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Intrinsic Fluorescence Studies of Metal Ion Binding Effects on the Thermodynamic Stability of Human α-Lactalbumin

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Abstract The stability of the protein human α-lactalbumin (HLA) after binding a series of metal cations (Mg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Sr^{2+}, Ca^{2+}, Na^{+} and K^{+}) has been examined and compared with that of α-lactalbumin from other species by monitoring the fluorescence of tryptophan residues upon thermal-induced denaturation. The melting temperature (T_m) was determined from the wavelength shift in λ_{max} data as well as the fluorescence intensity data as the protein unfolds. Mathematical expressions for determining thermodynamic parameters (ΔH, ΔG and ΔS) were introduced based on the assumption that the thermal denaturing process was a simple two-state model between the folded state and the unfolded state. These newly developed expressions are especially useful because they allow one to easily calculate the thermodynamic parameters at every temperature as long as the fitting parameters are known. The thermal-induced unfolding experiments revealed that the binding of metal ions to apo-α-lactalbumin increased its stability, but the degree of stabilization varied significantly for each metal ion. From the λ_{max} data and the fluorescence intensity data, the melting temperatures ranged from 28.15℃ to 63.59℃ and from 26.12℃ to 64.42℃, respectively. At T_m, ΔH and ΔS were determined to range from 129.19 kJ/mol to 273.69 kJ/mol and from 0.40 kJ/mol·K to 0.81 kJ/mol·K, respectively. At physiological temperature (37℃), ΔH and ΔS were determined to range from 121.84 kJ/mol to 238.65 kJ/mol and from 0.38 kJ/mol·K to 0.68 kJ/mol·K, respectively. At T_m, ΔG was 0 as expected, but it ranged from -5.29 kJ/mol to 19.91 kJ/mol at 37℃. Overall, the monovalent cations Na^{+} and K^{+} were found to destabilize HLA whereas the divalent cations Mg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Sr^{2+} and Ca^{2+} were found to stabilize HLA. As α-lactalbumin is a Ca^{2+}-binding protein, Ca^{2+} was found to have the greatest effect on the protein stability with the T_m value of 63.59℃.

Keywords Protein Folding, Metalloprotein, Thermal Denaturation, Fluorescence Spectroscopy

1. Introduction

α-lactalbumin is an acidic, globular protein that is produced in mammary glands during lactogenesis[1]. With a molecular weight of 14 kD and an isoelectric point of 4-5, it is made up of 123 amino acid residues except for variants in rabbit and rat, which contain 122 and 140 residues, respectively[2]. Its native structure is made up of two lobes: the α-domain (residues 1-34 and 86-123) consisting of four α–helices and two short 3_{10}-helices, and the β-domain (residues 35-85) consisting of a triple-stranded antiparallel β-sheet, one 3_{10}-helix and a series of loops[1]. One of the most interesting characteristics of α-lactalbumin is its ability to bind metal cations. It possesses a single distinct Ca^{2+} high affinity binding site which also binds other physiologically important metal cations including Mg^{2+}, Mn^{2+}, Na^{+} and K^{+}, and several distinct Zn^{2+} binding sites[3]. The binding of metal ions increases its stability against the action of heat and various denaturing factors such as urea and guanidine hydrochloride and has been extensively studied[4-9] in bovine and goat α-lactalbumin (BLA and GLA). From these studies, it was found that a series of divalent metal ions including Cd^{2+}, Sr^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+} and monovalent metal ions such as Na^{+} and K^{+} influence the conformation, stability and activity of α- lactalbumin upon binding, but the degree of stabilization varied for each metal cation. It is therefore reasonable to study the ability of human α-lactalbumin (HLA) to bind these metal ions.

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2. Materials and Methods

All chemicals were purchased from Sigma-Aldrich. All solutions were made using deionized water. Human α-lactalbumin in a lyophilized form with 95% purity was used. The buffer solution was prepared from Tris-HCl. For melting experiments of each metal ion, ultrapure trace metal

basis anhydrous $CaCl_2$, $ZnCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, $CdCl_2$ and $SrCl_2$ with purity of 99.99% or higher were used and stored in a dessicator. For pH adjustment, 0.1N HCl and 0.1N NaOH standards were used.

Only plastic labware was used for sample preparation and storage except for a graduated cylinder and a dialysis beaker which were treated with trace metal grade nitric acid (68~70%) to remove any trace contaminants prior to use. For demetallation procedures, ethylenediaminetetraacetic acid (EDTA) and Chelex-100 chelating ion exchange resin were used.

2.1. Buffer Solutions

Deionized water was first treated with Chelex-100 chelating ion exchange resin prior to use to remove any trace metal ions or impurities present. Chelex-100 was applied using a batch method where the addition of resin was directly made into the sample followed by stirring. This method was found to be extremely useful when the amount of sample was small. Approximately 5 grams of resin was used for every 100 mL of sample to be chelated. The mixture was then periodically swirled for about an hour, and the sample was carefully decanted out from the resin into a brand-new plastic container.

This treated water was then used for preparing 20 mM Tris-HCl buffer solution. In order to simulate physiological conditions, the pH was adjusted to approximately 7.5 by titrating with 0.1N HCl. An Accumet Basic (AB15 PLUS) pH meter manufactured by Fisher Scientific was utilized for the pH adjustment.

2.2. HLA Solutions/Demetallation

A 19.8 μM human α-lactalbumin (HLA) solution in 20 mM Tris-HCl buffer was prepared. Calcium metal ions bound to HLA were removed to obtain the apo form by mixing with 1 mM EDTA solution in a 1:1 volume ratio. All the chelated metal ions present in the mixture were separated by dialyzing against 20 mM Tris-HCl buffer solution. The dialysis device used for the separation process was Float-A-Lyzer G2 by Spectrum Laboratories. Its capacity was 5 mL for each dialysis run and had a 3.5-5 kD MWCO. Approximately 300 mL of the dialysate was used for each 5 mL of HLA / EDTA solution. For best results, the dialysate was replaced with a fresh batch of the same buffer solution three times over the course of a day and then left overnight at room temperature on a stir plate at 350 rpm using a StableTemp stirring hot plate manufactured by Cole-Parmer. After the dialysis was finished, apo-HLA solution that was left in the dialysis bag was transferred to multiple 0.5 mL Eppendorf tubes in aliquots of 0.26 mL in order to minimize the number of freeze-thaw cycles. After each dialysis, apo-HLA was confirmed and characterized by fluorescence spectroscopy as shown in Figure 1. The solid spectrum (blue) is apo-HLA and the dashed spectrum (red) is Ca^{2+}-saturated HLA. The results are in agreement with those obtained using BLA by Murakami *et al.*[3].

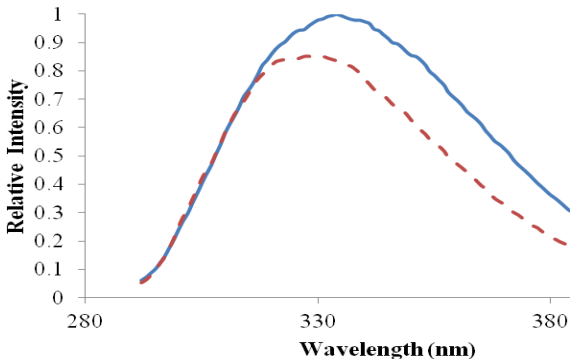

Figure 1. Intrinsic fluorescence spectra for characterization of apo-HLA (solid blue line) and Ca^{2+}-saturated HLA (dashed red line)

2.3. Metal Chloride Solutions

1 mM solutions of $MgCl_2$, $ZnCl_2$, $CdCl_2$, $CoCl_2$, $MnCl_2$, $SrCl_2$, $CaCl_2$, NaCl and KCl were each prepared in 20 mM Tris-HCl buffer.

2.4. Fluorescence Measurements

Fluorescence measurements were carried out using a Varian Eclipse fluorescence spectrophotometer. The spectra were recorded using a 10 mm by 10 mm semi-micro cuvette with an excitation wavelength of 280 nm and an emission wavelength ranging between 290 nm and 400 nm. Both the excitation and emission slit widths were set to 5 mm, and the voltage was adjusted to 700V. The scan rate and the signal averaging time during data collection were set to be medium (600 nm/min) and 0.1 second, respectively. The data interval was set to 1.0 nm. Under these conditions, the fluorescence intensity and noise-to-signal ratio were optimized. Furthermore, the Savitzky-Golay smoothing algorithm (using 5 points) was employed in order to obtain additional noise reduction with sharp peaks.

A single-cell Peltier block was thermostated by circulating water from an external water bath (PCB 150 water bath). Fluorescence measurements were carried out at predetermined fixed temperatures depending on the experiment. The rate of the cuvette heating was timed using a stopwatch at approximately 1℃ per minute. For fluorescence measurements, 500 μL of sample (250 μL of a metal chloride solution and 250 μL of apo-HLA) was used. For fluorescence measurements of apo-HLA, a solution containing 250 μL of the Tris-HCl buffer solution and 250 μL of apo-HLA was used. Prior to each scan, the spectrum was zeroed. For every newly prepared sample, the cuvette was thoroughly washed with the concentrated cuvette cleaner and was dried with compressed air. Non-linear regression analysis and curve-fitting were performed using Microcal Origin 8.0.

3. Results

Thermal denaturation was monitored via the wavelength shift in intrinsic tryptophan fluorescence for apo-HLA as well as for apo-HLA with a saturating amount of different

metal ions (Mg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Sr^{2+}, Ca^{2+}, Na^{+} and K^{+}). The temperature range was from 5℃ to 65℃-85℃, and the same sample was scanned four times separately but successively in order to confirm that the thermal equilibrium was reached at each temperature. The results of this experiment for apo-HLA are shown in Figure 2, and for the addition of nine different metal ions in Figure 3.

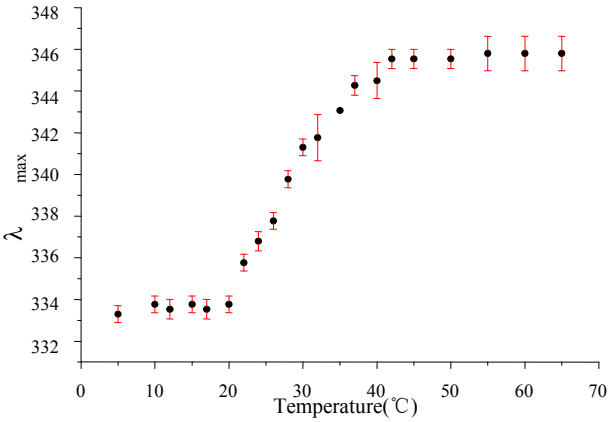

Figure 2. Temperature dependence of tryptophan λ_{max} for 0.5 mL of 5 μM apo-HLA

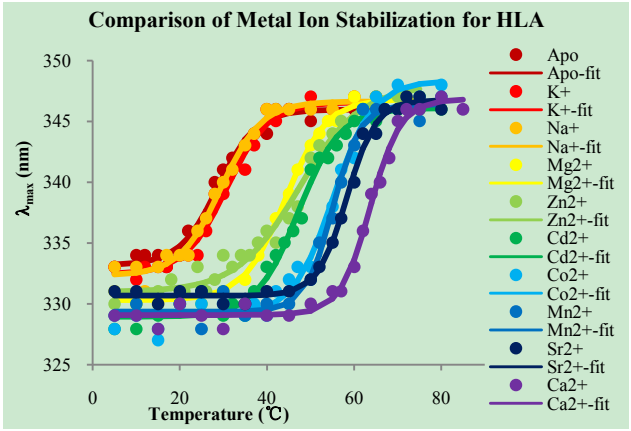

Figure 3. Temperature dependence of tryptophan λ_{max} for 0.25 mL of 10 μM apo-HLA + 0.25 mL of 1 mM metal chloride solutions

As an alternative, the intensity data as a function of temperature could be used to find the melting temperature (Section 3.2). Assuming a two-state model (folded state ⇌ unfolded state), the midpoint of the transition between the folded and the unfolded forms represents the melting temperature (T_m) of the protein. At this melting temperature, there is a 1:1 mixture of folded molecules and unfolded molecules. The melting temperature can be found by fitting the data into the dose-response equation. Since the protein denaturation processes produced a sigmoidal curve, the following mathematical dose-response relationship was used for the non-linear curve fitting of the data from fluorescence measurements:

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{(\mathrm{Log}\, x_o - x)\cdot p}} \quad (1)$$

A_1 and A_2 represent the bottom asymptote (folded state) and top asymptote (unfolded state), respectively, $\mathrm{Log}x_o$ is the parameter for the melting temperature (T_m) which is at the middle of the transition, and p is the Hill coefficient. Using this dose-response equation, expressions for the thermodynamic parameters (ΔH, ΔG and ΔS) of the conformation change upon thermal denaturation were derived (see Section 3.1). The results of this derivation are summarized in Table 1.

Table 1. Summary of mathematical expressions for derived thermodynamic parameters

Thermodynamic Parameter	Mathematical Expression
ΔH (J/mol)	$\Delta H = 2.303RT^2p$
ΔG (J/mol)	$\Delta G = 2.303RTp\ (T_m\text{-}T)$
ΔS (J/mol*K)	$\Delta S = 2.303Rp\ (2T_m\text{-}T)$

A summary of thermodynamic parameters (average melting temperature (T_m), ΔH, ΔG and ΔS) determined from each experiment is presented in Tables 2 and 3. The calculations for ΔH, ΔG and ΔS were performed at 37℃.

Table 2. Experimentally determined melting temperatures for apo-HLA and metal-ion bound HLA

Experiment	T_m (℃) – λ_{max} data	T_m (℃) – intensity data
Apo-HLA	28.15 ± 0.58	26.12
$MgCl_2$ + apo-HLA	44.31 ± 0.41	42.21
$ZnCl_2$ + apo-HLA	46.25 ± 0.61	47.55
$CdCl_2$ + apo-HLA	47.33 ± 0.17	51.25
$CoCl_2$ + apo-HLA	54.96 ± 0.38	56.31
$MnCl_2$ + apo-HLA	55.33 ± 0.15	55.84
$SrCl_2$ + apo-HLA	57.93 ± 0.28	57.30
$CaCl_2$ + apo-HLA	63.59 ± 0.33	64.42
NaCl + apo-HLA	28.99 ± 0.29	26.88
KCl + apo-HLA	29.75 ± 0.69	26.82

Table 3. Summary of thermodynamic parameters for apo-HLA and metal-ion bound HLA at 37℃

Experiment	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (kJ/mol*K)
Apo-HLA	184.92 ± 20.78	-5.29 ± 0.81	0.61 ± 0.07
$MgCl_2$ + apo-HLA	174.05 ± 5.72	4.10 ± 0.17	0.55 ± 0.02
$ZnCl_2$ + apo-HLA	121.84 ± 6.02	3.62 ± 0.13	0.38 ± 0.02
$CdCl_2$ + apo-HLA	181.79 ± 11.56	6.06 ± 0.46	0.57 ± 0.04
$CoCl_2$ + apo-HLA	184.69 ± 10.47	10.68 ± 0.44	0.56 ± 0.03
$MnCl_2$ + apo-HLA	238.65 ± 15.22	14.10 ± 0.88	0.72 ± 0.05
$SrCl_2$ + apo-HLA	223.41 ± 10.61	15.08 ± 0.91	0.67 ± 0.03
$CaCl_2$ + apo-HLA	232.16 ± 14.87	19.91 ± 1.33	0.68 ± 0.04
NaCl + apo-HLA	162.17 ± 6.27	-4.19 ± 0.18	0.54 ± 0.02
KCl + apo-HLA	154.02 ± 8.73	-3.58 ± 0.24	0.51 ± 0.03

3.1. Derivation of Thermodynamic Expressions for ΔH, ΔG and ΔS Using Dose-Response Curve Equation

Using the does-response equation discussed in the communication, the mathematical expressions for the thermodynamic parameters (ΔH, ΔG and ΔS) of the conformation change upon thermal denaturation were derived. In this derivation, A represents the folded state and B represents the unfolded state. Starting with the general dose-response equation where y is the λ_{max} or fluorescence intensity and x is the temperature in Kelvin:

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{(\mathrm{Log}\, x_o - x)\cdot p}} \quad (1)$$

If a two-state model (A ⇌ B) is assumed, the expression for the equilibrium constant can be written as follows:

$$K = \frac{B}{A} \quad (2)$$

The variables were normalized to yield the fraction (f) of unfolded protein using the following relationship,

$$f = \frac{y - y_{min}}{y_{max} - y_{min}} \quad (3)$$

where y_{min} and y_{max} represent the bottom asymptote and top asymptote of either λ_{max} or fluorescence intensity curve, respectively. Substituting y into the fraction expression and simplifying,

$$f = \frac{A_1 + \frac{A_2 - A_1}{1 + 10^{(T_m - T)\cdot p}} - A_1}{A_2 - A_1} = \frac{1}{1 + 10^{(T_m - T)\cdot p}} \quad (4)$$

The concentrations of A and B can be expressed in terms of the fraction and the total concentration C which remains constant.

$$A = (1 - f) \cdot C \quad (5)$$

$$B = f \cdot C \quad (6)$$

Substituting (5) and (6) into (4) and rearranging,

$$K = \frac{f \cdot C}{(1-f)\cdot C} = \frac{f}{1-f} = \frac{\frac{1}{1+10^{(T_m - T)\cdot p}}}{1 - \frac{1}{1+10^{(T_m - T)\cdot p}}} = \frac{\frac{1}{1+10^{(T_m - T)\cdot p}}}{\frac{10^{(T_m - T)\cdot p}}{1+10^{(T_m - T)\cdot p}}} =$$

$$\frac{1}{10^{(T_m - T)\cdot p}} = 10^{(T - T_m)\cdot p} \quad (7)$$

Taking logarithms on both sides,

$$\log K = (T - T_m) \cdot p \quad (8)$$

Knowing log K = ln K / ln 10 where ln 10 ≈ 2.303,

$$\mathbf{\ln K = 2.303 \cdot (T - T_m) \cdot p} \quad (9)$$

Differentiating with respect to T,

$$\frac{d\ln K}{dT} = 2.303 \cdot p \quad (10)$$

The differential form of the van't Hoff equation is,

$$\frac{d\ln K}{d\left(\frac{1}{T}\right)} = -T^2 \cdot \frac{d\ln K}{dT} = -\frac{\Delta H}{R} \quad (11)$$

Using (10), an expression for ΔH can be obtained by substituting and rearranging.

$$\Delta H = 2.303 \cdot R \cdot T^2 \cdot p \quad (12)$$

At equilibrium,

$$\mathbf{\Delta G = -R \cdot T \cdot \ln K} \quad (13)$$

By substituting the lnK expression from (9), an expression for ΔG can be obtained.

$$\mathbf{\Delta G = 2.303 \cdot R \cdot p \cdot (T \cdot T_m - T^2)} \quad (14)$$

Since ΔG = ΔH - T ΔS , an expression for ΔS can be developed.

$$\Delta S = \frac{\Delta H - \Delta G}{T} = \frac{2.303 \cdot R \cdot T^2 \cdot p - [2.303 \cdot R \cdot p \cdot (T \cdot T_m - T^2)]}{T} \quad (15)$$

which simplifies to

$$\mathbf{\Delta S = 2.303 \cdot R \cdot p \cdot (2 \cdot T - T_m)} \quad (16)$$

3.2. Determination of Melting Temperature Using Fluorescence Intensities

The following calculation was performed on apo-HLA + $ZnCl_2$ experimental data (Figure 4) to show that it is possible to determine the transition temperature (T_m) using the fluorescence intensity data instead of the λ_{max} data. The minimum fluorescence intensity was 105.1 a.u. at 40℃, and the maximum fluorescence intensity was 130.1 a.u. at 55℃ as shown in Figure 5. This indicates that the thermal transition takes place somewhere between these two temperature points. The top and bottom asymptotes can be generated by extending the local maximum towards the higher temperatures and by extending the local minimum towards the lower temperatures. A sigmoidal-shaped plot can then be produced, and a non-linear regression was then fitted to the plot using dose-response relationship as shown in Figure 5.

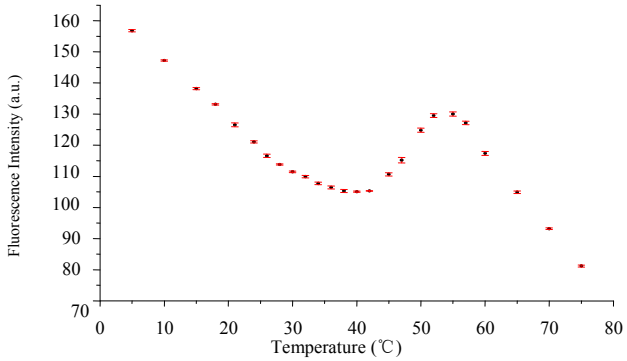

Figure 4. Sigmoidal transition curve of fluorescence intensity for Zn^{2+}-bound HLA

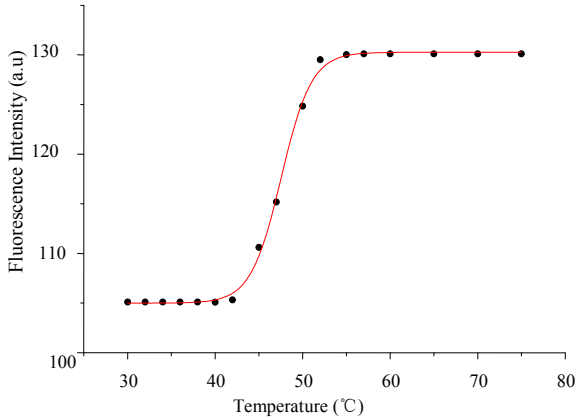

Figure 5. Sigmoidal transition curve of fluorescence intensity after data truncation

This shows an almost perfect sigmoidal transition with R^2 value of 0.998. Based on the fitting parameters generated by Origin data analysis software, the T_m was determined to be 47.55℃. The average of the T_m calculated from the λ_{max} data of two apo-HLA + $ZnCl_2$ experiments was 46.25℃ ± 0.61℃. These two values are almost the same, and it is reasonable to suggest that this method of calculating the T_m value is correct (Table 2).

4. Discussion

The substantial changes in the tryptophan fluorescence spectrum that occurred upon the removal of Ca^{2+} from human α-lactalbumin (HLA) suggested that a major change in molecular conformation took place. It was also found by Kronman, *et al.*[7] that the conformational change resulting from the removal of Ca^{2+} was reversible as the addition of Ca^{2+} to the apo-bovine α-lactalbumin (BLA) produced a

return to an emission spectrum indistinguishable from that of the native protein (i.e. Ca^{2+} saturated BLA). Considering the fact that the amino acid sequence of HLA differs from that of BLA and GLA only by a few amino acid residues, similar results were expected to be produced from HLA. The profound difference in the tryptophan emission spectra (blue shift of a few nanometers in λ_{max} values upon metal ion binding) for apo-HLA and the metal-bound as shown in Figure 1 allowed us to investigate the binding properties of HLA to various metal ions.

The thermal stability of HLA in its apo form and in its metal-bound form can be determined by monitoring the wavelength at which the tryptophan fluorescence reaches a maximum (λ_{max}) as well as by monitoring the change in fluorescence intensity at a given wavelength. From the thermal denaturation experiments, it has clearly been indicated that the different metal ions (especially the divalent metal ions) stabilize HLA in a different way with the transition temperatures ranging from 28.15 to 63.59℃. Upon heating, all the experiments performed with a different metal ion showed sigmoidal behavior, characteristic for a simple two-state transition with an increase in wavelength from 329 nm to 347.5 nm for divalent cation-bound HLA, from 332 nm to 346 nm for monovalent cation-bound HLA and from 333.5 nm to 346 nm for apo-HLA (Figure 3). The position of the shift in λ_{max} depended strongly upon the metal ion bound to the protein. All the divalent metal ions seemed to stabilize HLA because the T_m was significantly greater than that of apo-HLA.

Table 4. Comparison of T_m between HLA and GLA[4], as measured by fluorescence

	Average T_m (℃)	
Experiment	HLA	GLA [4]
Ca^{2+}	63.59 ± 0.33	66.71 ± 0.19
Sr^{2+}	57.93 ± 0.28	59.78 ± 0.17
Mg^{2+}	44.31 ± 0.41	55.68 ± 0.23
Mn^{2+}	55.33 ± 0.15	54.15 ± 0.15
Co^{2+}	54.96 ± 0.38	47.34 ± 0.16
Cd^{2+}	47.33 ± 0.17	47.14 ± 0.30
Zn^{2+}	46.25 ± 0.61	35.15 ± 0.48
Apo	28.15 ± 0.58	31.30 ± 0.33

As presented in Table 4, the melting temperatures obtained from this study on HLA have been compared to those obtained from the study done by Van Dael *et al*.[4] on GLA, which did not include experiments with monovalent cations. It is interesting to observe that some values of HLA are in good agreement with the corresponding values of GLA. There is a significant discrepancy in T_m between GLA and HLA for Mg^{2+}, Co^{2+} and Zn^{2+} metal ions. With respect to the apo-form, Mg^{2+} stabilizes GLA more than it does HLA whereas Co^{2+} and Zn^{2+} confer more stability to HLA than to GLA. As expected, for both studies, Ca^{2+} produced the largest T_m (63.59℃) whereas apo-HLA had the smallest T_m (28.15℃). This means that the Ca^{2+}/HLA complex is most stable one formed, and apo-HLA is relatively unstable. The results from the binding of the monovalent cations, Na^+ and K^+ were somewhat inconclusive as the T_m values were very close, and not significantly greater than that of apo-HLA (28.99℃ for Na^+ and 29.75℃ for K^+). Taking the thermodynamic parameters into consideration, it is difficult to conclude that Na^+ or K^+ stabilized apo-HLA just because the T_m was greater by about 1℃.

According to our data analysis, it is possible to explain how close the biological systems are to equilibrium (*i.e.* ΔG approaching zero). If ΔG is positive, the system favors the folded state, whereas if ΔG is negative, the system is likely in the unfolded state. Moreover, the greater the ΔG is, the greater the probability of finding the system in the folded state. It follows that either state is equally likely when ΔG is zero (at T_m), and the system spends 50% of its time in either state at equilibrium. Based on the analysis of the values of ΔG at 37℃, it can be concluded that Na^+ and K^+ would destabilize apo-HLA and are likely in the unfolded state. The complexes formed with the divalent metal ions would stabilize apo-HLA, and are likely to favor the folded state. The Ca^{2+}-bound protein had the greatest value of ΔG implying that the Ca^{2+}/apo-HLA complex is very stable, and it is likely that the system would be in the folded state. This conclusion is in good agreement with the T_m results.

In Table 5, the thermodynamic parameters (ΔH, ΔG and ΔS) obtained from this study on HLA have been compared to those obtained from the study done using the intrinsic fluorescence technique by Permyakov *et al*.[8] on BLA which examined the binding properties of Ca^{2+}, Mg^{2+}, Na^+ and K^+. It is important to note that for HLA, the thermodynamic parameters were calculated based on the mathematical analysis that was derived in Section 3.1, whereas for BLA, they were determined based on graphical analysis of the van't Hoff equation (see equation 11), which relates the change in temperature to the change in the equilibrium constant and allows one determine the thermodynamic parameters graphically. As shown in Table 5, the results for HLA are comparable to those for BLA, but there are several interesting differences that should be pointed out. Ca^{2+} produced the highest T_m in HLA. This implies that the degree of stabilization by Ca^{2+} binding is the greatest, and thus, it produced the largest ΔH. However, that is not the case in BLA. Although the T_m was found to be greatest for Ca^{2+} binding, ΔH was not necessarily the largest. The ΔH of Mg^{2+} binding, which produced a T_m of 45℃, was almost 20% larger than that of Ca^{2+}. This might suggest a different binding mechanism. In addition, the ΔH of the apo form was found to be greater than that of Na^+ and K^+ in HLA whereas the opposite results were observed in BLA. It means that it would require less energy to unfold Na^+/apo-HLA or K^+/apo-HLA complex than the apo-HLA itself. Therefore, these results can suggest that Na^+ and K^+ destabilize HLA whereas they stabilize BLA. However, the fact that the T_m value of Na^+ or K^+ -bound HLA was higher (though only by 1℃) than that of apo-HLA was unexplainable as mentioned before. Taking the errors into account, the ΔS values from both studies were also comparable.

Table 5. Comparison of T_m and thermodynamic parameters between HLA and BLA, as measured by fluorescence[8] and circular dichroism[5]

	HLA			BLA		
Experiment	T_m (℃)	ΔH (kJ/mol)	ΔS (kJ/mol*K)	T_m (℃)	ΔH (kJ/mol)	ΔS (kJ/mol*K)
Ca^{2+}	63.59 ± 0.33	273.69 ± 17.62	0.81 ± 0.05	62 ± 1 [8] 58.57 ± 0.12 [5]	200.83 ± 21 [8] 226.3 ± 2.3 [5]	0.73 ± 0.04 [8] 0.69 ± 0.07 [5]
Mg^{2+}	44.31 ± 0.41	182.34 ± 5.68	0.57 ± 0.02	45 ± 1 [8]	238.49 ± 21 [8]	0.53 ± 0.04 [8]
Na^{+}	28.99± 0.29	153.90 ± 6.06	0.51 ± 0.02	40 ± 1 [8]	167.36 ± 21 [8]	0.52 ± 0.04 [8]
K^{+}	29.75 ± 0.69	146.94 ± 8.87	0.49 ± 0.03	41 ± 1 [8]	158.99 ± 21 [8]	0.54± 0.04 [8]
Apo	28.15 ± 0.58	174.49 ± 19.35	0.58 ± 0.06	31 ± 1 [8]	154.81 ± 21 [8]	0.52 ± 0.04 [8]

The values of thermodynamic parameters were also compared to those reported in the study done on BLA using circular dichroism by Kuwajima *et al*.[5]. Their analysis was performed only on the Ca^{2+} bound BLA based on the two-state denaturation model. Although there is small discrepancy, each parameter is in decent agreement with the corresponding values of BLA. The comparisons made with other studies that used different analysis techniques confirm the validity of the experimental and analytical methods that were used in this study.

5. Conclusions

The effects of the binding of various metal ions including Mg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Sr^{2+}, Ca^{2+}, Na^{+} and K^{+} on the stability of the biologically important protein, human α-lactalbumin (HLA), have been examined by means of the intrinsic tryptophan fluorescence. Based on the assumption of a simple two-state model, thermal-induced denaturation experiments revealed that the binding of the different metal ions stabilizes HLA with varying degree of stabilization for each ion. Heating induced a pronounced change in the tryptophan fluorescence intensity and a red shift of the spectrum towards longer wavelengths, which suggested a conformational change. The thermal transition curves generated by the experimental data produced a sigmoidal behavior which confirmed the two-state model. The analysis was then performed utilizing nonlinear regression fitting which in turn was used to determine the thermodynamic parameters (ΔH, ΔG, ΔS and T_m) based on the newly reported mathematical relationships.

Based on the data analysis, among those metal ions tested in this study, the monovalent cations Na^{+} and K^{+} were found to destabilize HLA whereas all the divalent cations were found to stabilize HLA. The extent of the stabilization was the greatest when HLA was bound to Ca^{2+} with a T_m value of 63.59℃ and a ΔG of 19.91 kJ/mol at physiological temperature. The results of thermodynamic parameters obtained from the present analysis have also been compared with those obtained from other studies in order to confirm their validity.

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