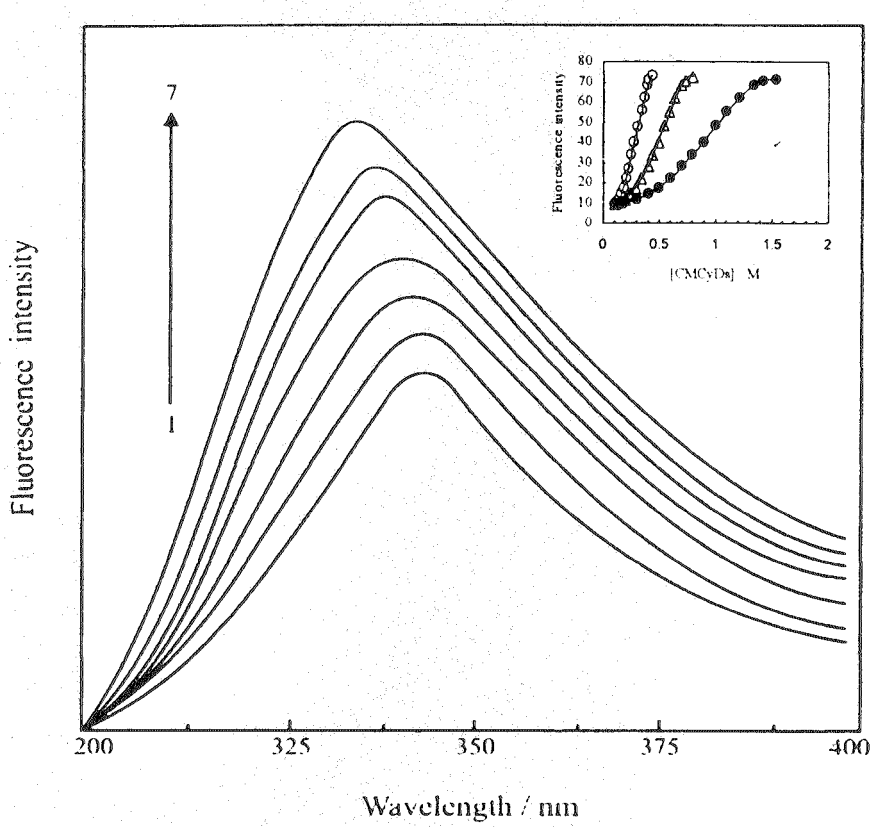

Fig. 2. Fluorescence spectra of β-LG conjugated with CM-α-CyD. Inset: Plots of fluorescence intensity *vs.* concentrations of CM-CyDs conjugates: CM-α-CyD conjugate (closed circle), CM-β-CyD conjugate (open triangle) and CM-γ-CyD conjugate (open circle). Arrow indicates the direction of the change.

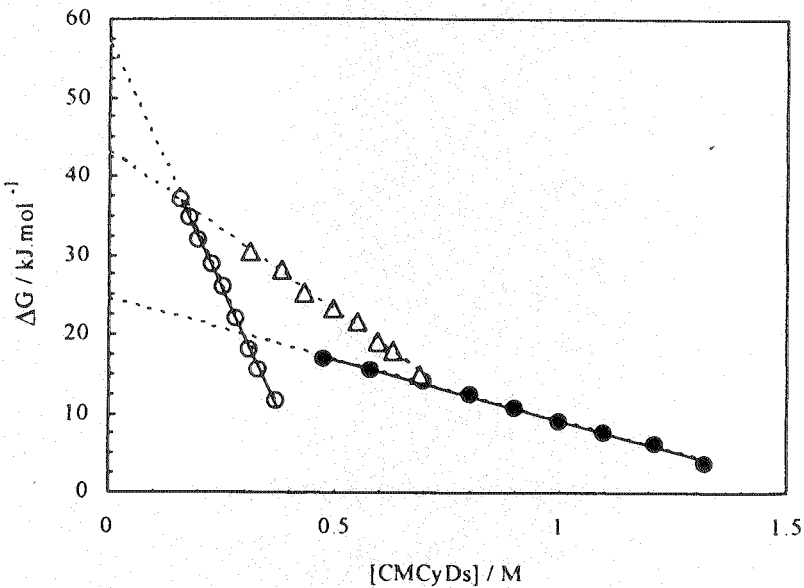

Fig. 3 Free energy values (ΔG°) *vs.* concentrations of CM-CyDs conjugates. CM-α-CyD conjugate (closed circle), CM-β-CyD conjugate (open triangle) and CM-γ-CyD conjugate (open circle).

heat capacity changes correlate well with each other, and also ΔC_p of the protein is linearly related to the fraction of hydrophobic residues[47]. Thus for proteins that undergo a simple two-state unfolding mechanism, the amount of surface exposed to solvent upon unfolding is a main structure determinant for m values, ΔC_p and hydrophobicity. The $\Delta G°$ (H_2O) and m values are tabulated in Table-1. The free energy values are shown as dotted linear lines in Fig. 3. It is apparent from Table-1 that the values of the midpoint concentration (C_m) and m values of the conformational transitions conjugated by CM-α-CyD, CM-β-CyD and CM-γ-CyD are not identical; as the glucose units of CM-CyDs increase, the values of C_m and m decrease and increase, respectively.

The effectiveness of three different kinds of CM-CyDs is compared by using the transition curves measured by the ellipticity at 222 nm. In order to close up the role of the glucose unit (as a hydrophobic unit), the effects of various CM-CyDs conjugated on native state of β-LG are compared. The insets of Fig. 1 and 2 show the dependence on CM-CyDs concentrations of the ellipticity at 222 nm and fluorescence intensity. The maximum ellipticities observed for various CM-CyDs conjugates are similar, indicating that the helical content, estimated by the method of Chen *et al.*[38], is independent on CM-CyDs conjugates species. It is evidence that CM-γ-CyD conjugate is much more effective in inducing the transition than CM-α-CyD and CM-β-CyD conjugates. The order of effectiveness of various CM-CyDs is CM-γ-CyD > CM-β-CyD > CM-α-CyD.

The spectroscopic properties of the αI state strongly support the view that CM-CyDs conjugates at different concentrations to stabilize the αI state of β-LG. The αI state in contrast to native state of β-LG shows high amounts of α-helix. The spectroscopic properties of the αI state closely resemble the ones reported by Hirota *et al.*[48], thus implying a close structural similarity. In conclusion, we show that the helical properties of β-LG in the presence of CM-CyDs conjugates are not related to its native structure. The results suggest a case of non-hierarchical protein folding, in which the α-helical intermediate accumulates during the formation of the native β-sheet structure.

One of the best criteria for determining protein stability is free energy in the absence of ligand, $\Delta G°$ (H_2O) or m values. We consider that m value is useful to further analyze the effects of CM-CyDs conjugates. From a comparison of m values of CM-α-CyD, CM-β-CyD and CM-γ-CyD, it is seen that for β-LG addition of one glucose unit to CM-CyDs enhances m values. This suggests that the contributions of glucose units can be explained additively. Differential scanning calorimetric (DSC) experiments confirm these results (data not shown). The m values for a given transition are generally interpreted as a measure of the change in solvent exposure for that transition[49], and this makes them useful estimates of the gross compactness of different states on the folding pathway relative to the two end-stations, the denatured state D and the native state N. Denaturant-induced unfolding is useful for understanding the mechanism of conformational stability. In particular, m value is important since, it is a measure of the cooperativity of the unfolding transition and is proposed to be approxi-

mately proportional to the difference in solvent-accessible surface area between the folded and unfolded states[50, 51]. The m values and ΔG° (H_2O) for the αI state of β-LG upon the conjugation of CM-CyDs such as CM-α-CyD, CM-β-CyD and CM-γ-CyD are shown in Table-1 and also the increase in ΔG° (H_2O) and m values that correspond to the glucose unit. It will be noted that m value for the αI state conjugated by CM-γ-CyD is significantly higher than other αI states, suggesting the ordering of the αI state conjugated by CM-γ-CyD than other αI states. On the other hand, m value is a sign of cooperativity; therefore αI state conjugated with CM-γ-CyD is more cooperative than other states. Therefore, the present results show a direct role of hydrophobicity to the stability and α-helical content induced by the αI state in β-LG.

ACKNOWLEDGMENT

The financial support of the Research Council of the Islamic Azad Univeity, Mashhad Branch is gratefully acknowledged.

REFERENCES

1. H.A. McKenzie, β-Lactoglobulin, in: Milk Proteins: Chemistry and Molecular Biology, Academic Press, New York, p. 257 (1972).
2. M.Z. Papiz, L. Sawyer, E.E. Eliopoulos, A.C.T. North, J.B.C. Findlay, R. Sivaprasadrao, T.A. Jones, M.E. Newcomer and P.J. Kraulis, *Nature*, **324**, 383 (1986).
3. S. Brownlow, C.J. Morais, R. Cooper, D.R. Flower, S.J. Yewdall, I. Polikarpov, A.C. North and L. Sawyer, *Structure*, **5**, 481 (1997).
4. S. Pervaiz and K. Brew, *Science*, **228**, 335 (1985).
5. D.R. Flower, *Biochem. J.*, **318**, 1 (1996).
6. M.D. Perez and M. Calvo, *J. Dairy Sci.*, **78**, 978 (1995).
7. D.M. Mulvihill and J.E. Kinsella, *Food Tech.*, **41**, 102 (1987).
8. E.A. Foegeding, P.R. Kuhn and C.C. Hardin, *J. Agric. Food Chem.*, **40**, 2092 (1992).
9. M. Shimizu, M. Saito and K. Yamauchi, *Agric. Biol. Chem.*, **49**, 189 (1985).
10. R.D. Waniska and J.E. Kinsella, *Food Hydrocolloids*, **2**, 439 (1988).
11. J. Spies, *J. Milk Food Technol.*, **36**, 225 (1973).
12. E.M. Akita and S. Nakai, *J. Food Sci.*, **55**, 711 (1990).
13. M. Hattori, Y. Okada and K. Takahashi, *J. Agric. Food Chem.*, **48**, 3789 (2000).
14. E.M. Akita and S. Nakai, *J. Food Sci.*, **55**, 718 (1990).
15. C.B. Harb, B. Charrier, M. Dalgalarrondo, J.M. Cobert and T. Haertle, *Lait*, **71**, 205 (1990).
16. C. Creuzenet, A. Touati, E. Dufour, Y. Choiset, J.M. Chobert and T. Heartle, *J. Agric. Food Chem.*, **40**, 184 (1992).
17. N. Kitabatake, J.L. Cuq and J.C. Cheftel, *J. Agric. Food Chem.*, **33**, 125 (1985).
18. N.L. Mattarella and T. Richardson, *J. Agric. Food Chem.*, **31**, 972 (1983).
19. N.L. Mattarella, L.K. Creamer and T. Richardson, *J. Agric. Food Chem.*, **31**, 968 (1983).
20. J. Szejtle, *Chem. Rev.*, **98**, 1743 (1998).
21. K. Nagasawa, K. Takahashi and M. Hattori, *Food Hydrocolloids*, **10**, 63 (1996).
22. A.H. Sehon, *Prog. Allergy*, **32**, 161 (1982).
23. M. Hattori, A. Ametani, Y. Katakura, M. Shimizu and S. Kaminogawa, *J. Biol. Chem.*, **268**, 22414 (1993).

24. M. Hattori, S. Imamura, K. Nagasawa and K. Takahashi, *Biosci. Biotech. Biochem.*, **58**, 174 (1994).
25. M. Hattori, K. Nagasawa, A. Ametani, S. Kaminogawa and K. Takahashi, *J. Agric. Food Chem.*, **42**, 2120 (1994).
26. M. Hattori, A. Ogino, H. Nakai and K. Takahashi, *J. Agric. Food Chem.*, **45**, 703 (1997).
27. M. Hattori, K.Y. Tsukamoto, H. Kumagai, Y. Feng and K. Takahashi, *J. Agric. Food Chem.*, **46**, 2167 (1998).
28. M. Hattori, K. Nagasawa, K. Ohgata, N. Sone, A. Fukuda and H. Matsuda, *Bioconjugate Chem.*, **11**, 84 (2000).
29. P.H. von Hippel and K.Y. Wong, *J. Biol. Chem.*, **240**, 3909 (1965).
30. R.P. Parodi, E. Bianchi and A. Ciferri, *J. Biol. Chem.*, **248**, 4047 (1973).
31. D. Hamada, S. Segawa and Y. Goto, *Nature Struct. Biol.*, **3**, 868 (1996).
32. P. Caliceti, S. Salmaso, A. Semenzato, T. Carofiglio, R. Fornasier, M. Fermeglia, M. Ferrone and S. Pricl, *Bioconjug. Chem.*, **14**, 899 (2003).
33. X. Ren, J. Liu, G. Luo, Y. Zhang, Y. Luo, G. Yan and J. Shen, *Bioconjug. Chem.*, **11**, 682 (2000).
34. M. Fernandez, L. Villalonga Mde, A. Fragoso, R. Cao and R. Villalonga, *Biotechnol. Appl. Biochem.*, **36**, 235 (2002).
35. T.Y. Tsong, *Biochemistry*, **14**, 1542 (1975).
36. M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Reberts and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
37. T. Takakuwa, T. Konno and H. Meguro, *Anal. Sci.*, **1**, 215 (1985).
38. Y. H. Chen, J.T. Yang and H.M. Martinez, *Biochemistry*, **11**, 4120 (1972).
39. A.A. Moosavi-Movahedi, J. Chamani, M. Gharanfoli and G.H. Hakimelahi, *Thermochim Acta*, **409**, 137 (2004).
40. P.L. Privalov and S.A. Potekhin, *Method Enzymol.*, **131**, 1 (1986).
41. J. Chamani, A.A.M. Movahedi, O. Rajabi, M. Gharanfoli, M.M. Heravi, G.H. Hakimelahi, A. N. Baghsiah and A.R. Varasteh, *J. Colloids Interface Sci.*, **293**, 52 (2006).
42. S. Kaminogawa, M. Shimizu, A. Ametani, M. Hattori, O. Ando, S. Hachimura, Y. Nakamura, M. Totsuka and K. Yamauchi, *Biochim. Biophys. Acta*, **998**, 50 (1989).
43. C.N. Pace, *Methods Enzymol.*, **131**, 266 (1986).
44. A.A.M. Movahedi, A.E. Wilkinson and M.N. Jones, *Int. J. Biol. Macromol.*, **9**, 327 (1987).
45. J.K. Myers, C.N. Pace and J.M. Scholtz, *Protein Sci.*, **4**, 2138 (1995).
46. D. Shorrtle, *Adv. Protein Chem.*, **46**, 217 (1995).
47. R.L. Baldwin, *Proc. Natl. Acad. Sci. USA*, **83**, 8069 (1986).
48. N. Hirota, K. Mizuno and Y. Goto, *Protein Sci.*, **6**, 416 (1997).
49. C. Tanford, *Adv. Protein Chem.*, **24**, 1 (1970).
50. J.A. Schellman, *Biopolymer*, **17**, 1305 (1978).
51. D.O.V. Alonso and K.A. Dill, *Biochemistry*, **30**, 5974 (1991).

(*Received*: 1 August 2005; *Accepted*: 1 March 2006) AJC-4673