Articles

Thermodynamics of Binding Interactions between Bovine β-Lactoglobulin A and the Antihypertensive Peptide β-Lg f142-148

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The binding capacity of bovine β-lactoglobulin variant A (β-Lg A) for six peptides derived from β-Lg was evaluated using an ultrafiltration method under the following conditions: pH 6.8, 40 °C, and a β-Lg A/peptide molar ratio of 1:5. Only peptides β-Lg f102-105, f142-148, and f69-83 bound in significant amounts to β-Lg A corresponding to 1.5, 1.1, and 0.7 mol of peptide per mole of β-Lg A, respectively. The interaction between β-Lg A and the antihypertensive peptide β-Lg f142-148 was investigated further by isothermal titration calorimetry. The binding isotherms at pH 6.8 and 25 °C confirmed that β-Lg f142-148 bound to β-Lg A and that the interaction followed a sequential three-site binding model with constants of association of 2×10^3, 1×10^3, and 0.4×10^3 M^{-1} for the first, second, and third binding sites, respectively. The enthalpy of binding was exothermic for the first and second binding sites and endothermic for the third binding site. Binding of the peptide to all three sites was spontaneous as shown by the negative free energy values. These results show for the first time that β-Lg A can bind bioactive peptides. This potential could be exploited to transport bioactive peptides and protect them in the gastrointestinal tract following their oral administration as nutraceuticals.

Introduction

Milk proteins are an important source of bioactive peptides with antihypertensive, opioid agonistic and antagonistic, immunomodulating, antithrombotic, antimicrobial, mineral-binding, and hypocholesterolaemic properties.[1−5] However, some of these peptides, which are active in vitro, are ineffective in vivo.[6−9] This suggests that they are degraded when they transit the gastrointestinal tract. To be used as nutraceuticals, bioactive peptides have to be administrated orally, cross the digestive tube, and reach target sites at the luminal side of the intestinal tract or specific peripheral organs without being degraded.[10−12] Some bioactive peptides thus need protection while they are transiting the gastrointestinal tract. β-Lg might be a suitable candidate for use as a carrier for these molecules because of its binding properties.[13]

Numerous studies have shown that β-Lg can bind a variety of ligands such as hydrophobic and slightly polar lipid-like molecules and different cations.[14−22] β-Lg, which is the major protein in the whey of ruminants and some non-ruminants, is a member of the lipocalycin family.[23] It has a relative molecular mass of 18.3 kDa and its 162-amino-acid polypeptide chain is folded into a compact globular conformation stabilized by two intramolecular disulfide bridges.[24,25] The core of the protein is a flattened β-barrel (calyx) composed of eight antiparallel β-strands (A to H). While the physiological function of β-Lg is unknown, its binding affinity for different molecules and the pH control of its inner binding site (calyx), which defines the so-called Tanford transition, suggest that it can act as a carrier for various ligands.[26] The Tanford transition involves the displacement of the EF loop (residues 85−90) at the entrance of the central cavity from an open (above pH 6.2) to a closed (acidic pHs) conformation as the pH drops.[23,27,28] These structural changes could have functional implications for the reversible binding and release of ligands ensuring their transport, protection, and release in the small intestine for absorption.[28]

Three potential binding sites have been proposed for ligand binding to β-Lg: the internal cavity of the β-barrel, the surface hydrophobic pocket in a groove between the α-helix and the β-barrel, and the outer surface close to $Trp_{19}-Arg_{124}$.[24,29−32] However, most evidence points to the β-barrel as the most important binding site. Binding at the other sites remains more controversial.[23,26] Retinol/protoporphyrin IX[33] and fatty acid/retinoid[15] mixtures bind simultaneously to β-Lg. Noiseux et al.[34] recently demonstrated that β-Lg can bind some peptides as a function of their intrinsic properties (charge, hydrophobicity, and molecular weight) and the physicochemical conditions of the medium. However, they studied the protein/peptide interaction using an ultrafiltration method and did not determine any thermodynamic parameters.

The purpose of the present work was to confirm the interactions between β-Lg and several peptides and to determine the thermodynamic parameters defining the interactions. Isothermal titration calorimetry (ITC) was used to characterize the thermodynamic changes accompanying the interactions between bovine β-Lg variant A and the antihypertensive peptide β-Lg f142-148. This technique has been successfully used in our lab[35] to characterize the formation of β-Lg/pectin complexes and is

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Table 1. Physicochemical Characteristics of the Six Peptides Studied

peptide	amino acid sequence	MW[a] (Da)	charge at pH 6.8	isoelectric point[a]	$H_{\varnothing av}$[b] (kcal per residue)
β-Lg f102-105	YLLF	555.3	0	5.5	2.58
β-Lg f142-148	ALPMHIR	837.5	+1	9.8	1.54
β-Lg f92-100	VLVLDTDYK	1065.2	−1	4.2	1.44
β-Lg f125-135	TPEVDDEALEK	1245.3	−4	3.8	0.85
β-Lg f69-83	KKIIAEKTKIPAVFK	1714.2	+4	10.2	1.68
β-Lg f41-60	VYVEELKPTPEGDLEILLQK	2313.7	−3	4.3	1.37

[a] Data determined from ExPASy molecular biology server. [b] Hydrophobicity scale calculated according to the method of Bigelow.[36]

regarded as one of the most rigorous methods for the accurate characterization of the binding interactions of biological macromolecules.

Experimental Section

Materials. Bovine β-lactoglobulin (β-Lg) variant A (no. L7880, >92% purity grade) was obtained from Sigma Chemical Co. (St. Louis, MO). The six peptides selected for this study are normal products of trypsin and trypsin/chymotrypsin hydrolysis of bovine β-lactoglobulin: f102-105, f142-148, f92-100, f125-135, f69-83, and f41-60. They were synthesized by the Service de Séquence de Peptide de l'Est du Québec (Sainte-Foy, PQ, Canada) using a 433A Applied Biosystems peptide synthesizer with fast-MOC chemistry (1.0 mmol scale) using a preloaded Fmoc-Arg(pbf) MBHA resin (Anaspec inc., San Jose, CA). The trityl side-chain-protecting groups were used for His. After synthesis, peptides were cleaved and deprotected 2 h using trifluoroacetic acid and a scavenger (reagent K) and precipitated with *tert*-butyl methyl ether. The precipitated peptides were then dissolved in water and freeze-dried. The crude peptides were purified by reverse-phase HPLC on a preparative C_{18} column. The purity and identity of the peptides were confirmed by analytical HPLC and mass spectrometry (matrix-assisted laser desorption ionization time-of-flight). The peptides were water soluble with a high degree of purity (>83%). Some of the physicochemical characteristics of the peptides are presented in Table 1. These peptides were chosen on the basis of their molecular mass (555.3−2313.7 Da), charge at pH 6.8 (positive, negative, or neutral), and average hydrophobicity (0.85−2.58 kcal per residue). All other chemicals were of analytical grade.

Binding of Peptides to β-Lg A. The binding of the peptides to β-Lg A was studied using the ultrafiltration method previously described by Noiseux et al.[34] Experiments were performed using the following physicochemical conditions: a β-Lg A/peptide molar ratio of 1:5 in 0.1 M phosphate buffer (pH 6.8) at 40 °C. Briefly, 150 μL of a β-Lg A solution (0.2 mM) and 150 μL of a peptide solution (1 mM) were mixed in an Eppendorf tube and incubated overnight at 40 °C. The solutions were then placed in a Microcon YM-10 centrifugal filter device with a MWCO of 10 kDa (Millipore, Bedford, MA), then centrifuged at 9500 × *g* for 35 min at 40 °C. Unbound peptide in the β-Lg A/peptide filtrate was estimated with respect to the peptide in the filtrate obtained from the pure peptide solution without β-Lg A (control sample). RP-HPLC analysis was used to determine the peptide content of the samples from the peak area of the peptide, while the percentage (%) of bound peptide was calculated as follows:

$$\text{bound peptide (\%)} = \frac{A - B}{A} \times 100 \qquad (1)$$

where A is the peak area for the peptide in the filtrate isolated from the pure peptide solution (control), and B is the peak area for the peptide in the Microcon filtrate isolated from the β-Lg A/peptide solution.

To verify the release of compounds from the Microcon membrane and the total retention of β-Lg A by the membrane and to estimate peptide adsorption onto the membrane, the following solutions were also analyzed by RP-HPLC: phosphate buffer filtered at 40 °C; a β-Lg A solution (0.2 mM, 150 μL) in phosphate buffer (0.1 M, 150 μL) incubated overnight at 40 °C and then filtered; solutions of each peptide (1 mM, 150 μL) in phosphate buffer (0.1 M, 150 μL) incubated at 40 °C then filtered. All measurements were carried out in triplicate.

RP-HPLC Analysis. RP-HPLC analyses were performed using a Beckman System Gold HPLC (Mississauga, ON, Canada) equipped with two pumps (model 126P), a variable UV−visible detector (model 166P) operating at 214 nm, and a Hewlett-Packard automatic injector (series 1100, Agilent Technologies, Palo Alto, CA). Data acquisition and chromatographic analyses were performed using Beckman System Gold Nouveau software (version 1.6). A Nova-Pak C_{18} column (4 mm, 3.9 i.d. × 150 mm) from Waters Co. (Millipore, Milford, MA) connected to a guard column (4 mm, 3.9 i.d. × 20 mm) filled with the same matrix was operated at a flow rate of 1 mL/min at 39 °C. Solvent A was composed of 0.11% (v/v) trifluoroacetic acid (TFA) in Milli-Q water, and solvent B was composed of 60% (v/v) acetonitrile, 40% Milli-Q water, and 0.1% (v/v) TFA. The column was equilibrated with solvent A. The sample (20 μL) was injected and eluted with a linear gradient of solvent B ranging from 0% to 60% for 10 min, 60% to 100% for 2 min, and 100% to 0% for 2 min. All buffers and mobile phases were prepared with HPLC grade water (18 MΩ, Modulab, Water systems, Continental corporation, San Antonio, TX), filtered through 0.22-μm filters (Millipore, Bedford, MA), and degassed.

Preparation of the Isothermal Titration Calorimetry Solutions. β-Lg f142-148 (purity of 92.7%) was selected for the ITC analysis. Excess salt in the β-Lg A and β-Lg f142-148 solutions was eliminated by dialysis against deionized water for 48 h at 4 °C using cellulose ester dialysis membranes (Spectra/Pro, Spectrum Laboratories Inc., Rancho Dominguez, CA) with MWCOs of 10 000 and 500 Da for β-Lg A and β-Lg f142-148, respectively. The contents of both membranes were then dialyzed together for another step (48 h, 4 °C) against a phosphate buffer (1 mM, pH 6.8) to equilibrate the pH and ionic strength of the solutions. The concentrations of the dialyzed β-Lg A and β-Lg f142-148 were determined by RP-HPLC analysis on C_{18} column using calibration curves ($R^2 = 0.999$) for each molecule. β-Lg A was eluted as a single peak before and after dialysis without peak related to aggregation or denaturation states of the protein. The resulting dialysis buffer was used as a solvent to adjust the β-Lg A and β-Lg f142-148 solutions to the appropriate concentrations for the ITC analysis. All solutions were degassed under vacuum for 15 min at 25 °C prior to the titration experiments.

Isothermal Titration Calorimetry Analysis. ITC was used to determine the enthalpy effects associated with the binding of β-Lg f142-148 to β-Lg A using a Microcal VP−ITC instrument (Microcal Inc., Northampton, MA). Detailed descriptions of the background to this method can be found in Ladbury and Chowdhry.[37] Data analysis was performed using ORIGIN software (version 5.0) from Microcal (Microcal Inc., Northampton, MA). All measurements were carried out in duplicate at 25 °C to ensure the native tertiary structure of β-Lg A.

After adjusting the temperature of the solutions to 25 °C, the apparatus was equilibrated at the same temperature for at least 30 min before beginning the measurements. The reference cell contained buffer, while the 1.345-mL reaction cell was filled with either buffer or an appropriately diluted solution of β-Lg A. A peptide solution (12.14 mM) prepared in the same buffer as the β-Lg A solution was put in

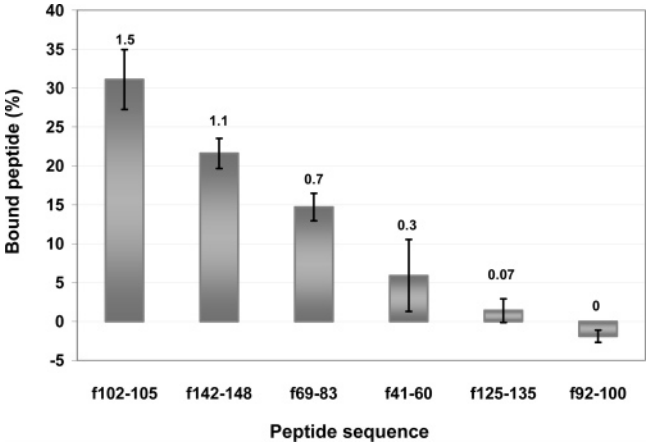

Figure 1. Percentage (% ± SD) of peptide bound to β-Lg A determined using the ultrafiltration method. Values above the bars indicate the amount (moles) of peptide bound per mole of β-Lg A.

the 250-μL stirrer−syringe assembly and added in 25 successive 10-μL injections to the β-Lg A solution. Each addition took 20 s with an interval of 300 s between consecutive injections. During the titration, the reaction mixture was stirred continuously at 260 rpm to ensure sufficient but not excessive mixing. The β-Lg A concentration was increased from 0.045 to 0.45 mM to investigate the relation of the binding isotherms to variable, increasing concentrations of β-Lg A. The heat of dilution of the β-Lg f142-148 solution in the buffer was measured and subtracted from the calorimetric data. The heat of dilution of the buffer in the β-Lg A solution was also measured. Because of the dilution across the injection needle tip during the initial equilibration period, the data from the first injections were discarded from the fit.

The Microcal ORIGIN software was used to analyze the titration heat profiles and to determine the site binding model that gave a good fit (low χ^2 value) to the experimental data. From the various binding models tested (single set of identical sites, two sets of independent sites, sequential two-site binding model), only the sequential three-sites binding model fitted adequately the binding isotherms. This model, extensively described in Hamman et al.[38] and Liu et al.,[39] allowed determination of the heat content after the *i*th injection from the following equations:

$$Q = M_t V_o(F_1\Delta H_1 + F_2[\Delta H_1 + \Delta H_2] + ... + F_n[\Delta H_1 + \Delta H_2 + \Delta H_3 + ... + \Delta H_n]) \quad (2)$$

and

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_o}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1) \quad (3)$$

where Q is the heat content after any injection (*i*th injection), M_t is the total concentration (moles/liter) of β-Lg A in the active cell volume (V_0), n is the number of binding sites on the β-Lg A, F is the fraction of sites occupied by the peptide for each binding site, and ΔH is the molar heat or enthalpy of peptide binding for each binding site. The binding constant (K) and molar reaction enthalpy ($\Delta H°$) then allows calculation of the standard free energy binding ($\Delta G°$) and entropy changes ($\Delta S°$), according to the following equation:

$$\Delta G° = -RT \ln K = \Delta H° - T\Delta S° \quad (4)$$

Results and Discussion

Binding of Peptides to β-Lg A. The binding capacities of β-Lg A for the selected peptides determined by the ultrafiltration method are summarized in Figure 1. The percentages of binding for peptides β-Lg f102-105, f142-148, f69-83, f41-60, and f125-135 were 31.1, 21.5, 14.7, 5.9, and 1.4, respectively. These values corresponded to 1.5, 1.1, 0.7, 0.3, and 0.07 mol of peptide bound per mole of β-Lg A. Peptide β-Lg f92-100 did not bind to β-Lg A (−1.9 ± 0.8%). The binding stoichiometry of β-Lg for various ligands is around 1:1.[17,40−42]

The peptides used in this study carried charges of 0 (f102-105), +1 (f142-148), +4 (f69-83), −3 (f41-60), −4 (f125-135), and −1 (f92-100). Positive charges thus seemed to favor binding to β-Lg A. However, the fact that β-Lg f102-105, which is neutral, bound to β-Lg A suggests that the charge effect was not the only factor affecting binding. The hydrophobicity of the peptides also appeared to be an important factor in the interaction. Peptides β-Lg f102-105, f142-148, f69-83, f41-60, f125-135, and f92-100 had average hydrophobicities of 2.6, 1.5, 1.7, 1.4, 0.9, and 1.4 kcal per residue, respectively, as calculated using the method of Bigelow[36] (Table 1). Highly hydrophobic peptides thus appeared to interact more strongly with β-Lg A. The interaction between the peptides and β-Lg A did not seem to be affected by the molecular weights of the peptides. Although hydrophobicity seems the most evident common feature of the other molecules which can bind to β-Lg, in our study both hydrophobicity and charge seem important for the interaction between β-Lg A and peptides.

At pH 6.8, β-Lg A is above its isoelectric point (pH 5.26) and is negatively charged.[43] This may explain the weak interactions with negatively charged peptides, which were probably due to electrostatic repulsions. The positively charged peptides β-Lg f142-148 and β-Lg f69-83 would be able to bind to any negatively charged patch on the surface of β-Lg A. The peptides that bound to β-Lg A also had relatively high hydrophobicities compared to the peptides that did not. Since β-Lg A has an internal hydrophobic calyx that adopts an open conformation at pH 6.8,[27,28,44,45] the hydrophobicity of the peptides may be another driving force behind binding to β-Lg. The calyx might be the binding site of the neutral/hydrophobic peptide β-Lg f102-105. However, it might also bind to the external surface of β-Lg.[46]

On the basis of the interactions of the peptides with β-Lg A, β-Lg f142-148 was selected for the ITC study to investigate the thermodynamic parameters of the interactions. β-Lg f142-148 is recognized as the most potent ACE-inhibitory peptide derived from bovine β-Lg and may be involved in the control of hypertension.[47] Despite the fact that β-Lg f102-105 bound the most efficiently to β-Lg A, it was not used because of its low solubility in phosphate buffer and the difficulties encountered with its synthesis.

Isothermal Titration Calorimetry (ITC). Figure 2A shows the heat flow versus time profile for β-Lg f142-148 injected into the reaction cell containing phosphate buffer. Initially, a series of relatively large endothermic peaks were observed. As the titration proceeded, the energy absorbed progressively decreased and tended to a state of relative thermodynamic stability toward the end of the injections. The changes in enthalpy might be due to micelle dissociation, as reported in previous studies.[48−51] The binding isotherm suggests that the peptide concentration used might have been above the critical micelle concentration (cmc), causing the peptide to form micelles. The micelles dissociate when they are diluted in the reaction cell. With increasing numbers of injections, the concentration of peptide in the reaction cell increases, equals, and then exceeds the cmc, which inhibits the dissociation of micelles[50] and leads to a decrease in the overall energy absorbed. The change in the pattern of dilution enthalpies occurs at the cmc,[48,50] which can be determined by the inflection of the titration curve.[52] Figure 2B shows the titration curve obtained

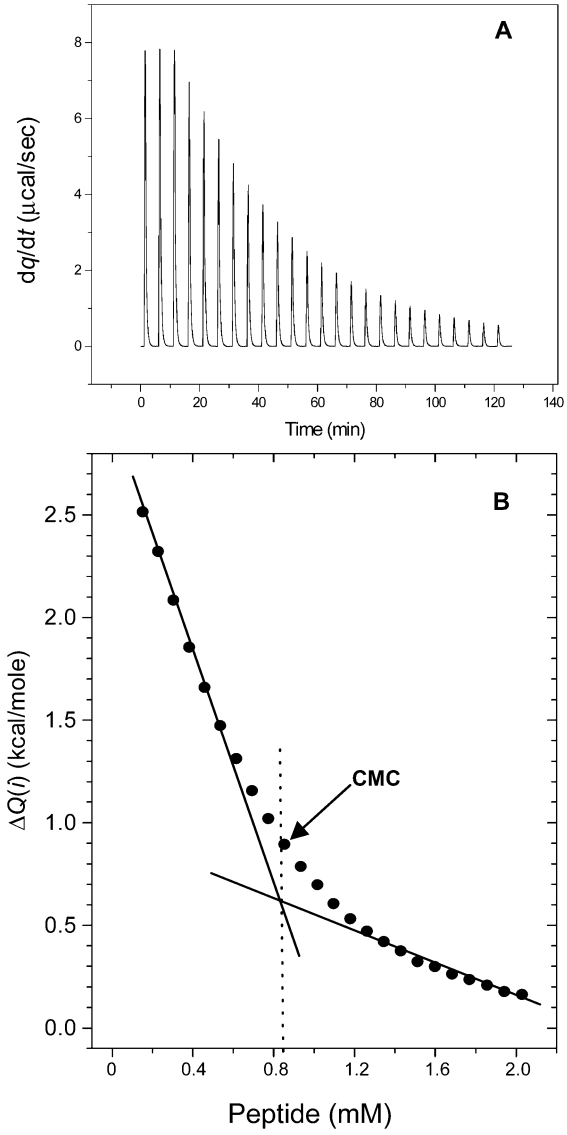

Figure 2. (A) Heat flow vs time profile resulting from injection of 10-μL aliquots of β-Lg f142-148 solution (12.14 mM) into the reaction cell containing phosphate buffer (1 mM, pH 6.8) at 25 °C. (B) Dependence of heat change $\Delta Q(i)$ per mole of peptide on the peptide concentration in the reaction cell for β-Lg f142-148 injected into phosphate buffer at 25 °C. The first injection was discarded from the fit. The apparent critical micelle concentration (cmc) was determined from the inflection point in the curve.

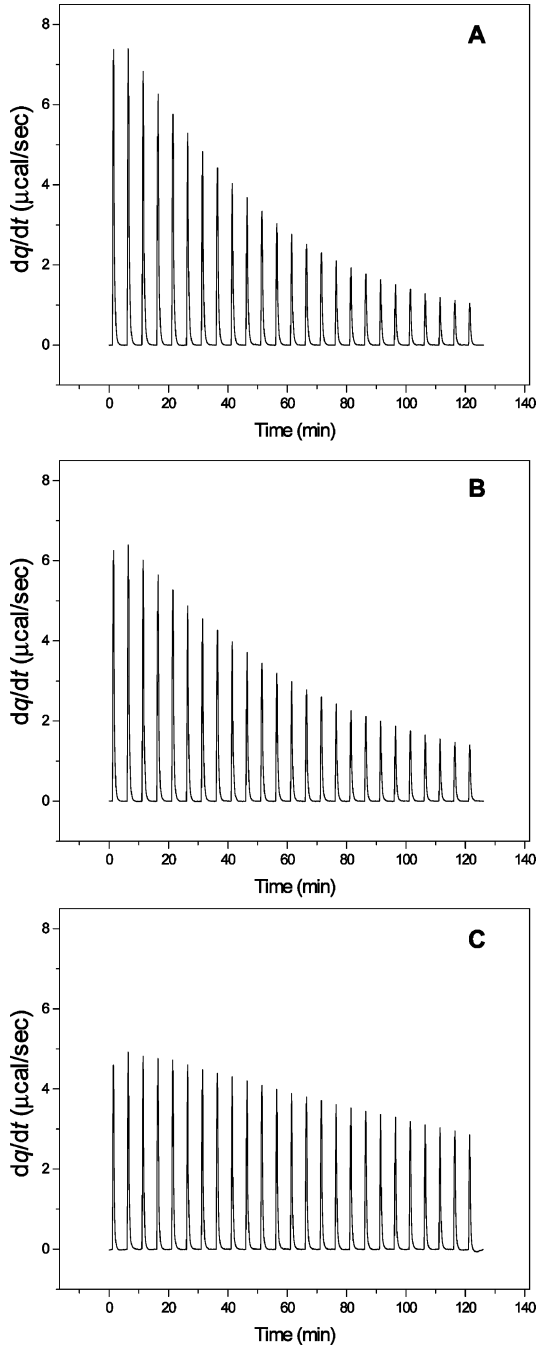

Figure 3. Heat flow vs time profiles of 10-μL aliquot injections of a 12.14 mM solution of β-Lg f142-148 into a reaction cell containing a (A) 0.045 mM, (B) 0.09 mM, or (C) 0.45 mM solution of β-Lg A.

by the integration of the isotherm peaks. This curve indicates that the cmc was reached at the 11th injection, which corresponded to a peptide concentration of 0.86 mM in the reaction cell.

Figure 3 shows the heat flow versus time profiles resulting from sequential injections (10 μL) of a β-Lg f142-148 solution (12.14 mM) into different β-Lg A solutions: 0.045 mM (Figure 3A), 0.09 mM (Figure 3B), and 0.45 mM (Figure 3C). The area under each peak represents the amount of heat absorbed with each injection. The energy absorbed when the peptide was injected into the β-Lg A solution was lower than that when the peptide was injected into buffer alone (Figure 2A). Also, the amount of energy absorbed decreased with the increase in the protein concentration in the reaction cell. The energy absorbed during the second injection was 7.4 (Figure 3A), 6.4 (Figure 3B), and 4.9 (Figure 3C) μcal per second for β-Lg concentrations of 0.045, 0.09, and 0.45 mM, respectively, compared to 7.8 μcal per second (Figure 2A) when the peptide was injected into buffer alone. The presence of the protein in the reaction cell thus caused an appreciable heat change, indicating that β-Lg A interacted with β-Lg f142-148.

The titration curves of peptide β-Lg f142-148 (12.14 mM) in buffer and in the β-Lg A solutions are compared in Figure 4. The titration curves obtained in the presence of β-Lg A were less endothermic than that obtained in the absence of β-Lg A. At the ninth−tenth injections, the heat absorbed was almost the same in the absence and presence of β-Lg A. Thereafter, the reaction became more endothermic in the presence of β-Lg A. In addition, the difference in heat change $\Delta Q(i)$ before and after the ninth−tenth injections increased with the increasing concentration of β-Lg A.

These observations indicate that, when the peptides were injected into the reaction cell containing the β-Lg A solution,

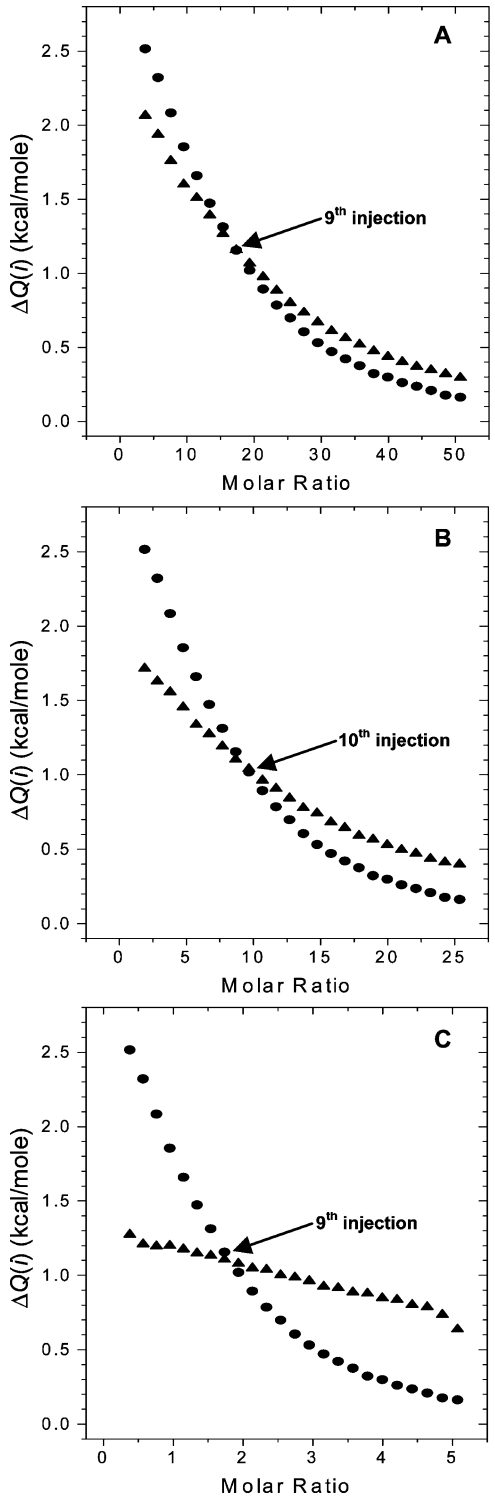

Figure 4. Comparison of the titration curves obtained following sequential injections of the β-Lg f142-148 solution (12.14 mM) into buffer (●) and (A) 0.045 mM, (B) 0.09 mM, and (C) 0.45 mM solutions of β-Lg A (▲). The first injections were discarded from the fits.

the dissociated micelles (peptides) interacted with the β-Lg A and released energy, reducing the endothermic energy in the reaction cell before the ninth−tenth injections. This effect increased with the rise in β-Lg A concentration in the reaction cell. At the ninth−tenth injections, the β-Lg A/β-Lg f142-148 molar ratios were 1:14, 1:8, and 1:1.5 for β-Lg A concentrations of 0.045, 0.09, and 0.45 mM, respectively, in the reaction cell. After the ninth−tenth injections, the reaction became more endothermic in the presence of β-Lg A. This may be because

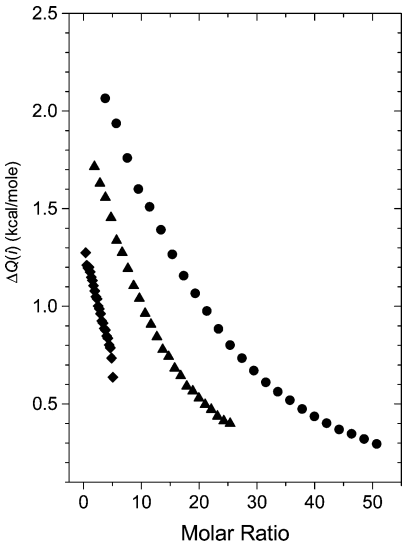

Figure 5. Superposition of the titration curves obtained following the sequential injections of a 12.14 mM solution of β-Lg f142-148 into the reaction cell containing the 0.045 mM (●), 0.09 mM (▲), and 0.45 mM (◆) solutions of β-Lg A. The first injections were discarded from the fits.

all the β-Lg A molecules were saturated and no more were available to interact with the dissociated micelles, or the concentration of free dissociated micelles (peptides) remained under the cmc, resulting in the absorption of more energy compared to when there was no β-Lg A in the reaction cell. Even though the determination of cmc in the presence of β-Lg A is much more complex than in the absence of β-Lg, the apparent cmc of the peptide in the presence of β-Lg A was determined. The apparent cmc in the presence of β-Lg A increased relative to the value obtained in the absence of β-Lg A (Figure 2B) and was reached at peptide concentrations of 1.0 and 1.07 mM when the concentration of the β-Lg A in the cell was 0.045 and 0.09 mM, respectively, compared to 0.86 mM in the absence of β-Lg A. For the highest β-Lg A concentration studied (0.45 mM), the apparent cmc was not reached, even after the 25th injection.

Figure 5 shows the evolution of the titration curves obtained following the injection of β-Lg f142-148 into the reaction cell containing increasing concentrations of β-Lg A. The energy absorbed in the reaction cell decreased with the increase in β-Lg A concentration, indicating that the energy released by the interaction between β-Lg A and β-Lg f142-148 increased with higher amounts of β-Lg A. When the titration curves of β-Lg f142-148 injected into the buffer were subtracted from those obtained in the presence of the β-Lg A, the resulting curves (Figure 6) confirmed that the complexation reaction between β-Lg A and β-Lg f142-148 was exothermic for the first 9 to 10 injections and then became endothermic.

An analysis of the isotherms using the Microcal ORIGIN software indicated that models of a single set of identical sites, two sets of independent site, and sequential two-site binding models did not adequately fit the binding isotherm of the β-Lg A/β-Lg f142-148 complex. Only the sequential three-site binding model adequately fit the calorimetric data. In this model, there was no fitting parameters equivalent to *N*, and the best fit was determined by only two parameters (*K* and *H*) at each site. The thermodynamic parameters obtained for the first, second, and third sites are listed in Table 2 and indicate that the association constant (K_a) decreased from about 2×10^3 to 1×10^3 M^{-1} then to 0.4×10^3 M^{-1} for the first, second, and third binding sites, respectively. However, K_a was 1.6×10^3 M^{-1} for the third

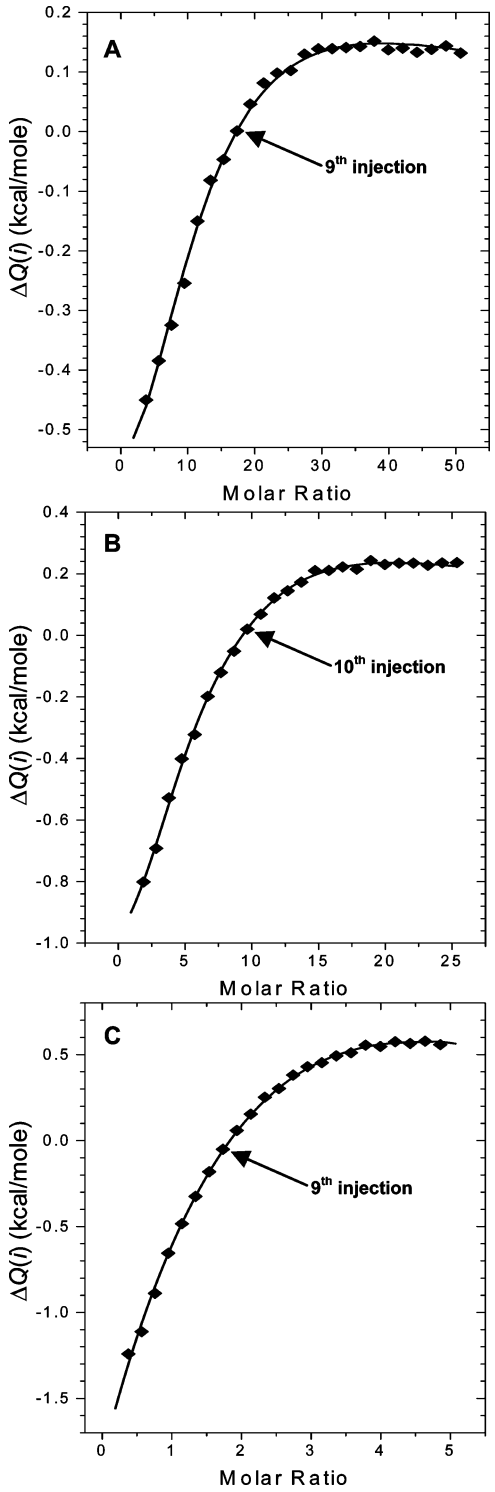

Figure 6. Titration curves of β-Lg f142-148 in 0.045 mM (A), 0.09 mM (B), and 0.45 mM (C) β-Lg A solutions. The heat of dilution of β-Lg f142-148 in the buffer was subtracted from all the curves. The first injections were discarded from the fits.

binding site at the highest β-Lg A concentration (0.45 mM). The binding constant of β-Lg can be affected by many factors such as pH, concentration, temperature, conformation, or shape of ligand. In fact, the published K_d values are in the range from 1×10^{-3} to 4.91×10^{-9} M.[42,53−56] In our work, K_d for the β-Lg A/β-Lg f142-148 complex is in the range of 0.5×10^{-3} M for the first binding site. This value is lower than those measured for some endogenous ligands (e.g., retinol) but similar to the values determined for 1-8-anilinonaphthalene sulfonate (ANS)[55,56] and Ca^{2+},[42] which are 1×10^{-3} and 3×10^{-3} M, respectively.

The free energy values of the three binding sites were negative (−3.5 to −4.6 kcal mol^{-1}), indicating that complexation at these sites was spontaneous. For both first and second binding sites, ΔG values were relatively constant (4−4.6 kcal mol^{-1}), while the enthalpic and entropic contributions show much greater variation. As is commonly observed in macromolecular systems,[38,57] large changes in ΔH and ΔS can be tolerated because of the relatively much smaller effects on ΔG, which is the only parameter that really matters for the function of a system.

For the first binding site, the enthalpy was negative at all β-Lg A concentrations, indicating that the interaction was exothermic. However, at the highest protein concentration (0.45 mM), the interaction was less exothermic and the entropy was positive. For the lower β-Lg A concentrations (0.045 and 0.09 mM), ΔS values were negative. For the second binding site, the interaction was also exothermic except at the highest β-Lg A concentration (0.45 mM). In this case, the interaction was almost endothermic, if not athermic. At this second binding site, the ΔS values increased significantly with the increase in β-Lg A concentration. The negative values of both ΔH and ΔS for the first and second binding sites at the lower β-Lg concentrations indicate that specific interactions dominated. Complexation at these sites seemed well-ordered (negative ΔS values) but was negatively affected by high protein concentrations (increase in ΔS values). The interaction at the third binding site was endothermic at all β-Lg A concentrations. However, the energy absorbed during the interaction decreased with increasing β-Lg A concentrations. Moreover, ΔS values were high for all the β-Lg A concentrations but decreased with the increase in protein concentration. The interaction at this site thus seemed to be favored by an increase in β-Lg A concentration. In addition, the positive ΔH and ΔS values indicate that the interaction of the peptide with β-Lg at the third binding site was dominated by the hydrophobic effect. The highly positive ΔS values were also a strong indication that water molecules had been released from the complex surface.

The ΔH and ΔS values point to a possible binding mechanism between β-Lg f142-148 and β-Lg A. For example, the binding of the peptide at the first site on β-Lg A could occur through the calyx of the protein, which is the most specific binding site on β-Lg A. With increasing concentrations of β-Lg A, the vicinity of proteins might affect the approach of the peptide, blocking access to the calyx and preventing complexation. This hypothesis was confirmed by the increase in entropy at a high β-Lg A concentration (0.45 mM). Complexation at this site might occur through the insertion of the nonpolar side-chain at the N-terminus of the peptide into the hydrophobic core of β-Lg A.

The binding of the peptide to the second site on β-Lg A, which has a lower K_a value than the first site (Table 2), likely occurred through electrostatic interactions between positively charged residues (arginine) on the peptide and negatively charged (at pH 6.8) acidic residues (aspartic acid, glutamic acid) on the surface of β-Lg A. As for the first site, surrounding proteins could affect the approach of the peptide because of steric hindrance, making complexation more difficult. This possibility is suggested by the increase in entropy with the increase in β-Lg A concentration. However, since binding at the second site seems to be greatly affected by increasing concentrations of β-Lg A, this suggests that the second binding site may be located at or near the region of β-Lg A involved in dimer formation (β-I strand). In fact, at physiological pH and

Table 2. Thermodynamic Parameters of β-Lg f142−148 Binding to β-Lg A Calculated from the Sequential Three-Site Binding Model

thermodynamic parameters	β-Lg A (mM)								
	first binding site			second binding site			third binding site		
	0.045	0.09	0.45	0.045	0.09	0.45	0.045	0.09	0.45
K_a (10^3 M^{-1})	1.99 ± 0.64	2.17 ± 0.77	1.91 ± 0.39	1.29 ± 0.27	0.90 ± 0.18	0.98 ± 0.36	0.46 ± 0.13	0.37 ± 0.10	1.63 ± 0.58
ΔH (kcal mol^{-1})	−5.33 ± 2.05	−5.95 ± 1.90	−3.37 ± 0.38	−7.90 ± 3.27	−4.47 ± 3.95	0.27 ± 0.92	31.78 ± 3.95	29.44 ± 3.63	6.92 ± 0.51
ΔS (cal mol^{-1} K^{-1})	−2.8	−4.7	3.7	−12.2	−1.5	14.6	118.8	110.5	37.9
ΔG (kcal mol^{-1})	−4.50	−4.55	−4.47	−4.25	−4.02	−4.08	−3.64	−3.50	−4.38

ambient temperature, there is a equilibrium of native β-Lg monomers and dimers.[25,58−60] The shift from one form to the other depends on protein concentration, ionic strength, pH, and temperature.[59] Since the last three parameters were kept constant during the ITC analyses, the protein concentration most likely affected the equilibrium, resulting in a shift from the monomer to the dimer conformation with increasing protein concentrations. On the basis of the mass action law, the equilibrium between dimers and monomers should depend on protein concentration.[61−63] The dimer is the predominant species of β-Lg at neutral pH regions and at protein concentration above 1 mg mL^{-1} (0.054 mM). However, because the dimerization constant is not large ($\sim 5 \times 10^4$ M^{-1}), the population of monomers increases at low protein concentrations.[64] Aymard et al.[59] reported that dissociation is complete at infinite dilution. Apenten and Galani[65] demonstrated that at pH 2.6 and low protein concentrations (≤0.4 mg mL^{-1}; 0.022 mM), β-Lg is more weakly associated than at pH 7 and exists as monomer (>70% dissociation). At levels exceeding 4 mg mL^{-1} (0.22 mM), differences in dimerization tendency at pH 2.6 and pH 7 disappear, and the predominant form in both cases is dimer. Our results indicate that β-Lg A probably exists mainly as a monomer at a protein concentration of 0.045 mM and that the proportion of dimers increases at higher protein concentrations. The formation of dimers would eliminate a proportion of the second binding site, which may explain the decrease in enthalpy and the increase in entropy for this binding site at a protein concentration of 0.09 mM (Table 2).

The binding of the peptide to the third site on β-Lg A likely occurs through interactions with protein aggregates. Gottschalk et al.[66] reported that, below room temperature in the pH range from 3.7 to 5.2, β-Lg self-associates into larger oligomers with maximum around pH 4.6. Verheul et al.[25] reported that structures larger than dimers form at high β-Lg concentrations (pH 6.9) and that β-Lg A associates more strongly than β-Lg B. The substitution of Gly_{64} by Asp_{64} on the surface of the molecule is a prime suspect in the ability of β-Lg A dimers (but not β-Lg B dimers) to oligomerize into octamers in the 3.5 to 6.5 pH range.[67] Using static light scattering, Kumosinski and Timasheff[68] showed the presence of intermediate oligomers (tetramers and hexamers) in significant amounts at 8 °C and 15 °C at β-Lg concentrations below 1 mM. High concentrations of β-Lg A would thus favor aggregation and create the third binding site. As shown in Table 2, both the enthalpy and the entropy seemed to favor interactions at the third site at a high protein concentration (0.45 mM). Moreover, K_a was higher for the third site (1.63×10^3 M^{-1}) at the highest β-Lg A concentration (0.45 mM) than for the second site (0.98×10^3 M^{-1}). This lends support to the hypothesis that the second binding site is located at or near the region involved in dimer formation, since oligomerization of β-Lg A would eliminate much of this interface. This might also explain the decrease in enthalpy and the increase in entropy at the second binding site at a protein concentration of 0.45 mM. Last, the interaction at the third binding site was endothermic (Table 2), indicating that energy was required to reorganize the β-Lg A molecules before binding could occur.

Wang et al.[41] demonstrated that palmitate binding to β-Lg is significantly affected by increasing the protein concentration from 0.001 to 0.2 mM and suggested that two sets of independent sites are present, one on the monomer and one on the dimer. They reported that the monomer−dimer equilibrium is affected by the protein concentration. At high concentrations, β-Lg self-associates into dimers, eliminating much of the surface responsible for palmitate binding. However, a smaller, higher-affinity binding site is created. By analogy, we suggest that the sequential three-site binding occurs in the following sequence: In step 1, the peptide binds to the inside of the calyx of the protein; in step 2, the peptide binds to the surface of the protein at or near the region involved in dimer formation; and in step 3, the peptide binds to the third site created by the formation of the oligomers. This hypothesis is supported by the change from the exothermic reaction accompanying the interactions at the first and second binding sites to the endothermic reaction accompanying the interaction at the third binding site. Our results thus offer convincing evidence that peptide binding occurs at more than one site on β-Lg A.

Conclusion

It was believed for a long time that β-Lg could only bind hydrophobic ligands. The present study demonstrates that β-Lg A can bind hydrophobic as well as positively charged bioactive peptides. To our knowledge, this is the first time that the thermodynamics of the interaction between β-Lg A and the antihypertensive peptide β-Lg f142-148 have been studied. It has been shown that the dissociation of the peptide micelles was immediately followed by the interaction between the peptide β-Lg f142-148 and β-Lg A. The interaction seemed to follow a sequential three-site binding model that was entirely driven by exothermic enthalpy at the first and second binding sites while the interaction at the third binding site was endothermic. The K_a values were determined for all sites, and binding sites have been proposed in this study. However, these results need to be confirmed by other methods. Front-face fluorescence spectroscopy is currently being used in our laboratory to confirm the identity of the binding sites on β-Lg A.

The demonstration of complex formation between β-Lg A and the antihypertensive peptide β-Lg f142-148 is relevant, since recent studies have shown the degradation of this peptide during simulated gastrointestinal digestion[69,70] and the loss of its activity following oral ingestion by two human volunteers.[69] These data thus suggest that the peptide β-Lg f142-148 and probably other bioactive peptides may need protection against gastric or intestinal enzymatic degradation in order to exert their physiological effects in vivo. Since β-Lg A is stable at acid pH and resists the gastric digestion, it could be used to transport bioactive peptides and protect them in the gastrointestinal tract, allowing them to reach their targets intact.[71,72]

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