

Conformational and Structural Analysis of Bovine β Lactoglobulin-A Upon Interaction with Cr^{+3}

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Thermodynamic studies have been made on the effect of Cr^{+3} on the conformation and structure of bovine β lactoglobulin-A, (Blg-A) in 50 mM sodium chloride solution at 27°C using isothermal titration calorimetry (ITC), circular dichroism (CD) and fluorescence spectroscopy. There is a set of six identical and independent binding sites for Cr^{+3} by a dissociation binding constant of 124 μM and the molar enthalpy of binding -17.8 kJ/mol. Circular dichroism studies do not show any significant change in the secondary structure of the protein after the binding of chromium ion on the Blg-A. Fluorescence spectroscopy studies do not show any considerable change in the tertiary structure of Blg-A due to the increasing of Cr^{+3} in low concentration. However, occupation of fourth and fifth binding sites for chromium ions induce partially unfolding in the tertiary structure of the protein resulted from solvent – exposed hydrophobic patches on BLG-A.

KEY WORDS: β lactoglobulin; Chromium ion; circular dichroism; fluorescence; isothermal titration calorimetry.

1. INTRODUCTION

One of the most widely studied and fascinating proteins of animal origin is beta lactoglobulin (Blg). This polypeptide, with 150–170 amino acids (depending on the species), is an abundant component of the milk of most mammals, although it has not been detected in human milk (Jankowski *et al.*, 2003). It is a globular protein with a monomer molecular weight of about 18,300 and exists in various oligomeric states (dimers and octamers) as a function of pH, temperature, concentration, ionic strength and genetic variant (Busti *et al.*, 2002). Bovine β lactoglobulin consists of 162 amino acid residues and contains two disulfide bonds (cys66-cys160 and cys106- cys119) and a free thiol (cys121) (Sakai *et al.*, 2000). To date, no clear physiological function for this protein has been defined. Blg may

be classified in the super family of the hydrophobic molecule transporters termed lipocalins (Busti *et al.*, 1998). This family includes transport proteins, such as the retinal binding protein, the odorant binding protein, and the major urinary protein, which all share the common structural feature of a β -barrel calyx, built from eight anti-parallel β -sheets, arranged as an ideal site for hydrophobic ligands (Collini *et al.*, 2000). This milk protein interacts strongly with various hydrophobic ligands such as fatty acids (Brown, 1984), hemin (Dufour and Haertle, 1990), ellipticine (Dodin *et al.*, 1990), aromatic hydrocarbons (Farrel *et al.*, 1987), and carcinogenic hydrocarbons (Ono *et al.*, 1975).

Although Blg exists as a homodimer at neutral pH, it dissociates into monomers at acidic pH, but still retains the native structure (Kontopidis *et al.*, 2002). Because of its stability under acidic pH conditions, Blg is resistant to digestion in the stomach

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Abbreviation: Blg-A, β lactoglobulin-A; Cr^{+3} , chromium ion; ITC, isothermal titration calorimetry; CD, circular dichroism; ANS, 1 – anilinonaphthalene-8 – sulfonate.

and is considered one of the allergens for human infant milk allergy (Mantylarvi *et al.*, 2000).

Blg is one of the few proteins, which bind sodium ions. Maximum number of Na^+ bound per molecule of Blg (A and B) is four (Powell Baker and Saroff, 1965). Studies show that Hg^{+2} leads to the formation of an insoluble aggregate at high pH in Blg (Sawyer and Kontopidis, 2000).

Metal ions play important roles in many biological systems for example, currently, at least one-third of all proteins appear to contain metal ions and all ribozymes (RNA enzymes) appear to be metalloenzymes. Naturally occurring metal ions add extra dimensions to the properties of proteins and ribozymes, which otherwise are constrained by finite number of building blocks that make up their primary structures (Lu and Valentine, 1997).

Trace elements, including the essential and the toxic, play an important role in the life and environmental science, which one of them is chromium (Cr). Chromium, as a trivalent organic or inorganic species, has been proposed to act as a potentiator of insulin action in animals and human beings (Chai *et al.*, 2004). The Cr deficiency includes symptoms resembling diabetes, such as glucose intolerance impairment with the requirement of increasing insulin, etc., and Cr supplement can alleviate these symptoms. However, up to now, the biological function of Cr in an organism is still unclear and the concentration of Cr in many biological materials is usually at ultratrace level (Chai *et al.*, 2004). The epidemiological studies show that the hexavalent Cr causes increased risk of bone, prostate, lymphomas, Hodgkins, leukemia, stomach, genital, renal, and bladder cancer, reflecting the ability of hexavalent chromium to penetrate all tissues in the body. A high accumulation of Cr (III) in all tissues and organ is a strong indication of the wide toxic potential of exposure to soluble hexavalent Cr in the drinking water and in the ambient environment (Costa, 1997). The median effective concentration (Ec_{50}) reducing rates of embryogenesis and larval (chordata, Ascidiacea) attachment by 50% is 10.318 microg Cr/l (198 μM) (Bellas *et al.*, 2001).

It has been reported that Cr (III) can bind to Blg but cannot displace by ANS (Lange *et al.*, 1998), but the number of binding sites and its binding strength for this metal ion have not been known. There are eight negative charges on the surface of Blg in neutral condition (Simons *et al.*, 2002), which they may adsorb metal ions. There is

not any precise report on the binding of chromium ion to Blg. Here, we present the results of a systematic thermodynamical analysis of the effect of Cr^{+3} on the structural and conformational properties of bovine Blg-A, as a carrier protein, in aqueous solution at room temperature.

2. MATERIALS AND METHODS

2.1. Materials

Bovine beta lactoglobulin (Blg-A) was obtained from sigma. Cr (III) nitrate was purchased from Merck and ANS (1 – anilinonaphthalene-8 – sulfonate) was purchased from Sigma. All other materials and reagents were of analytical grades, and solutions were made in double-distilled water. NaCl solution, with 50 mM concentration, was used as a solvent. Concentrations of Blg-A were determined spectrophotometrically by using, for the calculation, a molecular absorption coefficient of $\epsilon_{278\text{ nm}} = 17,600\text{ M}^{-1}\text{ cm}^{-1}$ (Dufour *et al.*, 1992).

2.2. Methods

2.2.1. Calorimetric measurements

The isothermal titration microcalorimetric experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277, Thermometric, Sweden. The insertion vessel was made from stainless steel. The chromium solution (2 mM) was injected by a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.8 ml Blg-A, 10 μM , including a sodium chloride (50 mM). Thin (0.15 mm inner diameter) stainless steel hypodermic needles (permanently fixed to the syringe) reached directly into the calorimetric vessel. The injection of the chromium solution into the perfusion vessel was repeated 50 times. Each injection included 20 μl reagent. 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 Diagram3” software program. The heat of the dilution of the chromium solution was measured as described previously, except the protein was excluded. The enthalpy of the dilution was subtracted from the enthalpy of the protein– Cr^{+3} interaction. The microcalorimeter was frequently calibrated electrically during the course of

the study. The molecular weight of Blg-A was taken to be 18,364 (Piez *et al.*, 1961).

2.2.2. Isothermal titration UV-visible spectrophotometric method

UV-visible spectrophotometric titration curve was obtained by measuring the maximum absorbance (280 nm) of the 1.5 ml solution containing 14.1 μM Blg-A against 1.5 ml NaCl solution using a shimadzu model UV-3100 spectrophotometer and 3 cm cuvettes thermostated to maintain the temperature at $27 \pm 0.1^\circ\text{C}$. Appropriate volumes of 10 mM chromium nitrate solution were cumulatively added to both sample and reference cells. All measurements were made after Blg-A and Cr ions had been incubated for over 3 min, after which time the absorbance did not change.

2.2.3. Fluorescence measurements

Fluorescence intensity measurements were carried out on a Hitachi spectrofluorimeter model MPF-4. The excitation wavelength was adjusted at 290 nm and the emission spectra were recorded for all of the samples in the range of 300–400 nm. The binding of a hydrophobic fluorescent probe, ANS to Blg-A was monitored by exciting the ANS at 350 nm and recording the emission spectra in the range of 400–650 nm. Measurements were made by applying a 1-cm path length fluorescence cuvette. Samples of 5 μM Blg-A were in 50 mM sodium solution.

2.2.4. Circular dichroism (CD) measurements

CD spectra were recorded on Aviv-215 spectropolarimeter (USA). The solutions for far-UV investigations contained of a constant concentration for Blg-A (13.5 μM) with the several concentrations of Cr^{+3} (0, 22, 47.38, 113.4, 219.1, 436.45 and 1283.3 μM). The results were expressed in molar ellipticity $[\theta]$ ($\text{deg cm}^2 \text{dmol}^{-1}$) based on a mean amino acid residue weight of 114 (MRW). The molar ellipticity was determined as $[\theta]_\lambda = (100 \times \text{MRW} \times \theta_{\text{obs}}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/ml and l is the length of the light path in cm. The CD software was used to predict the secondary structure of the protein according to the statistical method (Marthasarathy and Johnson, 1987; Yang *et al.*, 1986).

3. RESULTS AND DISCUSSION

The raw data obtained from isothermal titration calorimetry of Blg-A interaction with chromium ion was shown in Fig. 1. Figure 1a is showing the heat of each injection and Fig. 1b is showing the heat of related to each total concentration of chromium ion, $[\text{Cr}^{3+}]_t$. The heat values in

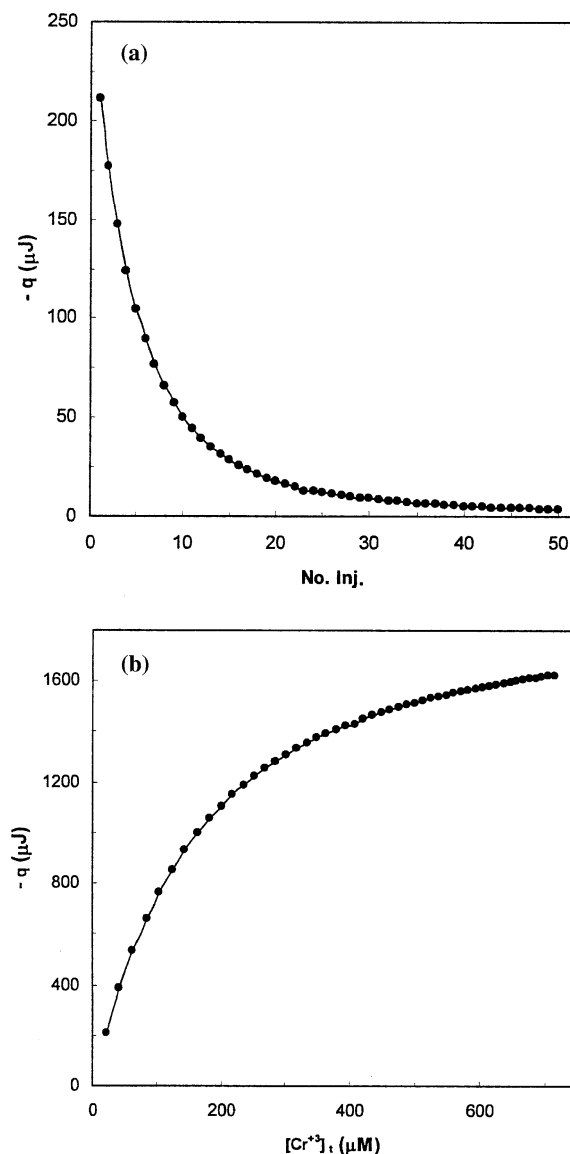
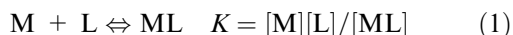


Fig. 1. (a) The heat of the Cr^{+3} binding on Blg-A for 50 automatic cumulative injections, each 20 μl of the Cr^{+3} solution, 2 mM, into the sample cell that contained 1.8 ml Blg-A solution at a concentration of 10 μM in 50 mM sodium chloride solution, pH 7, at 27°C . (b) The cumulative heat of the binding versus the total concentration of Cr^{+3} , calculated from (a).

these figures have been expressed in terms of total amount of protein (18 nmol) in the calorimetric sample cell.

Consider a solution containing ligand L, and a biomacromolecule (M_n) that contains n sites capable of binding the ligand. If the multiple binding sites on a biomacromolecule are identical and independent, the ligand binding sites can be reproduced by a model system of monovalent molecules ($M_n \rightarrow nM$) with the same set of dissociation equilibrium constant (K) values. Thus, the reaction under consideration can be written:



If α is defined as the fraction of free binding sites on the biomacromolecule, M_0 is the total biomacromolecule concentration and L_0 is the total ligand concentration, then the free concentrations of monovalent molecule, $[M]$, and ligand, $[L]$, as well as the concentration of bound ligand, $[ML]$, can be deduced as follow:

$$[ML] = n(1 - \alpha)M_0 \quad (2)$$

$$[L] = L_0 - [ML] = L_0 - n(1 - \alpha)M_0 \quad (3)$$

$$[M] = nM_0 - [ML] = nM_0 - n(1 - \alpha)M_0 = \alpha nM_0 \quad (4)$$

Substitution of free concentrations of all these components in Eq. (1) gives:

$$K = \left(\frac{\alpha}{1 - \alpha} \right) L_0 - \alpha nM_0$$

or

$$\alpha M_0 = \left(\frac{\alpha}{1 - \alpha} \right) \frac{1}{n} L_0 - \frac{K}{n} \quad (5)$$

The value of $1 - \alpha$ as the fraction of occupied binding sites on the biomacromolecule can be obtained from every desired point on the titration curve of heat value versus total concentration of ligand, as shown in Fig. 1b, using the relationship:

$$1 - \alpha = \frac{q}{q_{\max}} \quad (6)$$

where q represents the heat value at a certain L_0 and $q_{\max}$ represents the heat value upon saturation of all biomacromolecule. If q and $q_{\max}$ are calculated per mole of biomacromolecule then the molar enthalpy of binding for each binding site (ΔH) will be $\Delta H = q_{\max}/n$. Combination of Eqs. (5) and (6) yields:

$$\frac{\Delta q}{q_{\max}} M_0 = \left(\frac{\Delta q}{q} \right) L_0 \frac{1}{n} - \frac{K}{n} \quad (7)$$

where $\Delta q = q_{\max} - q$. Therefore, the plot of $(\Delta q/q_{\max})M_0$ versus $(\Delta q/q)L_0$ should be a linear plot by a slope of $1/n$ and the vertical-intercept of K/n , which n and K can be obtained. The related plot for the binding of chromium ions by Blg is showing in Fig. 2. The linearity of the plot has been examined by different estimated values for $q_{\max}$ to find the best value for the correlation coefficient (near to one). The best linear plot with the correlation coefficient value of 0.99 was obtained using a value of $-1920 \mu J$ (equal to -106.7 kJ/mol) for $q_{\max}$. The amounts of n and K , obtained from the slope and vertical-intercept plot, are 6 and $124 \mu M$, respectively. Dividing the $q_{\max}$ value of -106.7 kJ/mol by $n=6$, therefore, gives $\Delta H = -17.8 \text{ kJ/mol}$.

Figure 3 shows the absorbance change at 280 nm upon titration of a limiting concentration of Blg-A ($14.1 \mu M$) with increasing aliquots of a fixed stock concentration of Cr^{+3} ion (10 mM). As the aliquot solutions of Cr^{+3} ion are increased in the titration mixture, the absorbance has not any changes. Regarding to the Fig. 3, it might be concluded that increasing of Cr^{+3} concentration did not induce any considerable changes on the conformation and structure of Blg-A. We used other techniques (far UV-CD and fluorescence) that are capable of sensing different structural properties.

The effect of different concentrations of Cr^{+3} on the conformation of Blg-A was examined by far UV-CD at $27^\circ C$. Figure 4 shows the UV-CD spectra registered for Blg-A in the absence ($v=0$) and in the presence of different concentrations of Cr^{+3} (22, 47.38, 113.4, 219.1, 436.45, 1283.3 μM , from in which $v=0.5, 1, 2, 3, 4$ and 5 , respectively). v means the number of moles of ligand bound per mole of the protein. The recorded spectra represent a minimum at the wavelength of 217 nm. This wavelength can be utilized for monitoring of the secondary structural alterations for the protein, which might be occurred regarding to the conditional alterations. There are not any considerable difference between the spectra by increasing of Cr^{+3} concentration. Table 1 shows that increasing of Cr^{+3} concentration to $[Cr^{+3}] = 1283.3 \mu M$ (related to the binding of fifth Cr^{+3}) resulted in a small decrease in the content of α -helix of the native secondary structure of protein, which compensate with increasing in the content of β -sheet, so that binding

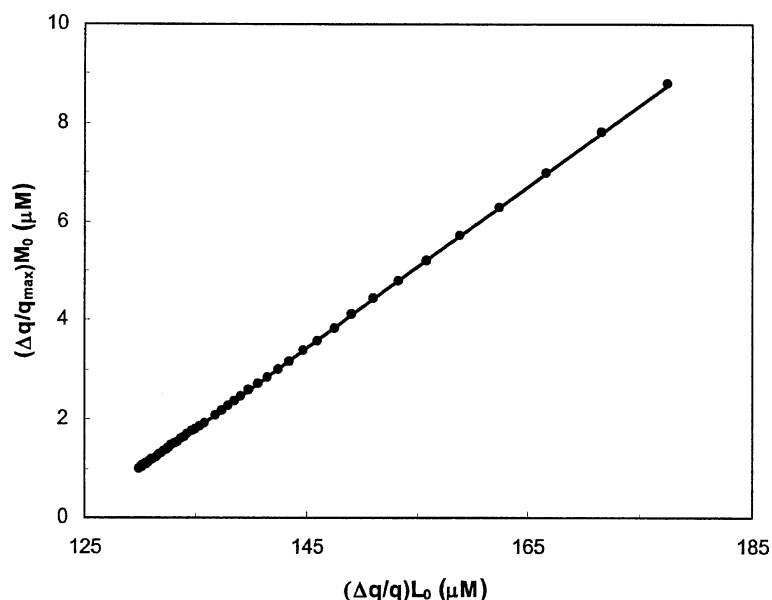


Fig. 2. The best linear plot of $(\Delta q/q_{\max}) M_0$ versus $(\Delta q/q)L_0$, according to the Eq. (7) and data shown in Fig. 1b, using a value of $-1920 \mu\text{J}$ (equal to -106.7 kJ/mol) for $q_{\max}$ to obtain the best correlation coefficient value ($R^2=0.99$). Values of n and K can be obtained from the slope and the vertical-intercepts, respectively.

of first to fifth Cr^{+3} ions have not induced any change in the content of the random coil of the Blg-A. Then it can be seen that increasing of Cr^{+3} concentration does not any significant effect on the secondary structure of Blg-A.

Bovine Blg contains two Trp residues, Trp-19 and Trp-61, which are in quite different environments. The lattice Z crystal structure (Qin *et al.*, 1998a, b) shows that Trp-19 is in an apolar environment within the cavity of Blg whereas Trp-61

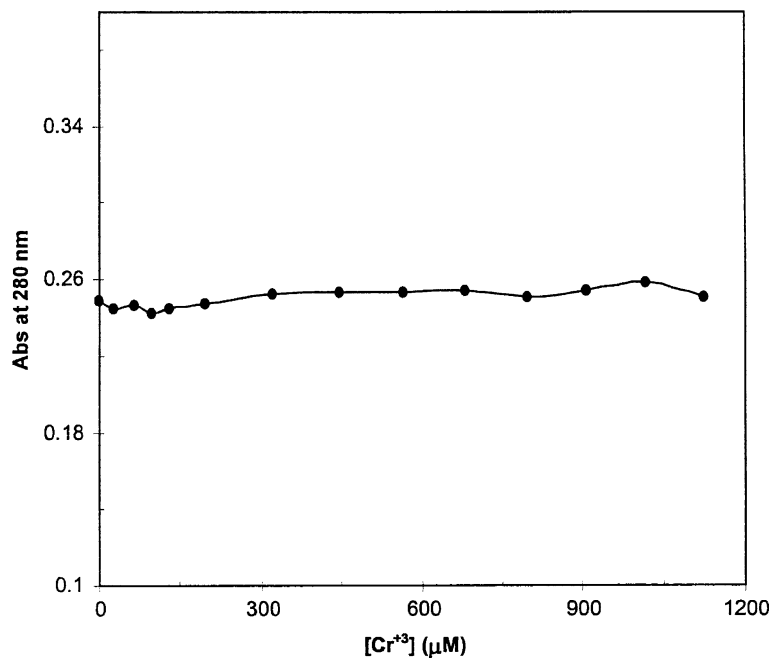


Fig. 3. The change in absorbance at 280 nm upon titration of a 1.5 ml Blg-A solution ($14.1 \mu\text{M}$) in 50 mM sodium chloride solution, pH 7, at 27°C with increasing aliquots of a fixed stock concentration of Cr^{+3} ion (10 mM).

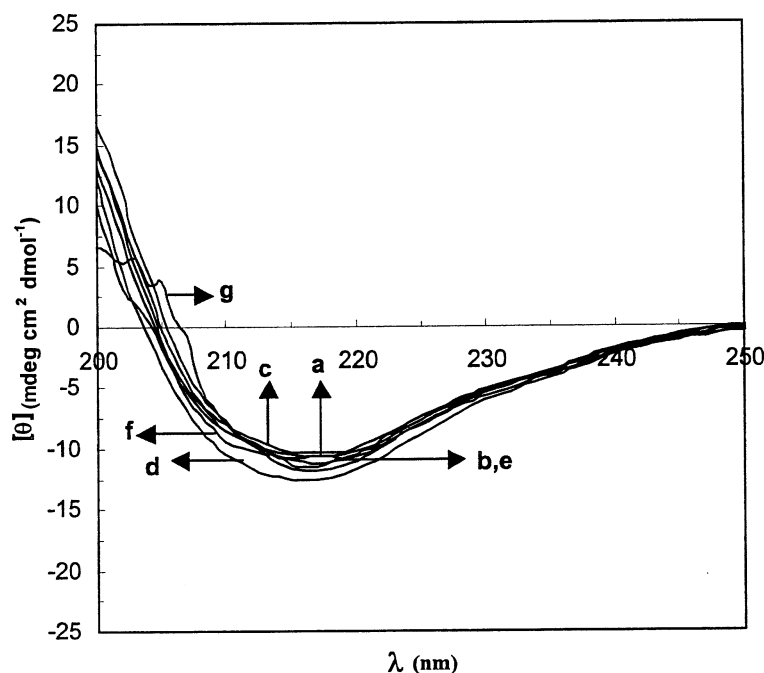


Fig. 4. Far-UV circular dichroism spectra of 13.5 μM BlgA measured in the absence (a) and presence of different concentrations of Cr^{+3} : 22 (b), 47.38 (c), 113.4 (d), 219.1 (e), 436.45 (f) and 1283.3 μM (g) in 50 mM sodium chloride solution, pH 7, at 27°C.

protrudes beyond the surface of the molecule and is quite close to the cys-66 to cys-160 disulfide bridge, which can be an effective Trp fluorescence quencher. The intrinsic fluorescence of the Blg is almost exclusively attributed to Trp-19 (Andrade and Costa, 2002). Figure 5 represents the effect of the addition of Cr^{+3} concentration on the intrinsic Trp fluorescence of Blg-A at 27°C. It shows that addition of Cr^{+3} induces a small change (approximately 6%) on the emission of native Blg. Then it can be seen that increasing of Cr^{+3} concentration does not any significant effect on the position of the two Trps of the Blg-A.

The ANS is a molecule that shares both hydrophilic and hydrophobic characters and whose fluorescence response appears as a convenient and

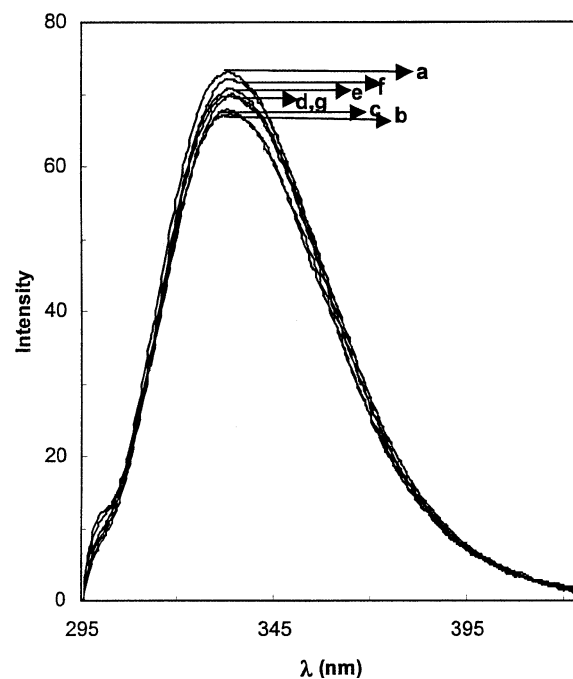


Fig. 5. The effect of different concentration of Cr^{+3} ions on the intrinsic fluorescence of Blg-A (5 μM) in 50 mM sodium chloride solution, pH 7, at 27°C. [Cr^{+3}] = 0 (a), 8.15 (b), 17.55 (c), 42 (d), 81.15 (e), 161.65 (f) and 475.5 μM (g).

Table 1. Content of the Secondary Structure of Blg-A Upon Interaction with Cr^{+3} in Different Concentrations

% Secondary structure	v=0	v=0.5	v=1	v=2	v=3	v=4	v=5
α -helix	17.4	18.7	19.1	18.8	17.8	16.1	13
β -sheet	44.2	43	42.4	43.8	43.1	45.9	49.5
Random coil	38.4	38.3	38.5	37.4	39.1	38	37.5

sensitive tool apt to investigate the nature of proteins binding regions. Small, albeit significant, changes in ANS fluorescence, when changing the protein environment, appear to reflect a modified ligand-protein interaction, which can be due to changes in the probe response (Matulis *et al.*, 1999). ANS is frequently used to demonstrate the presence of partially unfolded conformations of globular proteins. This is because ANS binds to solvent-exposed hydrophobic clusters, which results in a considerable increase in ANS fluorescence intensity and in a blue shift of the fluorescence emission maximum (Uversky *et al.*, 2001). Figure 6 shows the ANS spectra in the presence of Cr^{+3} concentrations ($v=1, 2, 3, 4$ and 5) and the absence of them on Blg-A at 27°C . Although chromium ions enhance a little in the ANS fluorescence intensity, but higher concentrations of 161.65 and 475.5 μM of Cr^{+3} (related to the $v=4$ and 5) induce considerably enhancement in ANS

fluorescence intensity. All concentrations of Cr^{+3} , induce a pronounced blue shift (~ 23 to ~ 39 nm) on the λ_{max} of ANS. Hence, binding of chromium ions lead to a partially unfolding in the protein resulted from solvent-exposed hydrophobic patches.

4. CONCLUSIONS

A simple graphical linear method was applied for isothermal titration calorimetric data analysis to find thermodynamic parameters (n , ΔH and K) on the study of the interaction of Blg-A with trivalent chromium ion. There is a set of six identical and independent binding sites for Cr^{3+} ions on the surface of Blg-A. Negative charges on the surface of the protein are positions, which they may adsorb the metal ions. The more accessible negative charged amino acids on the surface of the protein are as follow: Glu 112, Asp 64, Glu 134,

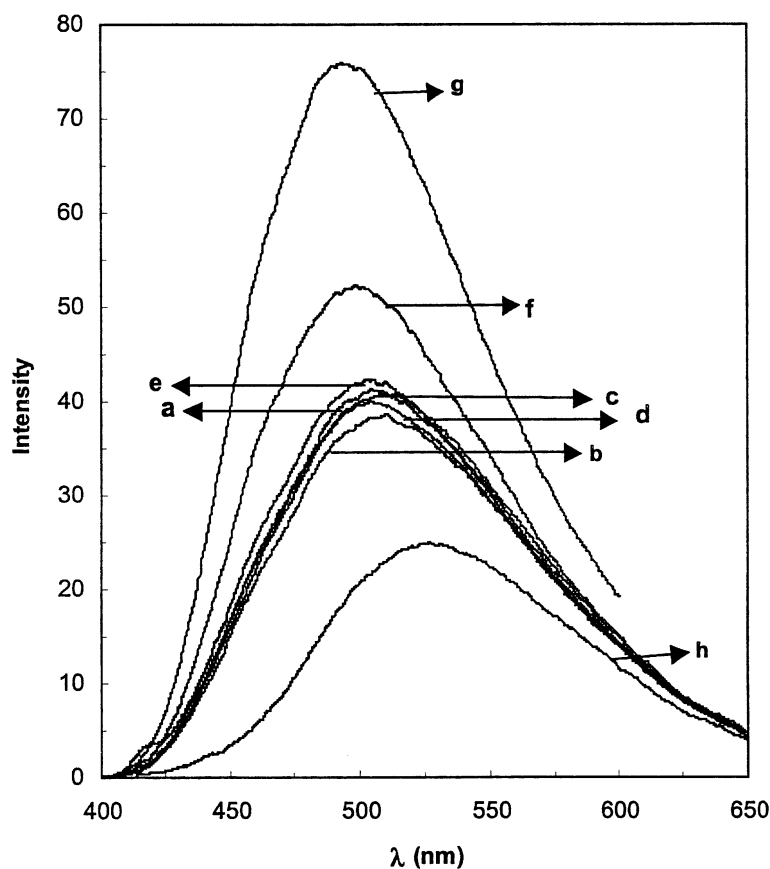


Fig. 6. ANS fluorescence spectra measured for free dye (h), in the presence of $5 \mu\text{M}$ Blg-A (a) and Blg-A ($5 \mu\text{M}$) incubated with 8.15 (b), 17.55 (c), 42 (d), 81.15 (e), 161.65 (f) and 475.5 μM (g) concentration of Cr^{+3} at 27°C . The solvent used is 50 mM sodium chloride solution, pH 7.

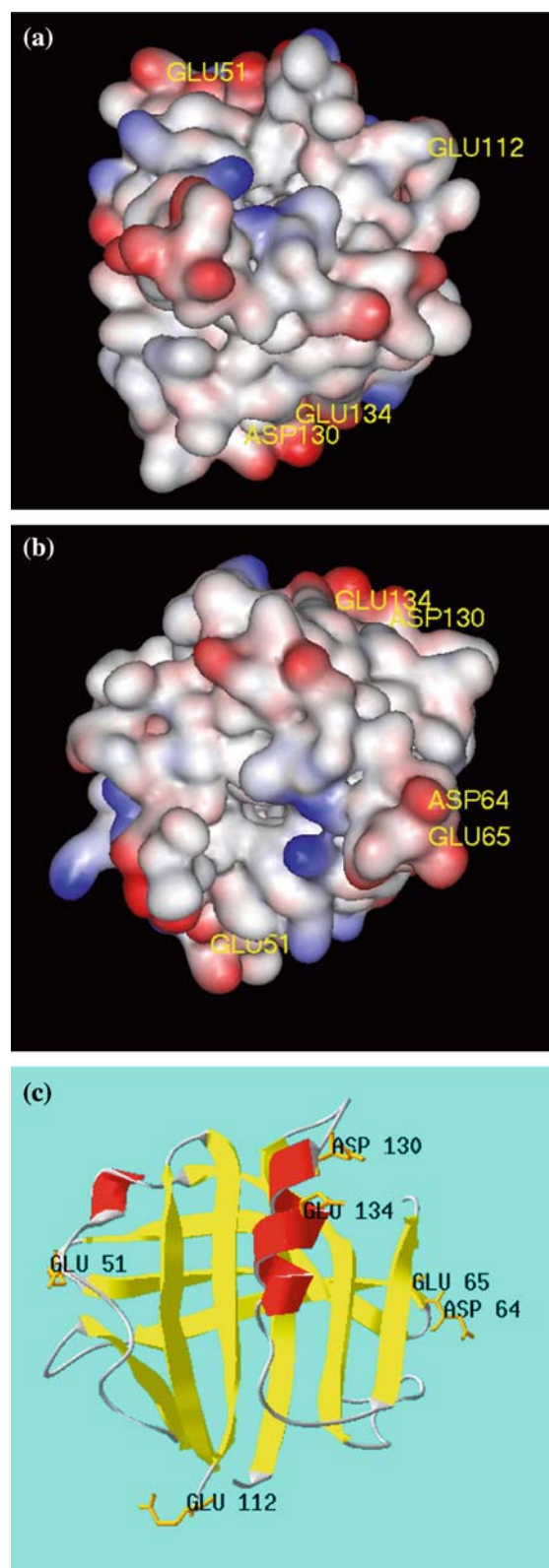


Fig. 7. Representations of the 3-D structure of the surface charge distribution of β lactoglobulin-A in space filling (a and b) and in ribbon models (c). The area with negative charge has been presented in red. The more solvent accessible negative charged amino acids on the surface of the protein (Glu 112, Asp 64, Glu 134, Glu 65, Asp130 and Glu 51) have been displayed. (This figure has been drawn by using WebLab ViewerPro Software 4.0 and Deep View-spdbv 3.7 Software downloaded from <www.expasy.org/spdbv> and PDB code: 1QG5).

Glu 65, Asp130 and Glu 51 with the average solvent accessible surface area of 93.9, 89.1, 72.9, 70.8, 70.2 and 69.2%, respectively. For other negative charged amino acids, the average solvent-accessible surface area is less than 60% (Fraczkiewicz and Braun, 1998). Cr^{+3} ions may be bound to these solvent accessible negative charged amino acids (Fig. 7). The intrinsic dissociation equilibrium constant and the molar enthalpy of binding are $124 \mu\text{M}$ and -17.8 kJ/mol , respectively. Far CD do not show any considerable change in the secondary structure of Blg-A upon binding of six chromium ions in the secondary structure of the protein. A blue shift in the λ_{max} of ANS and considerable increasing of ANS fluorescence intensity related to binding of fourth and fifth chromium ions show that chromium ions can induce partially unfolding on the tertiary structure of the protein.

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