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Conformation and Stability of the α-Helical Intermediate of Intact and Thiol-modified β-Lactoglobulin Induced by Sodium Dodecyl Sulfate

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β-Lactoglobulin is a predominantly β-sheet protein, although it has a markedly high intrinsic preference for α-helical structure. β-Lactoglobulin assumes a monomeric native conformation at acidic condition. It has a free thiol at Cys 121, which is buried between the β-barrel and the C-terminal major α-helix. This thiol group was specifically reacted with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at pH 2, producing a modified β-lactoglobulin (TNB-β-LG) containing a mixed disulfide bond with 5-thio-2-nitrobenzoic acid. The formation of non-native α-helical intermediate of intact and thiol modified β-lactoglobulin was induced by sodium dodecyl sulfate. The conformation and stability of non-native α-helical intermediate (αI) state of TNB-β-LG were studied by circular dichroism and fluorescence techniques. The effect of sodium dodecyl sulfate on the structure of αI state at acidic condition was utilized to investigate the contribution by hydrophobic interactions to the stability of αI intermediate. The present results suggest that modification of the buried thiol group destabilizes the rigid hydrophobic core, producing a monomeric state that is native-like at pH 2. Therefore TNB-β-LG will become a useful model to analyze the conformation and stability of the intermediate of protein folding.

Key Words: β-Lactoglobulin, Thiol modified protein, Non-native intermediate, Sodium dodecyl sulfate, α-Helical state, Electrostatic force, Hydrophobic interaction.

INTRODUCTION

Bovine β-lactoglobulin (β-LG) is a major whey protein of bovine milk with known primary structure and three-dimensional structures but with still unknown biological function[1]. Its polypeptide chain is composed of 162 amino acid residues including two disulfide bonds (Cys 66 - Cys 160 and Cys 109 - Cys 119) and one free cysteine (Cys 121)[1]. The molecule of β-LG is constituted by nine antiparallel β-strands and one α-helix[2,3]. Cys 121 is buried at the interface between β-sheet and α-helix. The core of

β-LG molecule includes a structural motif similar to that found in retinol-binding protein (RBP)[3]. Like RBP, β-LG is able to bind a wide variety of hydrophobic molecules[1,4].

Despite numerous studies of stability and unfolding transitions of β-LG[5-9], the key factors stabilizing its native folding have not been determined. Irreversibility of β-LG unfolding is one of the main reasons which makes thermodynamic analysis of conformational stability of β-LG difficult. It was suggested that irreversibility of β-LG denaturation results mainly from the formation of intermolecular and non-native interamolecular disulfide bonds initiated by a single sulfhydryl group during unfolding of β-LG[5,6]. This SH-group can be chemically modified with different reagents. Most of the traditionally used thiol blocking reagents such as iodoacetic acid, iodoacetamide, N-ethylmaleimide or mercurials react very slowly with the thiol of β-LG in native conformation (reaction times of order 5-80 h were reported)[10-12]. This suggests low accessibility of β-LG Cys 121 thiol to blocking reagents in folded conformation and agrees well with available structural data showing location of Cys 121 well inside of a protein core[3]. Substitution of this β-LG SH-group resulted in effective suppression of aggregation of unfolded polypeptide chains and allowed maximum yield of native-like refolding[5,6]. Many studies have observed that β-LG subjected to thiol modification differs from unmodified β-LG by various physico-chemical characteristics such as optical rotation[13], dimer-monomer equilibrium[14,15], stability to denaturants[6,16] and stability to pH[17,18]. These observations raised questions about conformational changes induced in β-LG by substitution of this thiol and about the role of free cysteine in the maintenance of integrity of β-LG tertiary structure. The questions still remain unanswered. Site-directed mutagenesis, often used to analyze the importance of well defined amino acid residues, was in the case of Cys 121 substitution, unproductive because of difficulty in purifying the mutant protein[19].

A free thiol of bovine β-LG at Cys 121 of βH strand is completely buried under the C-terminal α-helix. However, it has been known that, under certain conditions, this thiol group can be chemically modified by thiol-specific reagents such as 2-mercaptoethanol, mercaptopropionic acid, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) or 4,4′-dithiopyridine[20-24]. The modification of β-LG by relatively small reagents (*i.e.*, 2-mercaptoethanol, mercaptopropionic acid) results in destabilization of the native structure, while retaining its native fold[20,23]. Intriguingly, the thiol modification improves the reversibility from denaturation because the thiol disulfide exchange reaction after unfolding is suppressed[23]. DTNB consists of two molecules of 2-nitro-5-thiobenzoic acid (TNB)[25]. Reaction of a protein thiol with DTNB by a thiol-disulfide exchange reaction produces one free TNB

molecule showing a strong absorption at 412 nm and a protein derivative in which the thiol group forms a mixed disulfide bond with TNB. The formation of the mixed disulfide bond can be detected from the absorption of bound TNB with a maximum at 325 nm. This mixed disulfide bond is easily cleaved by a reducing reagent such as dithiothreitol (DTT), regenerating the intact β-LG. In this study, we characterized the conformation and stability of intact β-LG and TNB-β-LG upon interaction with sodium dodecyl sulfate at pH 2, suggesting a major role of electrostatic force in inducing of non-native α-helical intermediate of β-LG.

EXPERIMENTAL

Bovine β-LG and sodium dodecyl sulfate (SDS) were purchased from Sigma. The concentrations of *n*-alkyl sulfates used in all experiments were under the critical micelle concentration (CMC) and critical aggregation concentration (CAC)[26,27]. TNB-β-LG was prepared by titrating the thiol group of β-LG at 0.4 mg mL^{-1} with 0.4 mM DTNB in 1.0 M Gdn-HCl and 20 mM HCl at pH 2. The titration was followed by measuring the absorption increase at 412 nm and it was confirmed that 1.0 mol of thiol group reacted with DTNB. The molar absorption of DTNB and TNB were assumed to be 18000 $M^{-1}cm^{-1}$ at 325 nm and 13600 $M^{-1}cm^{-1}$ at 412 nm, respectively[28]. The excess reagents were removed by dialysis. The purity of TNB-β-LG was checked by HPLC ion-exchange chromatography with a DEAE 3 SW column (Tosoh, Tokyo, Japan). First, the intact β-LG or TNB-β-LG was adsorbed to the column equilibrated with 20 mM HCl at pH 2 and then the protein was eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. TNB-β-LG was separated from the intact β-LG. TNB-β-LG used in the present studies was more than 95 % pure. DTNB, DTT, Gdn-HCl and other reagents were purchased from Merck.

Absorption measurements: The absorption spectrum of titration of the thiol group of β-LG with DTNB at pH 2 was measured with a spectrophotometer; model Perkin Elmer, at protein concentrations of 0.4 mg mL^{-1} with 1 cm path length cells. The protein concentration was determined spectrophotometrically. Extinction coefficient was used to calculate the concentration of the native form. The protein concentration was determined from the absorption at 278 nm using the absorption coefficient[29] of E_{278} = 9.6. The pH was measured using a Beckman Φ 50 pH-meter at 20°C.

Circular dichroism: All measurements were carried out at 20°C with thermostatically controlled cell holders. Far-UV CD spectra were measured with a Jasco spectropolarimeter (Japan), model J-720, equipped with an interface and a personal computer. The instruments were calibrated with ammonium d-10-camphorsulfonic acid[30]. The data were expressed as

molar residue ellipticity [θ], which is defined as $[\theta] = 100\ \theta_{obs}/cl$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue mol cm^{-3} and l is the length of the light path in cm. CD spectra were recorded with a time constant of 4s, a 2 nm band width and a scan rate of 5 nm min^{-1}, were signal-averaged over at least five scans and baseline corrected by subtracting a buffer spectrum. The CD spectra were measured with 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 fractions of the helix (f_H), β 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 least-squares method, which defines the X_H, X_β, X_R. The CD for the helix, β 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 follows[31,32]:

$$f_H = -([\theta]_{222} + 2340/30300) \quad (1)$$

Fluorescence measurements: Fluorescence measurements were performed by using a Hitachi F-2500 spectrofluorometer. The intensity at 480 nm was detected after excitation at 350 nm. Sodium dodecyl sulfate significantly affect the fluorescence of free tryptophan under the experimental conditions used. The temperature of the cell compartments was kept constant at 20°C by water circulation.

Measurements of viscosity and Stoke's radius: The viscosity was measured using a Haake D8 (W. Germany) microviscometer. The intrinsic viscosities, [η] and Stoke's radii, R_s, of the different states of β-LG upon interaction with n-alkyl sulfates were determined using the equation[33]:

$$\eta_{sp}/c \cong [\eta] = \lim_{c \to 0} [(\eta/\eta_o - 1)/c] = 2.5\ N_A/M\ (4/3\ \pi\ R_s^3) \quad (2)$$

where η_{sp} is the specific viscosities, c the protein concentration in g mL^{-1}, N_A Avogadro's number, M the molar mass of the protein and π is equal to 3.14.

RESULTS AND DISCUSSION

The free thiol of β-LG was titrated with DTNB manually adding the DTNB solution to the protein solution while the titration reaction of the buried thiol of β-LG with 0.4 mM DTNB at pH 2 was very slow, the reaction was accelerated by the addition of Gdn-HCl (data not shown). In the presence of 1.0 M Gdn-HCl, the reaction followed first-order kinetics with an apparent rate constant of 0.0023 s^{-1}. In the presence of 1.5 M Gdn-HCl, the burst phase, which occurred within a dead time of about 10 s, appeared

and the rate of the slow phase increased to 0.0036 s^{-1}. In the presence of Gdn-HCl at concentrations higher than 2.0 M, the titration was completed within the dead time (data not shown). The titration of the buried thiol with DTNB can be represented by the following scheme:

β-LG-SH (buried) $\rightleftharpoons$ β-LG-SH (exposed) $\rightarrow$ β-LG-SS TNB + TNB

where β-LG-SH (buried) and β-LG-SH (exposed) are the unreactive and reactive conformations of β-LG, respectively and β-LG-SS TNB is TNB-β-LG, a reactive product with a mixed disulfide bond. The reaction produces 1 mol of the TNB molecule with an absorption maximum at 412 nm and a TNB-β-LG at pH 2 with 1 mol of TNB bound that have absorption maximum at 325 and 340 nm, respectively[34]. In the absence of denaturant, the unfolding reaction by which the thiol group becomes reactive occurs rarely, so that the titration reaction is very slow. In the presence of a low concentration of denaturant (*e.g.*, 1.0 M Gdn-HCl), where β-LG still assumes the native structure, the unfolding reaction becomes significant and DTNB reacts more frequently with Cys 121. The increase in the reactivity of the thiol at Cys 121 toward DTNB by the addition of urea was reported by Kella and Kinsella[35]. From the absorption spectrum of TNB-β-LG with a peak at 340 nm (pH 2), it was confirmed that 1 mol of the TNB molecule was bound to 1 mol of β-LG. The attached TNB molecule can be removed by reduction of the disulfide bond with DTT, reversibly yielding the intact β-LG.

Figs. 1 and 2 show the effects of sodium dodecyl sulfate (SDS) on the far-UV CD spectra of the native state of β-LG and TNB-β-LG at pH 2. The native structure of β-LG is stable as a monomer[36] even at pH 2. In the absence of SDS in 20 mM HCl, the far-UV CD of the intact β-LG and TNB-β-LG showed a spectrum with a minimum at around 218 and 212 nm, respectively, consistent with the abundance of β-sheets (Figs. 1 and 2, curve 1). The far-UV CD spectrum of TNB-β-LG at pH 2 was not so different from that of the native spectrum, although the intensity below 220 nm was notably increased. This suggests that TNB-β-LG retains significant native-like secondary structures at pH 2. The CD spectrum of TNB-β-LG at pH 2 with sharp peaks is consistent with this interpretation, indicating the presence of rigid tertiary structures (data not shown). The broad and large positive peak with a maximum at 350 nm probably represents the CD of the tightly fixed TNB group (data not shown). The addition of SDS at different concentrations induced substantial α-helical structure as seen by the lower minima (lower $[\theta]_{208}$, $[\theta]_{222}$) at 208 and 222 nm relative to the native and thiol modified β-LG spectrum (Figs. 1 and 2, curve 5). The α-helical content was estimated from the ellipticity value at 222 nm, using method reported by Chen *et al.*[37] (Table-1). The α-helix content of TNB-β-LG at pH 2 is slightly higher than of the intact β-LG, suggesting

TABLE-1
INTRINSIC VISCOSITY ([η]), STOKES RADIUS (R_s) AND MOLAR ELLIPTICITY AT 222 nm ($[\theta]_{222}$) FOR THE αI STATES OF β-LG AND TNB-β-LG AT pH 2 UPON INTERACTION WITH SDS

Proteins state	$[\eta]/M^{-1}$	R_s/nm	$[\theta]_{222}$ (deg cm^2/mol)
Native β-LG	39.03 ± 0.3	1.83 ± 0.02	-6170
TNB-β-LG	40.95 ± 0.3	1.92 ± 0.02	-5500
αI state of β-LG induced by SDS (0.25 mM)	53.50 ± 0.3	2.04 ± 0.02	-48430
αI state of TNB-β-LG induced by SDS (0.25 mM)	56.40 ± 0.3	2.15 ± 0.02	-12850

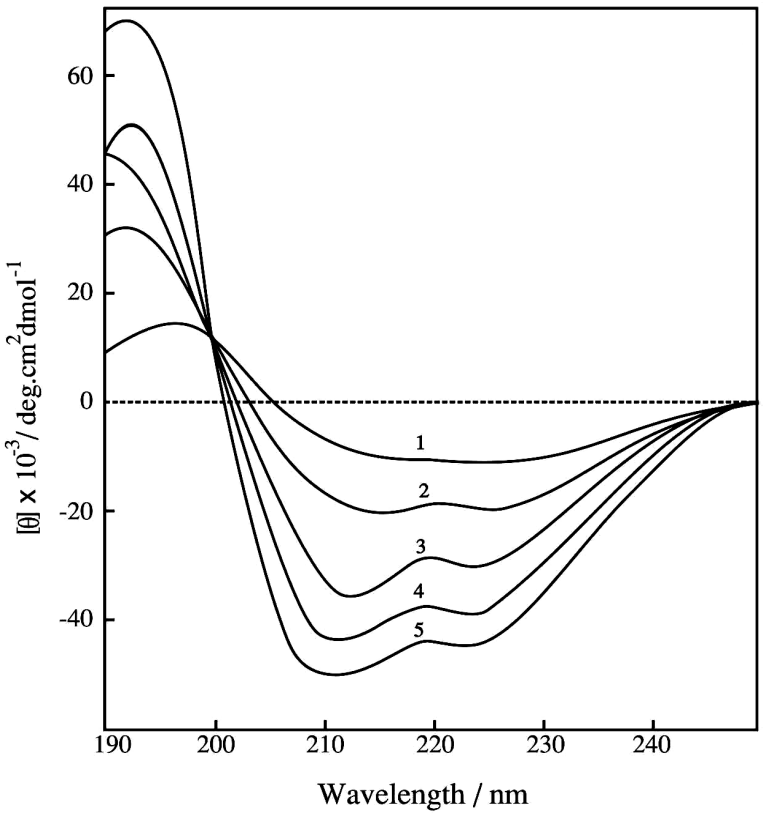

Fig. 1. Far UV CD spectra of β-LG (ellipticity, [θ]) as a function of sodium dodecyl sulfate concentration at pH 2 and 20°C. (1) 0 mM SDS (native state at pH 2 (2) 0.05 mM SDS (3) 0.1 mM SDS (4) 0.2 mM SDS (5) 0.25 mM SDS. Protein concentration was 27 mM

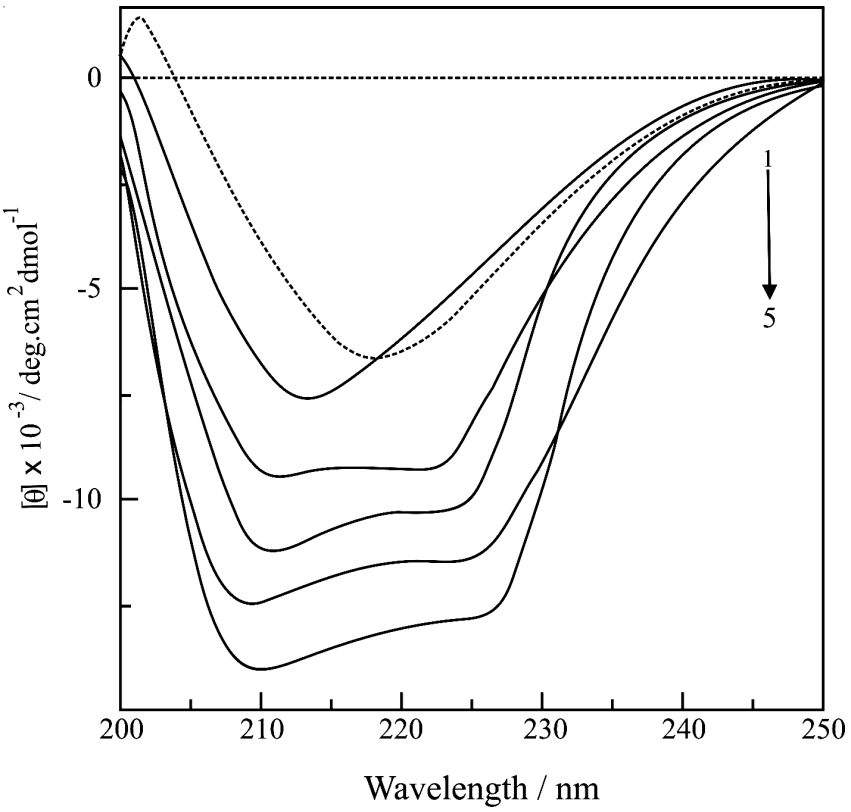

Fig. 2. Far-UV CD spectra of intact β-LG (dashed curve) and TNB-β-LG (ellipticity, [θ]) as a function of SDS concentration at pH 2 and 20°C. (1) 0 mM SDS (TNB-β-LG) (2) 0.05 mM SDS (3) 0.1 mM SDS (4) 0.2 mM SDS (5) 0.25 mM SDS

the formation of a small amount of non-native helical structure as observed for the kinetic intermediate[38,39]. The CD spectra show the non-native α-helical intermediate (αI) state for β-LG and TNB-β-LG upon the addition of SDS (0.25 mM). When [SDS] is larger than those values, the baselines increase, due to precipitation (data not shown).

Considering these findings, the SDS-induced conformations are not an indication of the ordinary intermediate. In fact, they are regarded as αI states of the protein with different secondary structures. The helical content in the native state of β-LG and TNB-β-LG are 7.0 and 14.7 %, respectively, on the basis of the ellipticity values at 222 nm as calculated by the method of Chen *et al.*[37]. The far-UV CD spectra in the presence of various concentrations of SDS show that, whereas TNB-β-LG at pH 2 was not so different from that of the native spectrum, the addition of SDS

stabilizes the helical conformation. This indicates that the denaturant of SDS is closely correlated with its potential for stabilizing the helical conformation in the TNB-β-LG. Although the anionic head of SDS is an important factor determining the SDS effects. We consider that the direct interaction between hydrophobic tail of SDS and hydrophobic groups of proteins is responsible for the SDS effects. When SDS at high concentrations as denaturant ligand interacts with native state of TNB-β-LG at pH 2, the interior hydrophobic groups of protein expose to solvent and then the polarity around the TNB-β-LG will decrease. This leads to stabilization of the intermolecular hydrogen binds and consequently the formation of a helical conformation. As in previous studies, changes in the accessibility of hydrophobic regions of the molecule were monitored through the binding of the fluorescence hydrophobic probe ANS. Figs. 3 and 4 show the effect of SDS on the fluorescence spectra of the β-LG-ANS and TNB-β-LG-ANS complexes at pH 2. According to Figs. 3 and 4, the addition of different concentrations of SDS to the β-LG-ANS and TNB-β-LG-ANS complexes cause an increase in the fluorescence intensity. Here, the

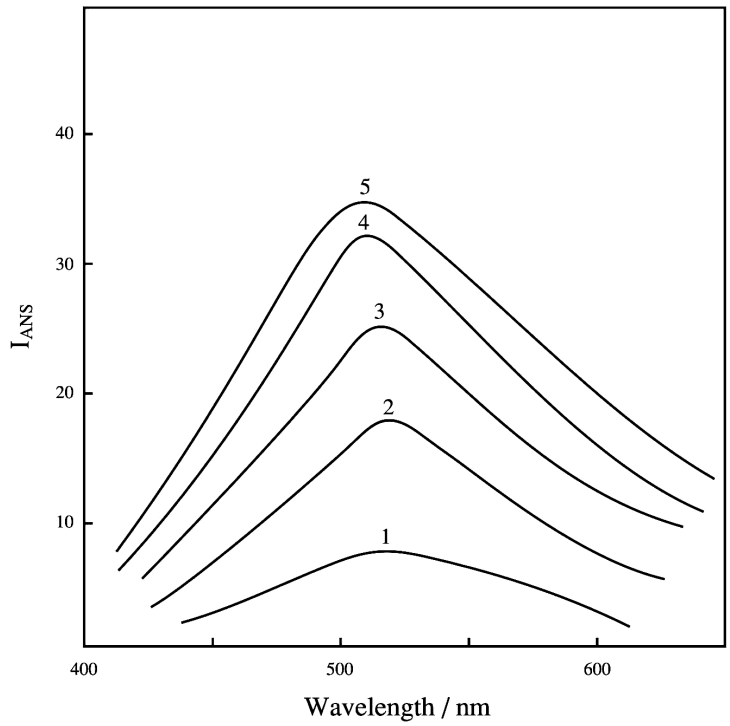

Fig. 3. Fluorescence spectra of 250 mM ANS in the present of following: (1) free ANS at pH 2 (2) native state of β-LG at pH 2 (3) β-LG upon the addition of 0.05 mM SDS (4) β-LG upon the addition of 0.1 mM SDS (5) β-LG upon the addition of 0.25 mM SDS. The ratio of molar concentrations [ANS]/[protein] = 250/1

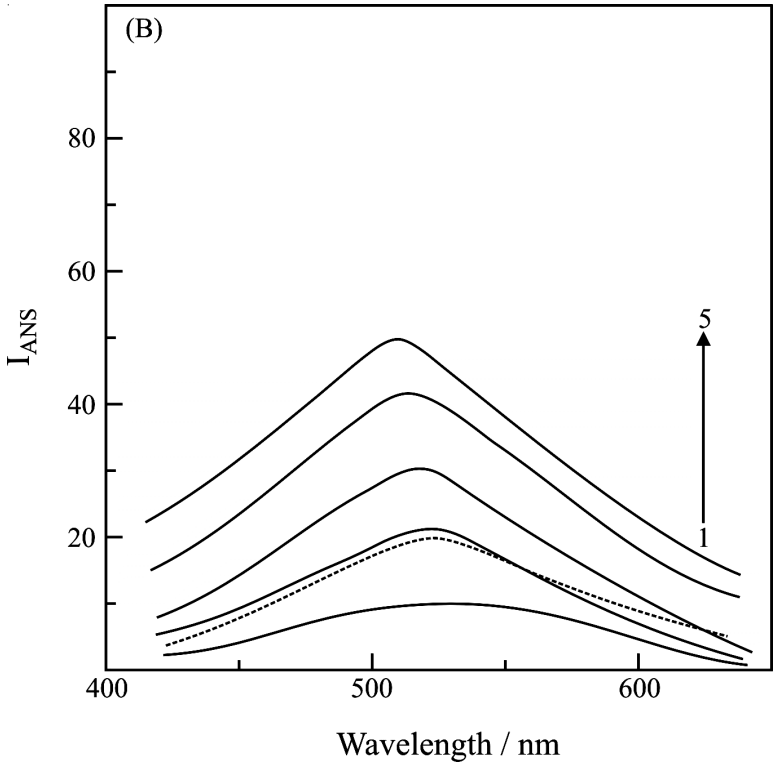

Fig. 4. Fluorescence spectra of 250 mM ANS in the present of the following: (1) free ANS (2) TNB-β-LG upon the addition of 0.05 mM SDS (3) TNB-β-LG upon the addition of 0.1 mM SDS (4) TNB-β-LG upon the addition of 0.2 mM SDS (5) TNB-β-LG upon the addition of 0.25 mM SDS. Dashed curve is intact TNB-β-LG. The ratio of molar concentrations [ANS]/[protein] = 250/1

interaction of β-LG-ANS and TNB-β-LG-ANS complexes with SDS at different concentrations are consistent with results obtained for β-LG in the presence of TFE as previously reported by Hamada *et al.*[38]. These indicate that β-LG and TNB-β-LG in the absence of SDS assume a compactly folded structure in which the tryptophan residues and quenchers are adjacent. Nevertheless the fluorescence intensities in the unfolded state were less than those of the intact β-LG in the unfolded state, indicating that the TNB group still quenches the tryptophan fluorescence. The fluorescence intensity observed for the same SDS concentration upon interaction with TNB-β-LG is more than β-LG at pH 2, indicating that the rigidity of protein tertiary structure of TNB-β-LG is less than β-LG, therefore the addition of SDS to the TNB-β-LG causes an increase in the fluorescence intensity than to β-LG. The blue shift of the emission maximum in both

structures suggests that the buried tryptophan (Trp 19) is preferentially dequenched, consistent with the proximity of Cys 121 and Trp 19.

Table-1 shows intrinsic viscosity and Stokes radius of the different structural states induced by various concentrations of SDS and the intact β-LG and TNB-β-LG at pH 2 and 20°C. The Stokes radii indicate different values for the native and non-native α-helical intermediate states induced by SDS at different concentrations. These results show the compaction of the native state of β-LG (R_s = 1.83 nm) relative to other states. The R_s value of β-LG is nearly consistent with the gyration radius (R_g) value[39]. The R_s values in Table-1 show the most compact state is the native form. The αI state of β-LG induced by SDS is more compact than the TNB-β-LG. It is noteworthy that the R_s values indicate the unfolding of the β-LG and TNB-β-LG during the addition of SDS. The pI value of β-LG is 4.6 and the net charge at pH 2 was calculated to be + 20, on the basis of the amino acid composition. Therefore, the net charge repulsion can not explain the increased stability at pH 2 and the exact reason for the increase stability of β-LG at pH 2 was unclear so far. In this paper, the spectroscopic properties of the αI state support the view that SDS at different concentrations stabilize the αI state of β-LG and TNB-β-LG. The αI state in contrary to native state of β-LG shows high amounts of α-helix. In conclusion, the helical propensity of the β-LG and TNB-β-LG in the presence of SDS must be related to the intrinsic helical propensity as was observed for proteins. The α-helical content of αI state of β-LG induced by SDS is more than TNB-β-LG at pH 2, therefore, there are net positive charges in the β-LG, electrostatic force is the main engine of hydrophobic interactions, which causes stability of β-LG.

Conclusion

β-LG has a buried thiol at Cys 121 that can be titrated by DTNB at two different pH. Modification of the thiol group with DTNB at pH 2 destabilizes β-LG. The results described in this paper strongly indicate that the non-native α-helical intermediate (αI) of β-LG and TNB-β-LG is in a stable thermodynamic state as is the case for the native or unfolded state. Here this indicated that the α-helical content of αI state of β-LG induced by SDS is more than TNB-β-LG at pH 2, therefore although there are positive charges repulsion in the β-LG at pH 2, electrostatic force is the main factor of hydrophobic interactions inducing, that causes stability of β-LG.

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