

# A Novel method for Thermodynamic Study on the Binding of Milk Carrier protein of BLG-A with Cr<sup>+3</sup>

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Thermodynamics of the interaction between Cr<sup>3+</sup> with  $\beta$ -lactoglobulin type A (BLG-A) was investigated at pH 7.0 and 37°C by isothermal titration calorimetry. A new method to follow the effect of Cr<sup>3+</sup> on the stability of BLG-A was introduced. The new solvation model was used to reproduce the enthalpies of BLG-A + Cr<sup>3+</sup> interactions over the whole range of Cr<sup>3+</sup> concentrations. The solvation parameters recovered from the new equation are attributed to the structural change of BLG-A and its biological activity. The results obtained indicate that there is a set of two identical binding sites for Cr<sup>3+</sup> ions with positive cooperativity. The association equilibrium constants are 14.39 and 0.49 mM<sup>-1</sup> for the first and second binding site, respectively. The enthalpy of binding for one mole of Cr<sup>3+</sup> ion to one mole of the binding site on BLG-A ( $\Delta H = 104.60$  kJ mol<sup>-1</sup>) is obtained.

**Keywords:** BLG-A; Cr<sup>3+</sup>; isothermal titration calorimetry; binding parameters.

## INTRODUCTION

Whey protein products are important food ingredients because of their desirable functional properties such as gelation and emulsification. The major proteins in these whey protein products are  $\alpha$ -lactalbumin ( $\alpha$ -La; 20%) and  $\beta$ -lactoglobulin (BLG; 50%)<sup>1</sup>.  $\beta$ -Lactoglobulin is a small water-soluble protein that forms the major component of ruminant milk whey<sup>2</sup>. Seven different genetic variants have been identified but in industrial preparations the A and B variants are the most prevalent ones. Variant A (BLG-A) differs in amino acid sequence from variant B (BLG-B) at positions 64 (Asp<sub>A</sub>→Gly<sub>B</sub>) and 118 (Val<sub>A</sub>→Ala<sub>B</sub>)<sup>3-5</sup>.

BLG interacts strongly with various hydrophobic ligands such as fatty acids<sup>6-7</sup>, hemin<sup>8</sup>, ellipticine<sup>9</sup>, aromatic hydrocarbons<sup>10</sup>, and carcinogenic hydrocarbons<sup>11</sup>. This protein is one of the few proteins, which bind sodium ions. The maximum number of Na<sup>+</sup> bound per molecule of BLG (A and B) is four<sup>11</sup>. Studies show that Hg<sup>+2</sup> leads to the formation of an insoluble aggregate at high pH in BLG<sup>12</sup>.

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 a finite number of building blocks that make up their primary structures<sup>13</sup>.

Trace elements, including the essential and the toxic ones, play an important role in the life and environmental sciences; one of them is chromium<sup>14</sup>. Chromium is an important transition metal ion with diverse industrial applications and is an essential micronutrient required to promote the action of insulin in body tissues so that the body can use sugars, proteins and fats<sup>15</sup>. Two of the most important oxidation states of chromium are Cr<sup>+3</sup> and Cr<sup>+6</sup><sup>16</sup>. The major non-occupational source of chromium for humans is food such as vegetables, meat, urban air and cigarettes<sup>16</sup>. The

reduction of Cr<sup>+6</sup> to Cr<sup>+3</sup> results in the formation of reactive intermediates that contribute to the cytotoxicity, genotoxicity and carcinogenicity of Cr<sup>+6</sup>-containing compounds<sup>17</sup>. The formation of reactive intermediates that together with the oxidative stress and oxidative tissue damage and a cascade of cellular events, including the modulation of apoptosis regulatory gene P53, contribute to the cytotoxicity, genotoxicity and carcinogenicity of the Cr<sup>+6</sup>-containing compounds. Cr<sup>+6</sup> is carcinogenic and mutagenic to the biological system<sup>14-15</sup>. On the contrary, Cr<sup>+3</sup> salts such as chromium polynicotinate, chromium chloride and chromium picolinate are used as micronutrients and nutritional supplements and have been demonstrated to exhibit a significant number of health benefits in animals and humans<sup>17</sup>. 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 an ultra trace level<sup>15</sup>. The epidemiological studies show that the hexavalent Cr causes increased risk of bone, prostate, lymphomas, Hodgkin's, leukemia, stomach, genital, renal, and bladder cancer, reflecting the ability of hexavalent chromium to penetrate all tissues in the body<sup>18</sup>. However, the reactivity of Cr<sup>+3</sup> can be tuned by complexing the metal ion with an appropriate ligand, such as in the case of [Cr(salen) (H<sub>2</sub>O)] (ClO<sub>4</sub>). This complex, unlike other Cr<sup>+3</sup> complexes undergoes facile aqua ligand substitution due to the ground state structural distortion. Cr<sup>+3</sup> complexes in the proper ligand environment have been shown to induce chromosomal aberrations, mutagenicity in Salmonella typhimurium, cytotoxicity and genotoxicity in cell lines. Direct effects on protein and DNA have been shown in terms of the DNA-protein cross-linking, DNA scission, plasmid cleavage and protein cleavage<sup>14-19</sup>. The relative importance of the chromium ions and of the free oxidizing radicals that may generate in causing cancers and

allergic sensitization, remain to be elucidated. Thus, in this paper, thermodynamic investigations of the interaction between  $\text{Cr}^{3+}$  and BLG-A, the carrier model protein, at the physiologic temperature of  $37^\circ\text{C}$ , were performed with the help of ITC.

## MATERIALS

Bovine  $\beta$ -lactoglobulin (BLG-A) was obtained from Sigma Chemical Company (USA) and  $\text{Cr}^{3+}$  nitrate was purchased from Merck. All other materials and reagents were of analytical grades, and solutions were made in the double-distilled water. 50 mM NaCl solution was used as a solvent. The concentration of BLG-A was determined spectrophotometrically, using the value of  $17600 \text{ M}^{-1} \text{ cm}^{-1}$  for the molar absorption coefficient, at  $287 \text{ nm}^{20}$ .

## METHOD

The isothermal titration calorimetric experiments were carried out on a VP-ITC ultra sensitive titration calorimeter (MicroCal, LLC, Northampton, MA). The microcalorimeter consists of a reference cell and a sample cell of 1.8 mL in volume, with both cells insulated by an adiabatic shield. All solutions were thoroughly degassed before use by stirring under vacuum. The sample cell was loaded with BLG-A solution ( $27 \mu\text{M}$ ) and the reference cell contained buffer solution. The solution in the cell was stirred at 307 rpm by the syringe (equipped with a micro propeller) filled with the  $\text{Cr}^{3+}$  nitrate solution ( $30 \mu\text{M}$ ) to ensure rapid mixing. The injections were started after baseline stability had been achieved. The titration of protein with the  $\text{Cr}^{3+}$  nitrate involved 30 consecutive injections of the ligand solution, the first being  $3 \mu\text{l}$ , and the remaining ones of  $10 \mu\text{l}$ . In all the cases, each injection was done in 6 s at 3-min intervals. To correct for the thermal effects due to  $\text{Cr}^{3+}$  nitrate dilution, control experiments were done in which identical aliquots were injected into the buffer solution. In the ITC experiments, the enthalpy changes associated with the processes occurring at a constant temperature are measured. The measurements were performed at a constant temperature of  $37.0 \pm 0.02^\circ\text{C}$  and the temperature was controlled using a Poly-Science water bath.

## RESULTS AND DISCUSSION

We have shown previously that the enthalpies of the solute-solvent (BLG-A +  $\text{Cr}^{3+}$  in this case) interactions in the aqueous solvent ( $\text{Cr}^{3+}$  + water in the present case) system, can be accounted for quantitatively in terms of three factors: preferential solvation by the components of a mixed solvent, weakening or strengthening of the solvent-solvent bonds by the solute and the change in the enthalpy of the solute-solvent interactions<sup>21-27</sup>. This treatment leads to:

$$\Delta H = \Delta H_{\text{max}} x'_B - \delta_A^0 (x'_A L_A + x'_B L_B) - (\delta_B^0 - \delta_A^0) (x'_A L_A + x'_B L_B) x'_B \quad (1)$$

The parameters  $\delta_A^0 = (\alpha n + \beta N)_A^0$  and  $\delta_B^0 = (\alpha n + \beta N)_B^0$  are the indexes of the BLG-A stability as a result of the interaction with  $\text{Cr}^{3+}$  in the low and high  $\text{Cr}^{3+}$  concentrations respectively, with  $\alpha n$  resulting from the formation of a cavity wherein  $n$  solvent molecules become the nearest

neighbors of the solute and  $\beta N$  reflecting the enthalpy change from strengthening or weakening of the solvent-solvent bonds of  $N$  solvent molecules ( $N \geq n$ ) around the cavity ( $\beta < 0$  indicates a net strengthening of the solvent-solvent bonds). The constants  $\alpha$  and  $\beta$  represent the fraction of the enthalpy of water +  $\text{Cr}^{3+}$  interaction associated with the cavity formation or restructuring, respectively. Cooperative binding requires that the macromolecule has more than one binding site, since cooperativity results from the interactions between binding sites. If the binding of the ligand at one site increases the affinity for the ligand at another site, the macromolecule exhibits positive cooperativity. Conversely, if the binding of the ligand at one site lowers the affinity for the ligand at another site, the protein exhibits negative cooperativity. If the ligand binds at each site independently, the binding is non-cooperative.  $p < 1$  or  $p > 1$  indicate positive or negative cooperativity of macromolecule for binding with the ligand, respectively;  $p = 1$  indicates that the binding is non-cooperative.  $x'_B$  can be expressed as follows:

$$x'_B = \frac{p x_B}{x_A + p x_B} = \frac{v}{g} \quad (2)$$

$x_B$  is the fraction of the  $\text{Cr}^{3+}$  needed for the saturation of the binding sites, and  $x_A = 1 - x_B$  is the fraction of unbounded  $\text{Cr}^{3+}$ . Now the model is a simple mass action treatment, with metal ions replacing water molecules, at the binding sites in the present case. We can express  $x_B$  fractions, as the total  $\text{Cr}^{3+}$  concentrations divided by the maximum concentration of the  $\text{Cr}^{3+}$  upon the saturation of all BLG-A as follows:

$$x_B = \frac{[\text{Cr}^{3+}]_T}{[\text{Cr}^{3+}]_{\text{max}}} \quad x_A = 1 - x_B \quad (3)$$

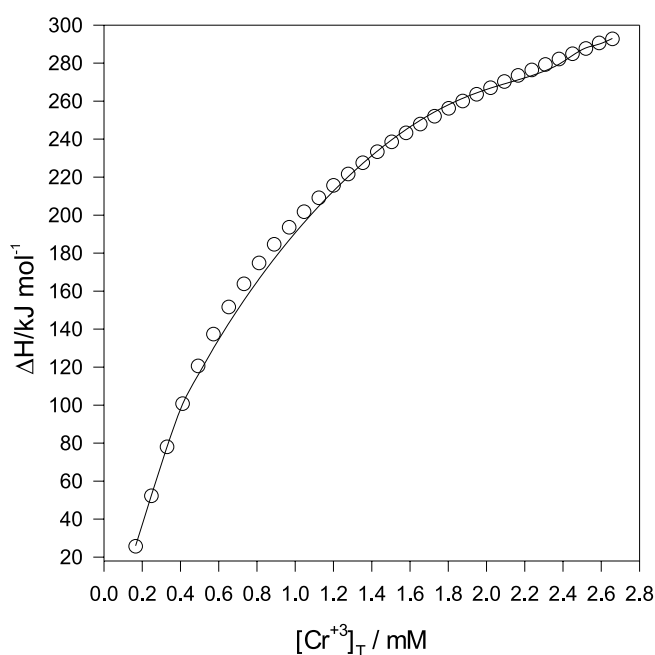
$[\text{Cr}^{3+}]_T$  is the total concentration of the surfactant and  $[\text{Cr}^{3+}]_{\text{max}}$  is the maximum concentration of the  $\text{Cr}^{3+}$  upon the saturation of all BLG-A. In general, there will be "g" sites for the binding of  $\text{Cr}^{3+}$  per BLG-A molecule and  $v$  is defined as the average moles of the bound  $\text{Cr}^{3+}$  per mole of the total BLG-A.  $L_A$  and  $L_B$  are the relative contributions of the unbounded and bounded  $\text{Cr}^{3+}$  to the enthalpies of dilution in with the exclusion of BLG-A and can be calculated from the enthalpies of the dilution of  $\text{Cr}^{3+}$  in the buffer,  $\Delta H_{\text{dilut}}$ , as follows:

$$L_A = \Delta H_{\text{dilut}} + x_B \left( \frac{\partial \Delta H_{\text{dilut}}}{\partial x_B} \right), \quad L_B = \Delta H_{\text{dilut}} - x_A \left( \frac{\partial \Delta H_{\text{dilut}}}{\partial x_B} \right) \quad (4)$$

The enthalpies of  $\text{Cr}^{3+}$ +BLG-A interactions,  $\Delta H$ , were fitted to Eq. 1 over the whole  $\text{Cr}^{3+}$  compositions. In the procedure the only adjustable parameter ( $p$ ) was changed until the best agreement between the experimental and calculated data was approached (Fig. 1). The  $\delta_A^0$  and  $\delta_B^0$  parameters have also been optimized to fit the data. The optimized  $\delta_A^0$  and  $\delta_B^0$  values are recovered from the coefficients of the second and third terms of Eq. 1. The small relative standard coefficient errors and the high  $r^2$  values (0.99999) support the method. The binding parameters for  $\text{Cr}^{3+}$ +BLG-A interactions recovered from Eq. 1 were listed in Table 3. The agreement between the calculated and the experimental results (Fig.1) is striking, and gives considerable support to the use of Eq. 1.

**Table 1.** Enthalpies of Cr<sup>3+</sup>+BLG-A interactions, ΔH, at 310 K. ΔH<sub>dilut</sub> are the enthalpies of dilution of Cr<sup>3+</sup> with water. Precision is ±0.002 kJ or better

[Cr <sup>3+</sup> ]/mM	ΔH/kJmol <sup>-1</sup>	ΔH <sub>dilut</sub> /kJmol <sup>-1</sup>
0.165	25.689	6.640
0.248	52.272	8.291
0.330	78.016	8.779
0.410	100.773	8.856
0.492	120.603	8.751
0.572	137.376	8.567
0.652	151.620	8.353
0.730	163.826	8.129
0.811	174.802	7.908
0.889	184.643	7.696
0.968	193.614	7.494
1.046	201.782	7.301
1.123	209.098	7.118
1.200	215.664	6.945
1.277	221.622	6.782
1.353	227.560	6.627
1.429	233.346	6.481
1.504	238.562	6.340
1.579	243.311	6.208
1.653	247.913	6.083
1.728	252.056	5.963
1.801	256.220	5.850
1.875	260.043	5.742
1.948	263.576	5.639
2.021	267.019	5.539
2.093	270.285	5.445
2.165	273.523	5.355
2.237	276.391	5.269
2.308	279.250	5.185
2.378	282.144	5.105
2.449	284.904	5.028
2.519	287.696	4.954
2.588	290.636	4.883
2.658	292.733	4.814

**Figure 1.** Comparison between the experimental enthalpies, ΔH, (O), for Cr<sup>3+</sup>+BLG-A interactions and calculated data (lines) via Eq. 6. [Cr<sup>3+</sup>]<sub>T</sub> is the total concentration of Cr<sup>3+</sup> solutions in mM

Φ is the fraction of the BLG-A molecule undergoing complexation with Cr<sup>3+</sup>, which can be expressed as follows:

$$\Phi = \frac{\Delta H}{\Delta H_{\max}} \quad (5)$$

ΔH<sub>max</sub> represents the heat value upon the saturation of all the BLG-A. The apparent association equilibrium constant, K<sub>a</sub>, as a function of Cr<sup>3+</sup> concentration can be calculated as follows:

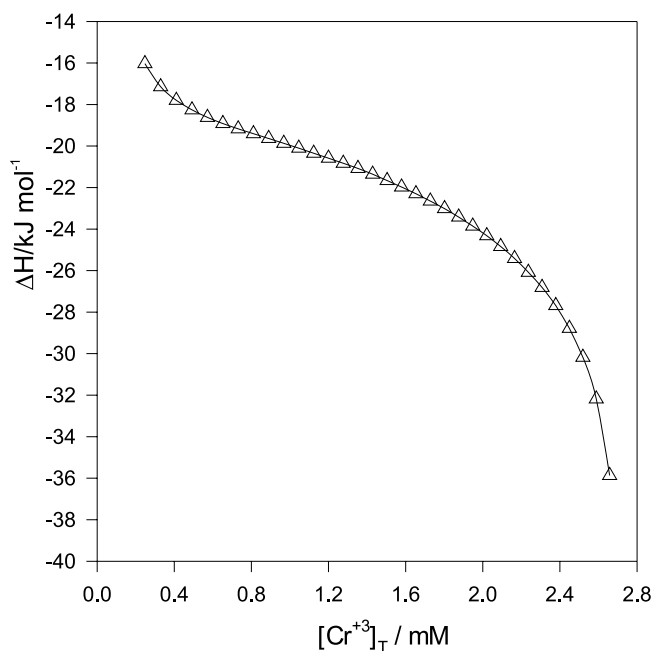
$$K_a = \frac{\Phi}{(1-\Phi)[Cr^{3+}]_f} \quad (6)$$

This is remarkable because the intrinsic association equilibrium constants all depend on the properties of the sites on the protein molecule, but the apparent equilibrium constant and the equilibrium composition do not. The effects of ionic strength, pH or ligand concentration on the unoccupied and the occupied binding site have to cancel in the calculation of the apparent equilibrium constant using the rate constants for the steps in the mechanism. It is possible to define the relationship between the apparent association equilibrium constant, K<sub>a</sub>, and the intrinsic association equilibrium constants, K<sub>i</sub>, for a successive replacement of the water molecules by Cr<sup>3+</sup> ions, as follows:

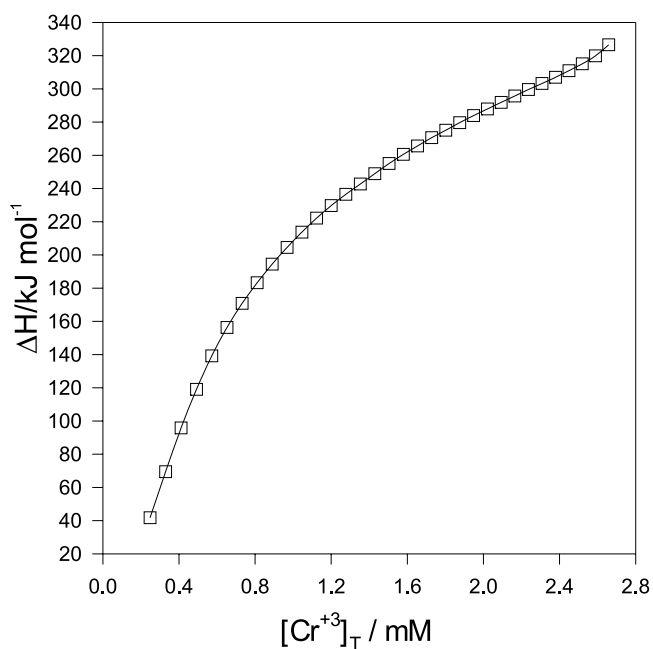
$$K_a = x_A^g - \sum_{i=1}^g K_i \frac{x_B^i}{x_A^{i-g}} \quad (7)$$

**Table 2.** The K<sub>a</sub> values for Cr<sup>3+</sup>+BLG-A interactions

[Cr <sup>3+</sup> ]/mM	K <sub>a</sub> /mM <sup>-1</sup>	v
0.1657	0.6229	0.3185
0.2479	0.9706	0.4531
0.3297	1.2587	0.5747
0.4110	1.5054	0.6849
0.4918	1.7327	0.7853
0.5722	1.9402	0.8771
0.6522	2.1357	0.9614
0.7317	2.3254	1.0391
0.8108	2.5286	1.1110
0.8895	2.7452	1.1776
0.9677	2.9826	1.2395
1.0456	3.2422	1.2972
1.1230	3.5194	1.3511
1.2000	3.8163	1.4016
1.2766	4.1391	1.4490
1.3528	4.5330	1.4936
1.4286	5.0027	1.5356
1.5040	5.5202	1.5752
1.5789	6.09861	1.6126
1.6535	6.7881	1.6480
1.7277	7.5636	1.6816
1.8016	8.5281	1.7135
1.8750	9.6588	1.7438
1.9481	11.0070	1.7727
2.0207	12.7019	1.8002
2.0930	14.8398	1.8264
2.1649	17.6825	1.8515
2.2365	21.3672	1.8754
2.3077	26.6583	1.8983
2.3785	34.8325	1.9203



**Figure 2.** Comparison between the experimental Gibbs energies values ( $\Delta$ ) for  $\text{Cr}^{+3}$ +BLG-A interactions and the calculated data (lines) via Eq. 8.  $[\text{Cr}^{+3}]_T$  is the total concentration of  $\text{Cr}^{+3}$  solutions in mM



**Figure 3.** Comparison between the experimental entropies,  $T\Delta S$ , ( $\square$ ) for  $\text{Cr}^{+3}$ +BLG-A interactions and the calculated data (lines) via Eq. 8.  $[\text{Cr}^{+3}]_T$  is the total concentration of  $\text{Cr}^{+3}$  solutions in mM

**Table 3.** Thermodynamic parameters for the  $\text{Cr}^{+3}$ +BLG-A interactions via Eqs. 1 and 7.  $p > 1$  indicates the overall positive cooperativity. In contrast  $K_2 < K_1$ , indicates negative specific cooperativity in the binding sites. As the  $p$  value shows the overall cooperativity, including the nonspecific interactions of the ligand (hydrophobic, hydrogen, and ionic interactions) with the polypeptide chain and the protein structural changes, we can attribute this inconsistency to the conformational changes of BLG-A

[BLG-A]	$p$	$\delta_A^\circ$	$\delta_B^\circ$	$K_1 / \text{mM}^{-1}$	$K_2 / \text{mM}^{-1}$
37 $\mu\text{M}$	2.59	-0.009	-0.020	14.396 $\pm$ 0.053	0.487 $\pm$ 0.121

The  $K_a$  values obtained from Eq. 6, have been fitted to Eq. 7 using a computer program for nonlinear least-square fitting. Therefore, we can approach the "g" value simply ( $g=2$  in this work). In fact,  $K_i$ 's are the equilibrium products for the individual equilibrium in the equilibria E1.  $\text{BLG} - A(\text{H}_2\text{O})_g + i\text{Cr}^{+3} \rightleftharpoons \text{BLG} - A(\text{H}_2\text{O})_{g-i}(\text{Cr}^{+3})_i + i\text{H}_2\text{O}$  (E1)

The  $\nu$  values can be calculated at any concentration of  $\text{Cr}^{+3}$  via Eq. 2. The Gibbs free energies as a function of  $\text{Cr}^{+3}$  concentrations can be obtained as follows:

$$\Delta G = -RT \ln K_a \quad (8)$$

Where  $K_a$  is the association equilibrium constant as a function of  $\text{Cr}^{+3}$  concentrations. Gibbs energies,  $\Delta G$ , calculated from Eq. 8 have been shown graphically in Fig. 2. The  $\Delta S$  values were calculated using the  $\Delta G$  values and have been shown in Fig. 3. Therefore, for the first time, we managed to calculate the  $\Delta S$  and  $\Delta G$  values while using one set of experimental data at one temperature. The binding parameters for BLG-A+  $\text{Cr}^{+3}$  interactions using the new model are listed in Table 3.

Eqs. 6 and 7 allow us to have the  $K_i$ 's values with the least standard deviations and correlation coefficients are so close to one. The low  $K_a$  values in the low  $\text{Cr}^{+3}$  concentrations reflect lower affinity of BLG-A for  $\text{Cr}^{+3}$  in this domain (Table 2). The positive Gibbs free energies in the low  $\text{Cr}^{+3}$  concentrations (Fig. 2) also indicate the lower affinity in this region.  $p > 1$  (Table 3) for BLG-A+  $\text{Cr}^{+3}$  interaction at 37°C, indicates overall positive

cooperativity. In contrast  $K_2 < K_1$ , indicates negative specific cooperativity in the binding sites. As  $p$  values recovered from Eq. 3 show the overall cooperativity, including the nonspecific interactions of ligand (hydrophobic, hydrogen, and ionic interactions) with the polypeptide chain and the protein structural changes, we can attribute this inconsistency to the conformational changes of BLG-A.

A nonpolar residue dissolved in water induces a solvation shell in which water molecules are highly ordered. When two nonpolar groups come together on the folding of a polypeptide chain, the surface area exposed to the solvent is reduced and part of the highly ordered water in the solvation shell is released to bulk solvent. Therefore, nonpolar moieties come together in aqueous solvent, resulting in formation of multimers and, in extreme cases, aggregation and precipitation. The most common mechanism of protein aggregation is believed to involve protein denaturation, via hydrophobic interfaces and often results in loss of biological activity. It is possible to introduce a correlation between the change in  $\delta_A^\circ$  and the increase in the stability of proteins. The  $\delta_A^\circ$  value reflects the hydrophobic property of BLG-A, leading to the enhancement of water structure. The greater the extent of this enhancement, the greater the stabilization of the BLG-A structure and the greater the value of  $\delta_A^\circ$ . The  $\delta_A^\circ$  value (Table 3) for the BLG-A+  $\text{Cr}^{+3}$  interaction is negative and small (-0.0009), indicating that small amounts of  $\text{Cr}^{+3}$  destabilizes the BLG-A structure.

At high  $\text{Cr}^{+3}$  concentrations, the  $\delta_B^0$  value is more negative (-0.002), indicates that the BLG-A structure is more destabilized in high  $\text{Cr}^{+3}$  concentration. The  $p$  value (2.85) indicates that there is positive cooperativity in two binding sites of BLG-A.

Previous studies have represented that there are three potential binding sites for ligand binding to  $\beta$ -LG: the internal cavity of the  $\beta$ -barrel, the surface hydrophobic pocket in a groove between the  $\alpha$ -helix and the  $\beta$ -barrel, and the outer surface near Trp19-Arg124. For example, polar aromatic compounds, such as p-nitrophenyl phosphate, 5-fluorocytosine, ellipticine, and protoporphyrin, bind to this outer surface site<sup>28</sup>. Various interactions, such as hydrophobic interaction, hydrogen bonding, and electrostatic interaction may occur between  $\beta$ -LG and the ligands<sup>4</sup>. These interactions are also the driving force for the structural transition of proteins<sup>28</sup>. Hydrophobic and amphiphilic ligands could therefore affect the native structure of  $\beta$ -LG. Cationic surfactants such as dodecyltrimethylammonium chloride and didodecyltrimethylammonium bromide and anionic phospholipids such as dimyristoylphosphatidylglycerol have been reported to loosen the tertiary structure and bring about the transition from the  $\beta$ -sheet to  $\alpha$ -helix in the secondary structure of  $\beta$ -LG<sup>28</sup> that is in good agreement with our results that show the destabilization of BLG-A structure in the presence of  $\text{Cr}^{3+}$  ions, as predicted by the negative values of  $\delta_A^0$  and  $\delta_B^0$  (Table 3).

Regarding genotoxic, mutagenic, and cytotoxic effects of chromium exposure and rapid absorption of them via the gastrointestinal and respiratory tracts and the skin, it seems that the present results obtained from the interaction of the novel chromium ion with whey carrier protein probably provide useful information to design better metal anticancer compounds in the future.

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