Self-assembled β -lactoglobulin–oleic acid and β -lactoglobulin–linoleic acid complexes with antitumor activities

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ABSTRACT

β -Lactoglobulin (β -LG) can bind to fatty acids such as oleic acid (OA) and linoleic acid (LA). Another whey protein, α -lactalbumin (α -LA), can also bind to OA to give the complex α -LA-OA, which has antitumor properties. Based on reports that the activity of α -LA-OA is highly dependent on OA, as well as the acquisition of similar complexes using other proteins, such as lysozyme and lactoferrin, we speculated whether β -LG could also kill tumor cells after binding to other fatty acids. Therefore, we prepared complexes of β -LG with OA (β -LG-OA) and LA (β -LG-LA) in the current study and evaluated them in terms of antitumor activity and thermostability using the methylene blue method and differential scanning calorimetry, respectively. The structural features of these complexes were also evaluated using fluorescence spectroscopy and circular dichroism. The binding dynamics of OA and LA to β -LG were studied using isothermal titration calorimetry. Cell viability results revealed that β -LG-LA and β -LG-OA exhibited similar antitumor activities. Interestingly, the binding of β -LG to LA led to an increase in its thermostability, whereas its binding to OA had very little effect. The environments of the tryptophan residues in the β -LG-OA and β -LG-LA complexes were very different, with the residues being blue- and red-shifted, respectively. Furthermore, the hydrophobic regions in β -LG were buried after binding of OA, which was slightly changed in β -LG-LA. Circular dichroism results showed that β -LG-OA enhanced the tertiary structure, which was partially lost in β -LG-LA. There were more binding sites for OA than for LA on β -LG, although the binding constants of the 2 fatty acids were similar, with both acids interacting with the protein through van der Waals and hydrogen bonding interactions. This study could help provide a deeper

understanding of the structural basis for formation of antitumor protein–fatty acid complexes.

Key words: structure, thermostability, binding kinetics, binding strength

INTRODUCTION

β -Lactoglobulin is the major whey protein found in cow milk, with an overall content of about 50% (Creamer and Sawyer, 2003). However, β -LG has been reported to be absent from human breast milk (Hambraeus and Lönnerdal, 2003). β -Lactoglobulin is a small globular protein consisting of 162 AA, with a monomer molecular mass of about 18 kDa (Wu et al., 1999). It is a predominantly β -sheet protein composed of 9 β -strands (A–I); β -strands A to H form an up-and-down β -barrel with a major α -helix at the C-terminal (Konuma et al., 2007). The AA sequence of β -LG shows several similarities to the retinol-binding proteins, and its tertiary structure shows some homology with lipocalin proteins (Sawyer et al., 1998). Because of these similarities, β -LG has been shown to bind to a variety of different hydrophobic molecules, including retinol and long-chain fatty acids (Ragona et al., 2000; Beringhelli et al., 2002; Barbiroli et al., 2010), which means that β -LG possesses the biological properties necessary to transport these binding ligands (Sawyer et al., 1998).

In contrast to α -LA, another whey protein, the biological function of β -LG has not yet been identified (Wu et al., 1999). α -Lactalbumin also binds oleic acid (OA), with the resulting complex exhibiting cytotoxic activity toward tumor cells (Zhang et al., 2009; Atri et al., 2011; Fang et al., 2012). It is noteworthy that this function has been observed in α -LA proteins of several different species (Pettersson et al., 2006; Atri et al., 2011), as well as in fragments of α -LA (Tolin et al., 2010) and proteins capable of assembling to α -LA, such as lysozymes (Vukojević et al., 2010). Although little evidence is available to support the idea that α -LA belongs to the lipocalin family of proteins, its 3-dimensional structure shares several similarities with β -LG, especially its triple-stranded anti-parallel β -sheets (Pet-

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tersson et al., 2006). Based on these similarities, we speculated whether the complex formed between β -LG and OA would also be cytotoxic toward tumor cells. Lišková et al. (2011) prepared a complex from β -LG and sodium oleate that exhibited apoptosis-inducing qualities comparable to those of the α -LA-OA complex.

The mechanism of the antitumor activity of HAMLET (human α -lactalbumin made lethal to tumor cells)-like complexes has been studied extensively (Hallgren et al., 2006; Aits et al., 2009; Zhang et al., 2013), and the toxicity of these complexes has been reported to be highly dependent on OA (Brinkmann et al., 2011; Nakamura et al., 2013). Furthermore, the antitumor properties of OA have been widely acknowledged (Brinkmann et al., 2011; Carrillo et al., 2012; Hoque et al., 2013; Fang et al., 2014). We envisaged that β -LG could bind other FA to form α -LA-OA analogs, which could also exhibit interesting biological properties. The aim of this study was to prepare complexes of OA and linoleic acid (LA) with β -LG using the method reported for the preparation of α -LA-OA (Fang et al., 2012) and to characterize the structural and biological properties of these complexes. The results of this study could therefore provide a theoretical basis for better understanding the physical processes involved in the formation of protein-fatty acid complexes, which represent a profound prospect in cancer therapy.

MATERIALS AND METHODS

Materials

β -Lactoglobulin, OA, LA, and 1-anilino-8-naphthalenesulfonate (ANS) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dulbecco's modified eagle's medium (DMEM) containing a high level of glucose, RPMI-1640 medium, fetal bovine serum (FBS), HEPES buffer solution, penicillin, and streptomycin were purchased from Gibco (Life Technologies, Waltham, MA). All other chemicals used in the study were purchased as analytical grade.

Preparation of β -LG-OA and β -LG-LA Complexes

The method was conducted in accordance with the reports provided by Fang et al. (2012, 2014). Briefly, β -LG was dissolved in PBS (10 mM, pH 8.0) to a final concentration of 100 μ M. Then, OA or LA was directly added into the protein solution at a 50 molar equivalent ratio (i.e., 50 mol of fatty acid per mol of protein), and the resulting mixture was vortexed for 30 s before being incubated in a water bath at 45°C for 20 min. Excess fatty acid in the complexes was removed by centrifugation at 12,000 $\times g$ at 4°C for 20 min, followed

by ultrafiltration using a 3,000-kDa cutoff membrane (Sartorius, Goettingen, Germany). A control β -LG solution was prepared in the same way but without the addition of either FA. Control solutions of OA and LA were prepared by adding these FA to PBS.

Cell Culture

Human cervical carcinoma cells (HeLa) and human breast cancer cells (MCF-7) were purchased from ATCC (Manassas, VA) and maintained in DMEM and RPMI-1640 media, respectively. The media were supplemented with 10% FBS, 20 U/mL of penicillin, 20 μ g/mL streptomycin, and 10 mM HEPES at 37°C under an atmosphere of 5% CO₂.

Cell Viability Assays

Cells were seeded into 96-well plates (Corning Inc., Corning, NY) at a density of 1×10^4 cells/well and grown for 24 h. The medium was then removed and replaced with new medium containing different concentrations of β -LG-OA without FBS; FBS was subsequently added into each well to a final concentration of 10% after 30 min. After 24 h of incubation at 37°C, cell viability was tested by methylene blue assay (Fang, et al., 2012). The control β -LG and control FA solutions were also added to separate wells at the same volume.

Apoptosis Assay Measured by Flow Cytometry

Cells were seeded into 12-well plates at a density of 1×10^5 cells/well and grown for 24 h. The β -LG-OA or β -LG-LA complex was added to the wells to the required final concentration, and the resulting mixtures were allowed to stand for 30 min. Fetal bovine serum was then added to each well, and the resulting mixtures were incubated for 24 h. The cells were then analyzed by flow cytometry using the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining apoptosis detection kit (eBioscience, Mountain View, CA). Cells were gated by dot plots and at least 15,000 cells were analyzed for each sample using a FACSCalibur instrument equipped with a FACS station running FACSCalibur software (BD Biosciences, San Diego, CA). Each experiment was performed in triplicate.

Differential Scanning Calorimetry

Two-milligram samples of lyophilized β -LG, β -LG-OA, and β -LG-LA were sealed in separate aluminum pans and heated from 0 to 175°C at a heating rate of 5°C/min and an excess pressure of 1.5 atm. The re-

sulting thermograms were measured using differential scanning calorimetry (DSC; TA Instruments, Crawley, UK).

Fluorescence Spectroscopy

The intrinsic fluorescence spectra of the complexes were measured at room temperature using an RF-5301PC spectrofluorometer (Shimadzu, Kyoto, Japan). The emission spectra were collected between 300 and 500 nm, with an excitation wavelength of 292 nm and a scan interval of 0.2 nm. The slit widths for excitation and emission were set at 3 nm. All of the experiments were repeated 3 times. The protein solutions were diluted with the corresponding buffer to a final concentration of 30 μ M.

For ANS-binding measurements, each protein sample was incubated with a 50-fold concentration of ANS for 15 min at room temperature in the absence of light. The emission spectrum of ANS was recorded between 400 and 600 nm at a scan interval of 0.2 nm and an excitation wavelength of 390 nm. The emission fluorescence intensities of ANS were also measured to exclude the influence of unbound ANS. The slit widths for excitation and emission were set at 3 nm. The protein concentration was set at 30 μ M for all of the measurements. All experiments were repeated 3 times.

Circular Dichroism Spectroscopy

Far-UV and near-UV circular dichroism (CD) spectra were measured using a J-810 instrument (Jasco Corp., Tokyo, Japan) between 200 and 240 nm and between 250 and 320 nm, respectively. A path length of 1 mm was used for all of the experiments. The scan rate was set at 50 nm per min with an interval of 0.5 nm, and the response time was set as 8 s. Each spectrum was averaged from 3 scans. Baseline spectra were recorded for the buffer and subtracted from the sample. The protein concentration was set at 30 μ M for all of the measurements. The secondary structure was calculated using the CD Pro software package (Jasco Corp.). All of the experiments were repeated 3 times.

Binding Dynamics Measured by Isothermal Titration Calorimetry

The binding dynamics of OA and LA to β -LG were evaluated by isothermal titration calorimetry (ITC; Nano Series, TA Corp., New Castle, DE) at pH 8.0 (10 mM PBS) and 45°C. Then, β -LG was dissolved in PBS (10 mM, pH 8.0) to final concentrations of 5.55 μ M (for OA titration) and 11.1 μ M (for LA titration). The titrated OA and LA solutions were initially dissolved in

alcohol to the required concentrations and then diluted with PBS to a final concentration of 1.68 mM. Samples (190 μ L) of the protein solutions were then injected into the sample cell and an automated sequence of 23 injections was performed, with each injection adding 2 μ L of an OA or LA solution into the protein solution at 200-s intervals to allow for complete equilibration. The stirring rate was set at 150 rpm. The data were collected automatically and the binding constant (**Ka**), stoichiometry of binding (**n**), standard enthalpy change (ΔH), standard Gibbs free energy change (ΔG), and standard entropy change (ΔS) were analyzed using Launch NanoAnalyze 2.0 software (TA Corp.).

RESULTS

Antitumor Activities of β -LG-OA and β -LG-LA

The antitumor activities of β -LG-OA and β -LG-LA were measured in HeLa and MCF-7 cells using the methylene blue assay (Figure 1). The results revealed that increasing the concentration of β -LG-OA or β -LG-LA led to a significant decrease ($P < 0.01$) in cell viability. According to the concentration–cell viability curve, the half-lethal dose (**LD₅₀**) values of β -LG-OA and β -LG-LA were 115.3 and 88.85 μ M toward HeLa cells, and 66.91 and 64.63 μ M toward MCF-7 cells. Under all of the conditions tested (Figure 1), only the control β -LG, OA, and LA solutions did not influence cell viability.

The abilities of β -LG-OA and β -LG-LA to induce cell apoptosis were also analyzed (Figure 2). As shown in Figure 2, at the concentrations corresponding to the LD₅₀ values calculated in Figure 1, β -LG-OA and β -LG-LA induced apoptosis rates of 30% (Figure 2A) and 35% (Figure 2B) in HeLa cells after 24 h of treatment.

Thermal Denaturation of β -LG, β -LG-OA, and β -LG-LA

The thermal denaturation profiles of β -LG, β -LG-OA, and β -LG-LA were measured by DSC (Figure 3), with peaks in the resulting thermograms corresponding to changes in the structure of the protein. Based on the thermograms, the onset temperature (**T_{onset}**) of the 2 peaks was calculated using the TA-60 software (TA Corp.). The **T_{onset}** values for the denaturation of β -LG, β -LG-OA, and β -LG-LA were 26.75, 26.69, and 42.17°C, respectively. Higher the **T_{onset}** values indicate higher thermostability of the material being tested. These results therefore revealed that the binding of LA to β -LG led to a significant increase in the heat resistance of β -LG, whereas the binding of OA had very little effect on its thermostability.

Figure 1. Cell viability of HeLa and MCF-7 cells treated with different concentrations of (A) β -LG complexed with oleic acid (β -LG-OA) or (B) β -LG complexed with linoleic acid (β -LG-LA; B); (C) cell viability of HeLa cells treated with β -LG, OA, and LA for 24 h; and (D) cell viability of MCF-7 cells treated with β -LG, OA, and LA for 24 h, as measured using a methylene blue assay. The formula inserted into each figure is the calculated concentration–cell viability equation. Data are expressed as mean values $\pm$ SD ($n = 3$).

Structural Changes of β -LG Induced by OA and LA

The intrinsic fluorescence (Figure 4A) and ANS-binding fluorescence (Figure 4B) emission spectra of β -LG, β -LG-OA, and β -LG-LA were measured to determine the differences induced by the binding of OA and LA to β -LG. A slight blue shift of 1.2 nm was observed in the intrinsic fluorescence going from β -LG (334.5 ± 0.14 nm) to β -LG-OA (333.3 ± 0.14 nm), which indicated that Trp residues were being directed out of the solvent and into the protein. However, the binding of LA (335.3 ± 0.14 nm) to β -LG led to a 0.8-nm red shift in the intrinsic fluorescence compared with β -LG, which suggested that Trp residues were being exposed to the solvent in this case.

For the ANS-binding fluorescence, β -LG-OA showed a significant red shift (505.0 ± 0.28 nm) and decreased intensity (86.83 ± 6.99) compared with β -LG (495.2 ± 0.01 nm, 162.62 ± 3.66), whereas β -LG-LA gave a red shift (495.4 ± 0.28) and a slight reduction in intensity (148.30 ± 4.56). These changes indicated that the hydrophobic regions of the β -LG protein were buried within the protein and directed away from the solvent.

Near-UV and far-UV CD spectroscopy were used to compare the secondary and tertiary structures of β -LG following the binding of OA (Figure 5). The peaks at 293 and 270 nm were attributed to Tyr and Trp residues, respectively, and reflect the rigid folding of the protein. As shown in Figure 5A, β -LG-OA exhibited increased signal intensities at 293 and 270 nm compared with the β -LG control solution, whereas the signals in β -LG-LA were lower than those in β -LG. These results indicated that the binding of LA to β -LG led to loss of the tertiary structure of β -LG, whereas OA strengthened the tertiary structure of β -LG.

Contents of the secondary structures were calculated based on their far-UV CD spectra (Table 1). Similar effects to those observed for the tertiary structures of β -LG, OA, and LA were observed in the contents of secondary structures. As shown in Table 1, the binding of LA to β -LG led to a decrease in the β -turn and random coil contents of the protein, and an increase in the β -sheet structure, although the α -helix content remained largely unchanged. Notably, however, we detected a significant difference in the content of the secondary structures in β -LG-OA compared with β -LG,

Figure 2. Apoptosis of HeLa cells treated with β -LG complexed with oleic acid (β -LG-OA; A); β -LG complexed with linoleic acid (β -LG-LA; B); β -LG (C), oleic acid (D), and linoleic acid (E) at the LD₅₀ (50% lethal dose) concentration for 24 h, as measured by flow cytometry using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis testing kit. Early apoptotic cells only bound to annexin V (the lower right D4 region), necrotic or late apoptotic cells bound to both annexin V and PI (the upper right D2 region), and viable cells did not bind to either annexin V or PI (the lower left quadrant D3 region). The upper left D1 region corresponds to dead cells. The number of cells undergoing apoptosis was the sum of D1, D2, and D4. Color version available online.

including an increase in the α -helix and β -sheet contents, as well as a decrease in β -turn and random coil contents.

Binding Dynamics of OA and LA Measured by ITC

The binding dynamics of OA and LA to β -LG were monitored by ITC (Figure 6). The upper panel shows the raw calorimetric data, which showed exothermic (upward) peaks in all cases. As titration progressed, the exothermic peaks decreased in size until they reached a heat of dilution corresponding to the protein alone in the buffer. The lower panel shows a plot of the total heat generated per injection as a function of the molar ratio of FA to protein. All of the binding isotherms were fitted to an independent binding site model and the corresponding thermodynamic parameters were obtained (Table 2). The heat of dilution of the free FA

has been subtracted from these data. The K_a value was used to indicate the binding affinity of the interaction. The K_a and n (i.e., number of binding sites) values for OA and LA on β -LG were $2.81 \times 10^5 M^{-1}$ and 40, and $2.21 \times 10^5 M^{-1}$ and 32, respectively. Furthermore, these exothermic binding processes were accompanied by negative ΔH and ΔS values for both OA and LA (Table 2).

DISCUSSION

As a lipocalin family protein, β -LG could play a vital role in the absorption and subsequent metabolism of FA and triglycerides (Kushibiki et al., 2001; Chatterton et al., 2006). The results of the current study revealed a new biological function for β -LG, involving the binding of FA. It can be seen from Figures 1 and 2 that β -LG-OA and β -LG-LA exhibit both antitumor and proapop-

Figure 3. Thermal denaturation profiles of β -LG, β -LG complexed with oleic acid (β -LG-OA), and β -LG complexed with linoleic acid (β -LG-LA), as measured by differential scanning calorimetry from 0 to 175°C at a heating rate of 5°C/min.

totic activities similar to those of α -LA-OA (Fang et al., 2012, 2014). Given that the control solutions of β -LG and FA did not exhibit any cytotoxic activity, it seems highly likely that the protein component of these complexes is not solely responsible for their antitumor activity, and that the protein could simply be acting as a vehicle for the cytotoxic FA component (i.e., OA or LA). The thermostability of β -LG-OA did not change significantly compared with that of β -LG, whereas the complexation of LA to β -LG led to a significant increase in thermostability of β -LG. These differences in the effect of OA and LA on the thermal stability of β -LG could be caused by differences in their binding sites.

β -Lactoglobulin has 2 potential FA-binding sites, including a typical interior calyx shaped by the 8-stranded antiparallel β -barrel and a cleft located in a groove between the helix and the barrel groove on the protein surface (Barbiroli et al., 2010). The 2 binding sites can be distinguished by the specificity and stabilization of the structure toward denaturants by the bound ligand (Barbiroli et al., 2011). Calyx-bound ligands make β -LG less sensitive to chemical and physical denaturation, whereas surface-bound ligands have a negligible effect on the stability of proteins toward denaturants

Figure 4. Intrinsic fluorescence (A) and 1-anilino-8-naphthalene-sulfonate (ANS) fluorescence (B) emission spectra of β -LG, β -LG complexed with oleic acid (β -LG-OA), and β -LG complexed with linoleic acid (β -LG-LA). The arrows indicate the position of the maximum emission wavelengths.

when they are saturated with ligands (Beringhelli et al., 2002; Considine et al., 2005; Lozinsky et al., 2006). In this context, it is likely that the binding site of OA could be on the surface of β -LG, whereas LA would be more likely to bind to the calyx of β -LG. These differences in binding sites could also explain the observed changes in the hydrophobic regions. Oleic acid would occupy the hydrophobic region around the surface-bound ligands, which would lead to the significant red shift (9.8 nm) observed in the ANS-binding spectra. In contrast, LA would bind to the calyx-bound ligands, leaving the surface-bound ligands unaffected, which would lead to the slight red shift observed for β -LG-LA (0.2 nm).

The changes in the shifts of the Trp residues in the β -LG-OA and β -LG-LA complexes were opposite, although the shifts themselves were only slight. β -Lactoglobulin has only 2 Trp residues, corresponding to the Trp19 and Trp61 residues in its AA sequence (Barbiroli et al., 2011; Hasni et al., 2011). The Trp19

Figure 5. Near-UV (A) and far-UV (B) circular dichroism (CD) spectra of β -LG, β -LG complexed with oleic acid (β -LG-OA), and β -LG complexed with linoleic acid (β -LG-LA).

residue is located at the bottom of the protein β -barrel (Sakurai and Goto, 2007) and contributes to 80% of the total fluorescence of β -LG (Hasni et al., 2011). In contrast, it has been reported that Trp61 is unlikely to have a significant effect on the fluorescence spectrum of β -LG, even if the protein structure were altered (Barbiroli et al., 2011). It can be speculated, therefore,

Table 1. The secondary structure contents¹ of β -LG and complexes of β -LG with oleic acid (β -LG-OA) and linoleic acid (β -LG-LA) calculated by the Jasco-810 software (Jasco Corp., Tokyo, Japan) using Yang's reference (algorithm for calculating the content of the secondary structures in proteins)

Content (%)	β -LG	β -LG-OA	β -LG-LA
α -Helix	11.73 $\pm$ 0.15	18.43 $\pm$ 0.74*	12.20 $\pm$ 0.23
β -Sheet	27.50 $\pm$ 1.57	33.40 $\pm$ 2.18*	32.50 $\pm$ 2.01*
β -Turn	22.73 $\pm$ 0.70	19.10 $\pm$ 1.48*	19.40 $\pm$ 1.27*
Random coil	38.03 $\pm$ 0.97	29.03 $\pm$ 1.15*	35.90 $\pm$ 1.11*

¹Data expressed as the mean values ($\pm$ SD; n = 3).
* $P \leq 0.05$.

that the binding of OA and LA to β -LG only influenced the environment of the Trp61 residue. The Trp61 residue is located on the surface of the β -LG protein close to a pH-sensitive EF loop, which controls access to the calyx-binding region (Sakurai and Goto, 2006, 2007; Tian et al., 2006). The EF loop exists in a closed conformation under acid conditions and it moves away from the calyx at pH >7.5 to form an open conformation, which favors ligand binding (Collini et al., 2003). The β -LG-OA and β -LG-LA complexes were prepared at pH 8.0, which would have led to the opening of the hydrophobic domain in the central barrel (Eberini et al., 2004). The blue shift observed in the intrinsic fluorescence spectrum of β -LG-OA therefore reflected the burying of the Trp61 residue in the core of the protein, which would have been induced by structural changes following binding of OA to the surface-bound ligands. In contrast, the red shift observed in the intrinsic fluorescence spectrum of β -LG-LA reflects exposure of the Trp61 residue to solvent following the binding of LA to the calyx. Furthermore, the different binding sites of OA and LA to β -LG could explain the differences in the secondary and tertiary structures of the complexes. It is clear from Figure 5A that the β -LG-LA complex exhibited a loss of tertiary structure, which was similar to the changes observed in the tertiary structure of α -LA induced by OA (Fang et al., 2012, 2014). This similarity could be caused by the binding sites of OA to α -LA and LA to β -LG both being in the calyx region. An enhanced tertiary structure of β -LG-OA would therefore be observed because of the binding of OA to the exposed hydrophobic regions on the protein's surface.

Isothermal titration calorimetry can be used to measure the kinetics and thermodynamic constants of a binding reaction (Ababou and Ladbury, 2006, 2007; Okhrimenko et al., 2008). It can be seen from the kinetic results that the binding strengths of OA and LA to β -LG were similar, even though there were more binding sites for OA on β -LG than there were for LA. Under the conditions in the ITC measurement, once OA or LA entered the binding sites in β -LG, the

Table 2. Dynamic and thermodynamic parameters¹ for the reactions of the oleic acid (OA) and linoleic acid (LA) titrates with β -LG according to the fitting of the data in Figure 6 to an independent binding site model²

Protein	K_a (M^{-1})	n	ΔH (kJ/mol)	ΔS (kJ/mol)	ΔG (kJ/mol)
OA	$(2.81 \pm 1.43) \times 10^5$	40.39 ± 0.43	-146.61 ± 0.97	-384.02 ± 2.59	-31.31 ± 1.06
LA	$(2.21 \pm 1.19) \times 10^5$	32.67 ± 0.36	-431.70 ± 2.15	$-1,335.13 \pm 8.41$	-30.72 ± 1.14

¹Data expressed as the mean values ($\pm$ SD; $n = 3$).² K_a = binding constant; n = stoichiometry of binding (i.e., number of binding sites); ΔH = enthalpy change; ΔS = entropy change; ΔG = Gibbs free energy change.

structure of β -LG changed. The increasing exposure of the hydrophobic regions on the surface of β -LG may enhance the interaction with OA and LA, leading to the formation of a micelle when the amounts of OA and LA continue to increase. Therefore, the binding site measured by ITC exhibited a much higher stoichiometric number. The Gibbs free energy change, ΔG , which is derived from K , did not differ significantly between β -LG and LA. A negative value for ΔG is indicative of spontaneous binding of OA and LA to the proteins (Liu et al., 2009; Singh and Mitra, 2011; Fang et al., 2014). The ΔH value reflects the amount of heat released or absorbed during the binding reaction (Cestaria et al., 2000; Leckband, 2000; Liu et al., 2009), whereas ΔS reflects the order of the system, which is also influenced by changes in the conformation of the macromolecules (Cestaria et al., 2000; Liu et al., 2009). Ligand-binding interactions not only involve electrostatic and hydro-

phobic interactions, but also involve hydrogen bonding and van der Waals interactions (Barbana et al., 2006; De et al., 2007). In this study, the negative ΔH and ΔS changes indicated the involvement of van der Waals forces as well as the formation of hydrogen bonding interactions during the binding of OA and LA (De et al., 2007; Singh and Mitra, 2011; Fang et al., 2014).

CONCLUSIONS

Although the FA-binding ability of β -LG had been studied extensively, the antitumor activities of the β -LG complexes formed after binding OA and LA to β -LG were largely ignored until the discovery of the α -LA-OA complex. In this study, the β -LG-OA and β -LG-LA complexes killed tumor cells by inducing apoptosis, although the changes induced in the structure of β -LG by OA and LA were very different. The binding of OA to

Figure 6. Calorimetric titration (upper figure) and plots of the integrated heat versus each injection (lower figure) fitted to an independent binding site model. Oleic acid (solid line) or linoleic acid (dotted line) at 1.68 mM was titrated to 5.55 or 11.1 μM β -LG, respectively, at 45°C and a stirring rate of 150 rpm. The protein, oleic acid, and linoleic acid were dissolved in 10 mM PBS (pH 8.0).

β -LG led to the burying of the tryptophan residues and hydrophobic regions in the core of the protein, and the tertiary structure of the protein was enhanced. In contrast, binding of LA to β -LG led to an open structure with the tryptophan residues exposed to the solvent. Furthermore, the binding of LA to β -LG led to a slight change in the hydrophobic regions of β -LG and to partial loss of its tertiary structure. Notably, β -LG-LA exhibited enhanced thermostability compared with β -LG, whereas the binding of OA had very little effect on the thermostability of β -LG. Furthermore, there were more binding sites for OA than for LA on β -LG, although the binding constants of the 2 FA were similar and they both interacted with the protein through van der Waals and hydrogen bonding interactions. The results of this study provide a theoretical basis for understanding the mechanisms through which proteins can acquire new biological activities following their complexation with small molecules such as FA.

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