JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Ethanol Effect on the Structure of β-Lactoglobulin B and Its Ligand Binding

SEYED HABIB-ALLAH MOUSAVI,[†] JEAN-MARC CHOBERT,[‡] ABDOL-KHALEGH BORDBAR,*[,†] AND THOMAS HAERTLÉ*[,‡]

Laboratory of Biophysical Chemistry, Department of Chemistry, University of Isfahan, Isfahan, 81746-73441 I.R., Iran, and UR1268, Biopolymères, Interactions Assemblages, INRA, F-44300 Nantes, France

The changes of structure and ligand binding properties of β-LG B have been studied by fluorescence and circular dichroism spectroscopy in ethanolic solutions. Fluorescence measurements of retinol/β-LG interactions at 480 nm in various ethanol concentrations show that the maximal fluorescence intensity induced by this interaction between retinol and β-LG is observed around 20% v/v of ethanol. It is reduced to zero at 40% and 50% of ethanol. These results suggest that there are two distinct structural changes in β-LG occurring between 20% and 30% and around 40% of ethanol. The first transition, which increases affinity and the apparent number of binding sites for retinol, may be related or similar to the Tanford transition. The strong quenching of retinol emission at 480 nm in 40% of ethanol indicates the radical transformation of β-LG tertiary structure and the release of retinol. CD spectra at the aromatic region show that secondary and tertiary structures of β-LG are not significantly affected between 0% and 20% of ethanol. In 30% of ethanol, β-sheet percentage of β-LG decreases with respect to native β-LG (from 55% to 46%). β-Sheet percentage in β-LG increases in 40% and 50% alcohol (51% and 53%) relative to 30% of ethanol, which also indicates the strong rearrangement of the secondary structure of β-LG, while its tertiary structure and β-LG interactions are radically changed.

KEYWORDS: β-Lactoglobulin; retinol; ethanol; circular dichroism; fluorescence spectroscopy

1. INTRODUCTION

β-Lactoglobulin (β-LG) is a small globular protein and the major whey protein component in the milk of ruminant species. Its properties have been frequently reviewed (*1*−*4*). Unlike the other major whey proteins, β-LG does not have any known functions. Its amino acid sequence and three-dimensional structure show that it is a member of the lipocalin superfamily (*5, 6*). It has been proposed that a similar function (hydrophobic ligand binding) must be the main physiological reason for the presence of significant quantities of β-LG in milk (*7*).

β-LG is known to bind tightly, *in vitro*, one retinol molecule per monomer (*8*). Advances in structural studies of β-LG (*9*−*11*), of retinol binding protein (*12*) and of bilin binding protein (*13*), show that these hydrophobic molecule transporters share a common three-dimensional structural pattern termed β-barrel. There is some evidence of the protrusion of the retinol hydroxyl group out of the binding site deduced from its susceptibility to an attack by dehydrogenase (*8*). This could indicate the external placement of the retinol binding site on the β-LG molecule (*10*).

Organic solvents have been used in many protein studies (*14*−*16*). The addition of weak protic solvents such as alcohols may have broad effects, changing solvent−solute interactions (*17, 18*). It significantly shifts the balance of the electrostatic charges on the protein molecule (*19*) and changes the organization of dipolar moments (*20*). The structure of hydration layers is a major factor responsible for the organization of hydrophobic moieties in the globulin core (*21*). The change of polarity of the protein medium achieved by the addition of amphiprotic solvent able to form hydrogen bonds and replace hydrogen-bonded water molecules in hydration layers (*22*) and in the protein structure displaces structural equilibria maintained by the hydrogen bonds and hydrophobic interactions. The reversibility of the secondary structure changes of β-LG has been studied under the influence of solvent polarity changes (*16*). Independent of the alcohol used, the midpoints of the observed structural $\beta \leftrightarrow \alpha$ transformation occur around dielectric constant $\varepsilon \approx 60$. The structural change destroying the hydrophobic core formed by the β-barrel leads to the dissociation of the retinol/β-LG complex. This happens when the polarity of the medium reaches the dielectric constant $\varepsilon \approx 50$. However, when the dielectric constant of the medium is increased back to $\varepsilon \approx 70$

* To whom correspondence should be addressed. (T.H.) Tel: +332 40 67 50 91. Fax: +332 40 67 50 25. E-mail: haertle@nantes.inra.fr. (A.-K.B.) Tel: +98-311-7932710. Fax: +98-311-6689732. E-mail: khalegh_bordbar@yahoo.com.

[†] University of Isfahan.

[‡] Institut National de la Recherche Agronomique.

10.1021/jf801383m CCC: $40.75
Published on Web 08/27/2008

(dielectric constant of water) by the decrease of the temperature, the refolding of β-LG and the recreation of the retinol/β-LG complex are observed. Apparently, the binding of retinol to β-LG has no influence (stabilizing or destabilizing) on folding changes induced by alcohol (*16*), but despite all these data, the quantitative changes in tertiary structure and binding characteristics of β-LG are still unknown.

Hence, the purpose of the present study was to analyze and quantify the ethanol effect on secondary and tertiary structures of β-LG and on its retinol binding properties.

2. MATERIALS AND METHODS

2.1. Materials. All chemicals used were of reagent grade. The β-LG variant B was obtained from homozygous cow's milk following the method of Mailliart and Ribadeau Dumas (*23*), and it was found to be more than 95% pure. The β-LG concentration was determined spectrophotometrically using the molecular absorption coefficient $\varepsilon_{278} = 17{,}600\ M^{-1}\ cm^{-1}$ in the calculations (*24*). β-LG stock solutions, in 50 mM phosphate buffer, pH 6.5, and in 20 mM glycine buffer, pH 1.8, were 57 and 73 μM, respectively.

Retinol used in this study was purchased from Sigma. In order to prevent oxidation and isomerization, retinol was dissolved in ethanol in darkness and under strictly anaerobic conditions to give stock solutions of 0.5 mM. Its concentration was confirmed by spectrophotometry using the molecular absorption coefficient $\varepsilon_{325} = 46{,}000\ M^{-1}\ cm^{-1}$ (*25*).

2.2. Fluorescence Spectroscopy. Fluorescence spectra between 300 and 400 nm (excitation: 295 nm) were recorded at 20 °C on a Hitachi F-4500 spectrofluorimeter in ratio mode. The binding of the ligand was measured by following the decrease of protein tryptophan fluorescence at 330 nm. The following procedure was used for titration of β-LG, with retinol: 2 mL of β-LG solution, ranging between 5 and 7 μM, was placed in a cuvette, and small increments (2 to 5 μL) of the ligand solution were injected in the cuvette with a Hamilton syringe. The experiments were performed in 50 mM phosphate buffer at pH 6.5. In order to eliminate the dilution of β-LG solution by the added ligand solution and tryptophan fluorescence changes induced by alcohol, a blank containing *N*-acetyl-tryptophan amide (NATA) solution titrated with ligand was monitored as described above. In all cases, before correction for the blank, tryptophan fluorescence intensity at 330 nm of free β-LG was normalized to 1.

Retinol fluorescence is very weak in aqueous solutions, but it is greatly enhanced after retinol binding to β-LG. Its complex with β-LG exhibits a typical fluorescence spectrum with a maximum emission at 480 nm (*26*). Fluorescence study of retinol/β-LG complex in molar ratio 1:1 was carried out under the influence of various alcohol concentrations at 20 °C and pH 1.8, 6.5, and 8.0 (excitation, 330 nm; emission, 400−600 nm).

2.3. Determination of the Apparent Dissociation Constants. Differences in fluorescence intensity at 330 nm between the complex and free protein (excitation at 295 nm) were analyzed according to Cogan et al. (*25*) (eq 1) in order to determine apparent dissociation constants, K_d', and apparent number of binding sites at saturation state of protein, *n*.

$$P_0\alpha = (L_0/n)(\alpha/(1-\alpha)) - K_d'/n \quad (1)$$

In eq 1, P_0 is the total protein concentration, α is the fraction of free binding sites, and L_0 is the total ligand concentration. The value of α was calculated for every point on the titration curve using the following equation:

$$\alpha = (F - F_{min})/(F_0 - F_{min}) \quad (2)$$

where *F* represents the fluorescence intensity (corrected for blank) at a certain L_0, F_{min} represents the fluorescence intensity upon saturation of all of the apoprotein molecules, and F_0 is the initial fluorescence intensity.

2.4. Circular Dichroism. CD spectra of β-LG in water−ethanol solutions were measured on a Jobin Yvon Mark III dichrograph, and data were recorded online using an Olivetti personal computer. Spectra were averages of 3 accumulated scans with subtraction of the baseline. The cylindrical cells used had a path length of 0.2 mm in the case of far-UV spectra (185−260 nm), and 10 mm in the case of near-UV spectra (240−340 nm). All spectra were measured at 20 °C using a β-LG concentration of 57 μM, and the results are expressed as molar ellipticity, θ (deg·cm^2·dmol^{-1}). The Fasman method (*27, 28*) was assayed in order to simulate the experimental spectra because it gave the best fit with the available β-LG X-ray structure data (*9, 10*). Ethanol and buffer were added to the β-LG solution to obtain the appropriated alcohol percentage (0−50%).

Table 1. Apparent Dissociation Binding Constants, K_d' and Apparent Number of Binding Site, *n*, at Various Ethanol Concentrations and at Neutral and Acidic pH (Results Expressed per Protein Monomer)

	ethanol content (%)	*n*	K_d
pH 6.5	0	0.98	1.41×10^{-7}
	10	0.93	1.05×10^{-7}
	20	1.24	9.46×10^{-8}
	30	0.69	undetermined
pH 1.82	0	0.87	1.28×10^{-7}
	10		
	20	1.17	6.87×10^{-8}

3. RESULTS

3.1. Ligand Binding Properties. The fluorescence of β-LG tryptophanyl residues was quenched from 100% to 54% in the presence of retinol indicating retinol/β-LG binding.

The fluorescence of retinol rises with the increase of retinol concentration, and it becomes flat at molar ratio 1:1. The excitation wavelength could be 295 or 330 nm. At 330 nm, retinol is the only excited molecule, which is bound by β-LG. Tryptophanyl residues are excited at 295 nm. This energy transfers from tryptophan indol to retinol, and it is emitted at 480 nm. **Table 1** shows that there is one binding site for retinol on the β-LG molecule. The apparent dissociation constant of this binding site is 1.41×10^{-7} M, which agrees well with the results obtained previously (*16*).

The fluorescence of the retinol/β-LG complex is quenched progressively by increasing ethanol concentration, and it disappears altogether around 40% ethanol (v/v). In contrast, the fluorescence intensity of β-LG tryptophan in the case of the palmitic acid/β-LG complex, molar ratio 1:1, moderately increases with rising ethanol concentration at 330 nm. The maximum of tryptophan emission is red-shifted between 7 and 10 nm at 40% and 50% of ethanol (**Figure 1A**). This red shift indicates a significant structural β-LG change between 30% and 40% of ethanol. Such a red shift was observed at all studied

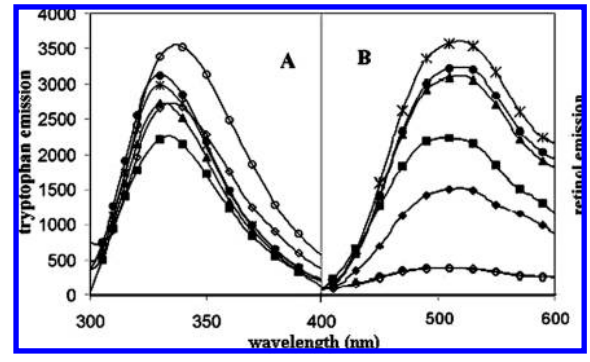

Figure 1. (**A**) Tryptophan emission spectra of a 1:1 solution of palmitic acid/β-LG and (**B**) retinol emission spectra of a 1:1 solution of retinol/β-LG at pH 6.5 and 0% (■), 10% (▲), 20% (∗), 25% (●), 30% (◆), 40% (◇), and 50% (○) of ethanol.

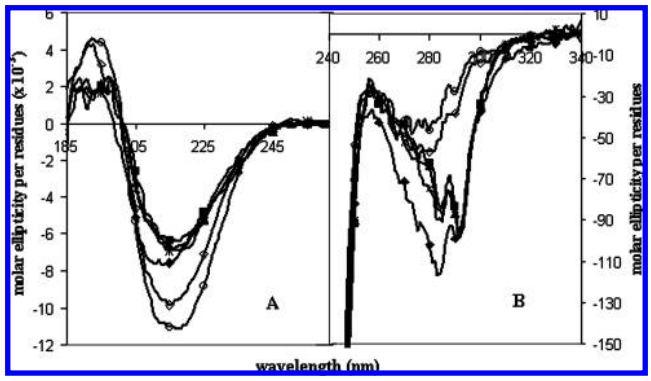

Figure 2. Far (**A**)- and near (**B**)-UV circular dichroism spectra of β-LG at 0% (■), 10% (▲), 20% (∗), 30% (◆), 40% (◇), and 50% (○) (v/v) of ethanol. Spectra were averages of 3 accumulated scans with subtraction of the baseline, which were measured at 20 °C using a β-LG concentration of 57 μM in 50 mM phosphate buffer, pH 6.5.

conditions (acidic, neutral, and basic pH, after Tanford transition). It was also confirmed by the disappearance of retinol fluorescence emission at 480 nm (**Figure 1B**), which decreased to zero at 40% and 50% (v/v) of ethanol. This indicates that secondary and tertiary protein structures were radically changed and also indicates that the hydrophobic retinol-binding site was completely destructured.

Titrations of native β-LG solution with retinol were investigated in 0%, 10%, 20%, and 30% (v/v) ethanol (**Table 1**). The computed number of retinol binding sites is about one. However, the small increase around 1/4 in number of binding sites is seen at 20% (v/v) of ethanol. Also, the affinity of the binding site rises with increasing ethanol content. On the one hand, this phenomenon could be due to the increased flexibility or accessibility of the binding site affected by ethanol. On the other hand, no retinol binding could be seen any more at 40% and 50% (v/v) of ethanol. Titration of native β-LG in 40% and 50% ethanolic solution with retinol has shown only very moderate changes in tryptophan emission. It seems that these changes are mostly due to the effect of ethanol concentration increase on β-LG structure. This indicates that at high concentration of ethanol, either the binding site has disappeared because of radical structural refolding of globulin or that the competition of ethanol has simply relinquished all retinol out of its binding site.

3.2. Circular Dichroism. Two minima of the ellipticity at ~285 and ~292 nm in the CD spectrum of native β-LG in water are mainly due to tryptophan (Trp19 and/or Trp61) absorbance (*29*). The negative peaks of the ellipticity at 208 and 222 nm are typical of the α-helical content of protein. There is a weak but broad $n \rightarrow \pi^*$ transition centered around 210 nm and an intense $\pi \rightarrow \pi^*$ transition at about 190 nm. CD spectra at the aromatic region (**Figure 2B**) show that secondary and tertiary structures of β-LG are not affected significantly between 0% and 20% of ethanol. The intensity of CD spectra changed significantly at 30%, and it decreased radically at 40% and 50% of ethanol (**Figure 3**).

The analysis of β-LG structure by CD spectroscopy shows changes of β-LG secondary structure in various ethanol concentrations compared with native β-LG conformations in aqueous solutions (**Figure 2**). According to the method of Fasman (*27, 28*) in solutions containing between 10% and 20% of ethanol, β-LG is composed of 54% of β-sheet, 5% of α-helix, and 41% of aperiodic structure. These values do not differ much from those observed in the case of native protein in neutral pH (55, 4, and 41%, respectively). The shifts in positions of the

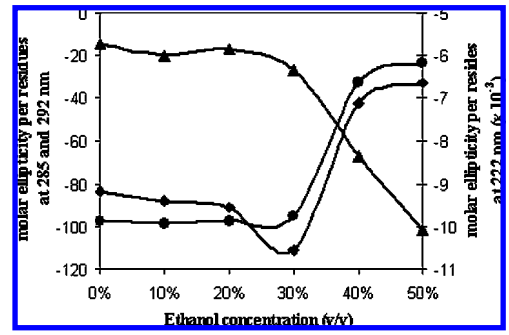

Figure 3. Ellipticity of β-LG (57 μM) at 285 (◆), 292 (●), and 222 (▲) nm in the presence of various ethanol percentages (v/v) at 20 °C in 50 mM phosphate buffer (pH 6.5).

major minima and change in magnitude of ellipticity indicate significant changes in structure of β-LG in 40% and 50% of ethanol. CD spectra of tryptophanyl residues have a minimum at 290 nm with fine structure between 290 and 305 nm. The disappearance of the chirality of aromatic amino acids side chains in the near-UV region indicates that they have acquired almost total freedom of rotation, which is characteristic of molten globule state or total denaturation provoking a loss of chirality. The CD spectra in the aromatic region (near-UV) demonstrate (**Figure 2B**) that the chirality of aromatic amino acid chromophores disappears in 40% and 50% of ethanol, which is characteristic of the molten globule state observed between 30% and 40% of ethanol. Hence, high concentrations of ethanol induce significant secondary and tertiary structure changes, which may be due to the increase of protein hydrophobic interactions with the medium and to the modifications of hydrogen bonds and dipolar moments. Since the secondary structure contents in 40% and 50% of ethanol differ much from those observed in the case of β-LG in 30% ethanolic solution, it is obvious that the β-LG tertiary structure has collapsed radically in solutions with high content of ethanol.

4. DISCUSSION

The β-LG fluorescence at 330 nm is dependent on the microenvironment of its Trp residues (*30*). β-LG contains two tryptophans, Trp19 and Trp61 (*31*). Trp19 is placed in an apolar environment in the hydrophobic pocket of β-LG, whereas Trp61 protrudes beyond the surface of the molecule and is quite close to the Cys66−Cys160 disulfide bridge (*32, 33*). As the disulfide bridge is an effective Trp fluorescence quencher, the intrinsic fluorescence of β-LG can be almost exclusively attributed to Trp19 (*34*). Fluorescence measurements of retinol/β-LG and palmitic acid/β-LG solutions (1:1) at 480 and 330 nm, respectively, in the absence and presence of ethanol at various pH show that the strongest interaction between β-LG and ligand is created around 20% of ethanol (**Figure 4**). After that, it decreases to zero at 40% and 50% of ethanol. These results confirm the structural changes in β-LG happening between 20% and 30% and around 40% of ethanol. They may indicate something similar to the Tanford transition between 20% and 30% of ethanol, where the affinity and apparent number of binding sites for retinol increase (**Table 1**). The intense quenching of retinol emission at 480 nm in 40% of ethanolic solution shows that the tertiary structure of β-LG is radically changed. The endoplasmic reticulum (ER) is an organelle found in all eukaryotic cells that is an interconnected network of tubules, vesicles, and cisternae. These structures are responsible for several specialized functions: protein translation, folding, and transport of proteins to be used in the cell membrane (e.g.,

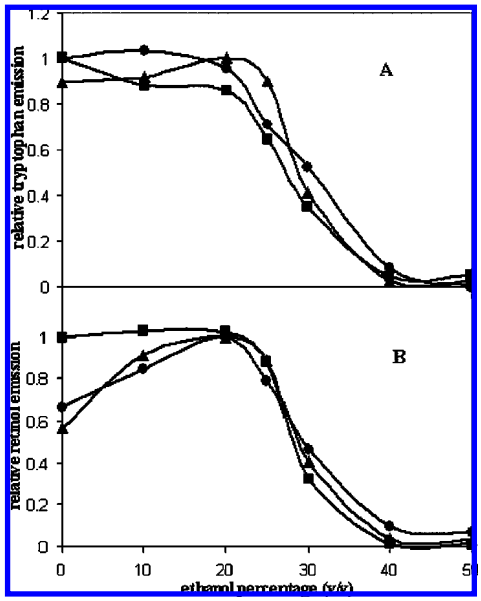

Figure 4. (**A**) Variation of fluorescence intensity (corrected for control) of tryptophan of native β-LG complexed with palmitic acid (excitation at 295 nm, emission at 330 nm) and (**B**) retinol complexed to native β-LG (excitation at 330 nm, emission at 480 nm) at pH 1.8 (▲), 6.5 (●), and 8.0 (■) as a function of ethanol percentages. The molar ratio of ligand to β-LG was equal to one.

Table 2. Estimation of α-Helix and β-Sheet Content in Native and Ethanol Treated β-LG Using the Fasman Method

ethanol concentration (%)	helix (%)	sheet (%)	others (%)
0	4	55	41
10	5	53	42
20	6	55	39
30	10	46	44
40	8	51	41
50	7	53	40
X-ray crystallography (*7*)	6.8	51.2	42

transmembrane receptors and other integral membrane proteins) or to be secreted (exocytosed) from the cell (e.g., digestive enzymes); sequestration of calcium; and production and storage of glycogen, steroids, and other macromolecules. The smooth ER is the site at which some drugs are detoxified (*35*). Hydrophobicity/polarity of the β-LG medium in 20% ethanolic solution may be in the neighborhood of membranes or inside of them. Hence, these results could have a biological meaning if and when β-LG would be bound in membranes of cytoplasmic reticulum (Golgi) or in milk close to fat globules.

The observed changes in CD spectra at 30%, 40%, and 50% of ethanol indicate that alcohol induces structural changes similar to the Tanford transition between 20% and 30% v/v. Calculated secondary structure of β-LG using the Fasman method (**Table 2**) shows that the α-helix content goes up with increasing ethanol concentration and that the β-sheet percentage does not change significantly between 0% and 20% of ethanol. It decreases at 30% of ethanol. This indicates structural transition between 20% and 30% of ethanol. β-Sheet percentage of β-LG increases again in the presence of 40% and 50% relative to 30% of ethanol, which indicates the rearrangement of the secondary structure of β-LG.

Figure 3 showed molar ellipticity of β-LG at 285 and 292 nm in the various percentages of ethanol. When ethanol was added to β-LG solutions, the ellipticity of β-LG at 285 and 292 nm decreased, around 30%, and then increased (see also **Figure 2B**). When the ethanol concentration increased to 40%, the minima at 285 and 292 nm disappeared. This indicates that the Trp residues are in a less constrained environment than in the native β-LG; therefore, the tertiary structure of β-LG was changed at and above 30% of ethanol. Moreover, ethanol significantly increased the absolute value of measured β-LG ellipticity at 208 and 222 nm at about 40% of ethanol (**Figures 2A** and **3**).

Increase of the apparent number of binding sites was perceived around 20% of ethanol, and also, the moderate decrease of molar ellipticities at 285 and 292 nm (**Figure 3**) shows that, between 20% and 30% of ethanol, β-LG is probably in the molten globule state. The balance of forces maintaining protein structure is changed significantly by the increase of ethanol concentration in solution. It causes a restructuring of β-LG and greatly influences its interactions.

5. ABBREVIATIONS USED

β-LG, β-lactoglobulin; CD, circular dichroism; NATA, *N*-acetyl-tryptophan amide.

ACKNOWLEDGMENT

S.H.-A.M. expresses his gratitude for the possibility to do a part of his research at Institut National de la Recherche Agronomique, Nantes, France.

LITERATURE CITED

(1) Tilley, J. M. A. The chemical and physical properties of bovine β-lactoglobulin. *Dairy Sci. Abstr.* **1960**, *22*, 111–125.
(2) Kinsella, J. E.; Whitehead, D. M. Proteins in whey: chemical, physical and functional properties. *Adv. Food Nutr. Res.* **1989**, *33*, 343–438.
(3) Sawyer, L. β-Lactoglobulin. In *Advanced Dairy Chemistry-I*, 3rd edition; Fox, P. F., McSweeney, P., Eds.; Kluwer: Amsterdam, The Netherlands, 2003; pp 319−386.
(4) Konuma, T.; Sakurai, K.; Goto, Y. Promiscuous binding of ligands by β-LG involves hydrophobic interactions and plasticity. *J. Mol. Biol.* **2007**, *368* (1), 209–218.
(5) Sawyer, L.; Richardson, J. S. Using appropriate nomenclature. *Trends Biochem. Sci.* **1991**, *16*, 11.
(6) Flower, D. R. Beyond the super family: the lipocalin receptors. *Biochim. Biophys. Acta* **2000**, *1482*, 327–336.
(7) Kontopidis, G.; Holt, C.; Sawyer, L. The ligand-binding site of bovine beta-lactoglobulin: evidence for a function. *J. Mol. Biol.* **2002**, *318*, 1043–1055.
(8) Futterman, S.; Heller, J. The enhancement of fluorescence and the decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin, β-lactoglobulin, and the retinol-binding protein of human plasma. *J. Biol. Chem.* **1972**, *247*, 5168–5172.
(9) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Fondlay, J. B. C.; Sivaprasadarno, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383–385.
(10) Monaco, H. L.; Zanotti, G.; Spadon, P.; Bolognesi, M.; Sawyer, L.; Eliopoulos, E. E. Crystal structure of the trigonal form of bovine beta-lactoglobulin and of its complex with retinol at 2.5 Å resolution. *J. Mol. Biol.* **1987**, *197*, 695–706.
(11) Jayat, D.; Gaudin, J.-C.; Chobert, J.-M.; Burova, T.; Holt, C.; Mcnae, I.; Sawyer, L.; Haertlé, T. A recombinant C121S mutant of bovine β-lactoglobulin is more susceptible to peptic digestion and to denaturation by reducing agents and heating. *Biochemistry* **2004**, *43*, 6312–6321.
(12) Newcomer, M. E.; Jones, T. A.; Aqvist, J.; Sundelin, J.; Eriksson, U.; Rask, L.; Peterson, P. A. The three-dimensional structure of retinol-binding protein. *EMBO J.* **1984**, *3*, 1451–1454.

(13) Huber, R.; Schneider, M.; Epp, O.; Mayr, I.; Messerschmidt, A.; Pflugrath, J.; Kayser, H. Crystallization, crystal structure analysis and preliminary molecular model of the bilin binding protein from the insect Pieris brassicae. *J. Mol. Biol.* **1987**, *195*, 423–434.
(14) Yang, J. T.; Doty, P. Polypeptides. XI. The optical rotary dispersion of polypeptides and proteins in relation to configuration. *J. Am. Chem. Soc.* **1957**, *79*, 761–775.
(15) Townend, R.; Kumosinski, T. F.; Timasheff, S. N. The circular dichroism of variants of beta-lactoglobulin. *J. Biol. Chem.* **1967**, *242*, 4538–4545.
(16) Dufour, E.; Bertrand-Harb, C.; Haertlé, T. Reversible effects of medium dielectric constant on structural transformation of β-LG and its retinol binding. *Biopolymer* **1993**, *33*, 589–598.
(17) Lee, L. L. Y.; Lee, J. C. Thermal stability of proteins in the presence of poly (ethylene glycols). *Biochemistry* **1987**, *26*, 7813–7819.
(18) Richards, W. G.; King, P. M.; Reynolds, C. A. Solvation effects. *Protein Eng.* **1989**, *2*, 319–327.
(19) Jukes, T. H.; Schmidt, C. L. A. The apparent dissociation constants of certain amino acids and related substances in water-ethanol mixtures. *J. Biol. Chem.* **1934**, *105*, 359–371.
(20) Pauling, L. *General Chemistry*, Dover Publication: New York, 1988; pp 420−440.
(21) Tanford, C. *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*; Wiley & Sons: New York, 1980; pp 139−146.
(22) Robinson, R. S.; Stokes, R. H. *Electrolyte Solutions*, 2nd ed.; Butterworths and Co.: London, 1970; pp 1−23.
(23) Mailliart, P.; Ribadeau-Dumas, B. Preparation of beta-lactoglobulin and beta-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH. *J. Food Sci.* **1988**, *53* (3), 743–745.
(24) Pace, N. C.; Tanford, C. Thermodynamics of the unfolding of beta-lactoglobulin A in aqueous solutions between 5 and 55 degrees. *Biochemistry* **1968**, *7*, 198–208.
(25) Cogan, U.; Kopelman, M.; Mokady, S.; Shinitzky, M. Binding affinities of retinol and related compounds to retinol binding proteins. *Eur. J. Biochem.* **1976**, *65*, 71–78.
(26) Fugate, R. D.; Song, P. S. Spectroscopic characterization of β-lactoglobulin-retinol complex. *Biochim. Biophys. Acta* **1980**, *625*, 28–42.
(27) Chou, P. Y.; Fasman, G. D. Empirical predictions of protein conformation. *Annu. Rev. Biochem.* **1978**, *47*, 251–276.
(28) Chou, P. Y.; Fasman, G. D. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1978**, *47*, 45–148.
(29) Gast, K.; Siemer, A.; Zirwer, D.; Damaschun, G. Fluoroalcohol-induced structural changes of proteins: some aspects of co solvent-protein interactions. *Eur. Biophys. J.* **2001**, *30*, 273–283.
(30) Eftink, M. R. The use of fluorescence methods to monitor unfolding transitions in proteins. *Biophys. J.* **1994**, *66*, 482–501.
(31) Wong, D. W. S.; Camirand, W. M.; Pavlath, A. E. Structures and functionalities of milk proteins. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 807–844.
(32) Nozaki, Y.; Reynolds, J. A.; Tanford, C. The interaction of a cationic detergent with bovine serum albumin and other proteins. *J. Biol. Chem.* **1974**, *249*, 4452–4459.
(33) Qin, B. Y.; Bewley, M. C.; Creamer, L. K. Structural basis of the Tanford transition of bovine β-lactoglobulin. *Biochemistry* **1998**, *37*, 14014–14023.
(34) Frapin, D.; Dufour, E.; Haertlé, T. Probing the fatty acid binding site of β-lactoglobulins. *J. Protein Chem.* **1993**, *12*, 443–449.
(35) Spurger, L. Endoplasmic reticulum: Structure and Function. University of Texas Medical Branch, 2002; http://cellbio.utmb.edu/cellbio/rer1.htm.

Received for review May 4, 2008. Revised manuscript received July 22, 2008. Accepted July 25, 2008. The financial support of Isfahan University, Ministry of Science, Research and Technology of Iran, and Franco-Iranian PHC Gundishapur project #10114VC and 18647RM awarded by the French Ministry or Foreign Affairs are gratefully acknowledged.

JF801383M