Denaturation of Bovine β-Lactoglobulin in the Presence of *n*-Octyl-, Decyl-, and Dodecyldimethylphosphine Oxides

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Denaturation of bovine β-lactoglobulin (β-L) in pH 2.9, 0.2 M glycine buffer was investigated by DSC in the presence of three nonionic surfactants, *n*-octyldimethylphosphine oxide (APO8), *n*-decyldimethylphosphine oxide (APO10), and *n*-dodecyldimethylphosphine oxide (APO12), and by ITC at temperatures from 25 to 61 °C. The thermal transition was eliminated when the molar ratio of surfactant to β-L was between 88 and 133, 21−25, and 11−22 depending upon the protein concentration for APO8, APO10, and APO12, respectively. A protocol was developed that may be used for future studies that involve ligands with large temperature-dependent heats of dilution. Approximately 30 mol of APO10 and APO12 per mole of β-L were bound at 45 °C and 37 °C, respectively, with an average affinity of $(2.5 \pm 0.7) \times 10^3$ M^{-1}. This amount of surfactant would cover about 50% of the protein surface and may correspond to a new class of nonspecific neutral ligand binding sites that facilitate the digestion of lipids by neonatal calves. Titration of β-L into 2% solutions of APO8, APO10, and APO12 at various temperatures between 25 and 61 °C yielded enthalpy changes with the same temperature dependence as for the thermal denaturation of β-L without surfactant at much higher temperatures.

Introduction

Members of the *n*-alkyldimethylphosphine oxide (APO) class of nonionic surfactants are water soluble, colorless in the near-UV and visible region of the spectrum, available in high purity,[1] and have a signature ^{31}P NMR signal that is sensitive to the polarity of their environment.[2] The temperature dependence of the thermodynamic changes that accompany micelle formation for five long-chain members of this family between 15 and 79 °C in H_2O and D_2O has been reported.[1,3] The phase diagrams for a homologous series of aqueous APO compounds have been determined over a broad concentration range.[4] The liquid−liquid phase separation behavior of several APO/phospholipid mixtures has been investigated, and the potential use of these mixtures for protein purification has been suggested.[5] However, the influence of APO surfactants on the native structure of proteins has not been reported. Therefore, we decided to investigate the binding properties of three members of the (APO) class of surfactants, *n*-octyldimethylphosphine oxide (APO8), *n*-decyldimethylphosphine oxide (APO10), and *n*-dodecyldimethylphosphine oxide (APO12), to a well-characterized[6] globular protein, bovine β-lactoglobulin (β-L).

Bovine β-L is a major component of milk and exists in nature as a dimer that dissociates into a monomer of 162 amino acid residues below pH 3.5.[7] It has been extensively studied during the past 60 years by essentially every biochemical technique available.[8] There are several isoforms, of which the most abundant are isoforms A and B. The amino acids Asp64 and Val118 in isoform A are replaced by glycine and alanine in isoform B. The secondary structure contains one strand of α-helix and eight antiparallel β-strands that form a binding pocket for small hydrophobic ligands. Organic solvents[9] and high pressure[10] have been reported to induce the formation of the molten globule state of β-L.

β-L was one of the first members of the lipocalin superfamily of proteins.[11] Although a number of biologically important ligands, including vitamin D[12] and palmitic acid,[13] have a strong affinity for the hydrophobic pocket, the biological role of β-L is still uncertain.[8,9] However, it has been suggested that β-L may act as a transporter of lipids through the acidic gastric tract and facilitate the digestion of milk consumed by neonatal calves.[13] It has also been suggested that nonnative molten globule-like states of proteins may facilitate their ability to penetrate biological membranes,[14] although this does not seem to be the case for porcine β-L.[15] The knowledge that β-L interacts with neutral bilayers formed from milk phospholipids supports this notion.[16] Since fatty acids in the stomach are protonated, a knowledge of the binding of medium-chain-length nonionic surfactants to β-L in an acid medium might provide some insights regarding the biological function of the protein,

Solution calorimetry and equilibrium dialysis have been used to study the binding of sodium dodecyl sulfate to β-L, and three stages in the binding process were identified.[17−19] There have not been any previous calorimetric studies of the binding of nonionic surfactants to β-L to our knowledge. Differential scanning calorimetry (DSC) studies have shown that the thermal denaturation of β-L may be modeled as a simple two-state process,[20,21] although baseline issues have been raised.[22] Results on binding-induced denaturation obtained at constant temperature by isothermal titration calorimetry (ITC) may be compared with those obtained from temperature scanning experiments. Therefore, both calorimetric methods were used to study the interaction of three members of the APO class of surfactants with β-lactoglobulin. All three of the surfactants investigated decrease the thermal stability of the protein, but the thermal transition could be restored by the removal of APO10 that was bound to the protein at 60 °C and below.

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Experimental Section

Materials. The sample of β-lactoglobulin used in this study was obtained from mixed herd milk. The protein was purified by trichloroacetic acid precipitation and ammonium sulfate fractionation followed by precipitation and lyophilized after exhaustive dialysis against distilled water. The white, fluffy material was stored in a tightly sealed container at −10 °C. Equal amounts of the A and B forms of the protein were the only proteins detected by gel electrophoresis. The circular dichroism spectrum of the native form of the protein was in excellent agreement with spectra previously reported.[23] The protein concentration was determined from the absorbance at 278 nm with an extinction coefficient of 0.96 cm^3/mg.[24] The samples of *n*-octyldimethylphosphine oxide (APO8), *n*-decyldimethylphosphine oxide (APO10), and *n*-dodecyldimethylphosphine oxide (APO12) used in this study (BioAffinity Systems, Rockford, IL) were the same samples previously used[1,3] and were free of impurities as determined by gas chromatography. Deionized water (17 MΩ·cm) was used to prepare all solutions. Buffers containing 0.2 M glycine were adjusted to pH 2.9 ± 0.1 with 0.1 M HCl. The solid protein and surfactant samples were dissolved with gentle shaking or stirring with a magnetic stirrer to minimize foaming, and finally degassed prior to placing into the calorimeter cell. Solutions were stored at 5 °C after they were prepared and were normally used within 2−3 days.

Methods. The isothermal titration calorimetry experiments were performed with a MicroCal Omega isothermal titration calorimeter (MicroCal, Inc., Northampton, MA) with a 300 μL syringe unless stated otherwise. The Origin software package, version 2.9, supplied by MicroCal, Inc., was used for data collection, analysis, and plotting. Differential scanning calorimetry studies were performed with the same batch of protein investigated by ITC. An MC-2 differential scanning calorimeter (MicroCal, Inc.) was interfaced to the desktop computer with a second analog/digital board (Data Translation, DT 2801, Marlboro, MA). This allowed the ITC and DSC to be operated simultaneously. A nominal scanning rate of 60 K/h was used for the collection of the data unless stated otherwise. The reference cells for both ITC and DSC experiments contained the same buffer that was used for the protein samples. Details of the experimental protocol for the ITC and DSC experiments that was previously described[1,25] were followed.

The thermal stability of β-L was determined by differential scanning calorimetry in the presence of various concentrations of APO8, APO10, and APO12 and titration calorimetry with a limited number of injections of β-L into high concentrations of surfactants at fixed temperatures. A binding profile that described the concentration dependence of surfactant binding to the protein was obtained from an analysis of the isothermal calorimetric titration curves.

Theory. The model used to fit the ITC data in this study assumes that the titration curves can be represented as the sum of two independent processes, but as will be shown later, APO binding to the protein corresponds to only one process. The protein is located in the cell and the surfactant is in the syringe for this analysis. Reverse titrations were also conducted, but the data were not fitted to this model. In this model, n_1 and n_2 are the number of moles of APO molecules binding independently at each hypothetical site:[26]

$$n_1\text{APO} + \beta\text{-L} \leftrightarrow \beta\text{-L}(n_1\text{APO}) \quad (1)$$

$$n_2\text{APO} + \beta\text{-L} \leftrightarrow \beta\text{-L}(n_2\text{APO}) \quad (2)$$

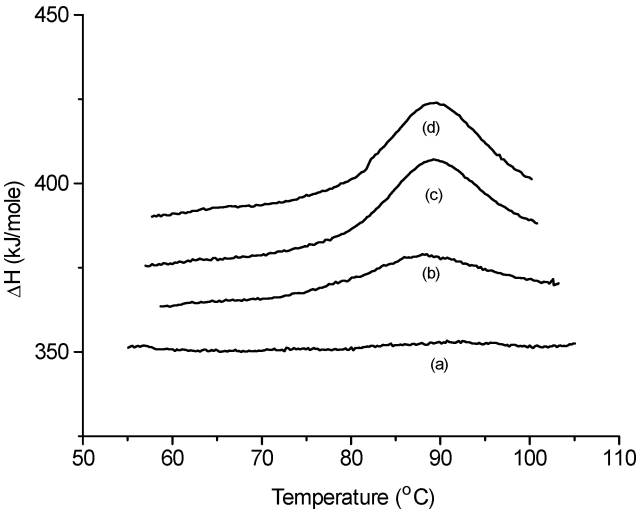

Figure 1. Offset DSC curves for thermal denaturation of β-L in the presence of 5 mM APO10 (a), 2.85 mM APO10 (b), buffer (c), and a dialyzed sample that originally contained 5.7 mM APO10 (d).

The equilibrium constant for each step, K_i, is defined in terms of the fraction of occupied sites (θ_i) and free molar concentration of APO, $[\text{APO}]_f$, by the equation

$$K_i = (\theta_i/(1 - \theta_i))[\text{APO}]_f \quad (3)$$

The free concentration of APO is related to the total APO and β-L concentrations, $[\text{APO}]_T$ and $[\beta\text{-L}]_T$, respectively, by the equation

$$[\text{APO}]_T = [\text{APO}]_f + [\beta\text{-L}]_T(n_1\theta_1 + n_2\theta_2) \quad (4)$$

The observed heat change after any injection, Q, is related to the total cell volume, V, and the enthalpy change for each step, ΔH_i, by the equation

$$Q = [\beta\text{-L}]V(n_1\theta_1\Delta H_1 + n_2\theta_2\Delta H_2) \quad (5)$$

The best values for n_i, K_i, and ΔH_i were obtained from repeated trials to fit the data until the chi-square values reached a stable minimum using the Marquardt algorithm with software provided by MicroCal.

Results

DSC Studies. *APO10.* Typical results are presented in Figure 1. The enthalpy change for the thermal transition was reduced by the addition of 2.85 mM APO10 and completely eliminated by 5 mM APO10. A 1:4 dilution of a β-L solution originally containing 5.0 mM APO10 produced the same thermogram as a sample containing 1.25 mM APO10. Holding β-L samples at either room temperature for various lengths of time or heating to 60 °C in the presence of 5 mM APO10, and once again diluted 1:4 at room temperature with buffer, did not affect the results for the thermal midpoint, T_m, calorimetric enthalpy change, ΔH_c, and van't Hoff enthalpy change, ΔH_{vH}. Lower values of ΔH_c and $\Delta H_c/\Delta H_{vH}$ were obtained for second scans which demonstrate the partially irreversible nature of thermal denaturation.[27] The extent of recovery of the native structure after exposure to APO10 was determined by the removal of the surfactant by overnight dialysis at room temperature against pH 2.9, 0.2 M glycine buffer with a solution of β-L that initially contained 5.7 mM APO10. The thermogram in Figure 1 for the dialyzed sample demonstrates complete recovery of the native form of the protein. These results indicate that the native

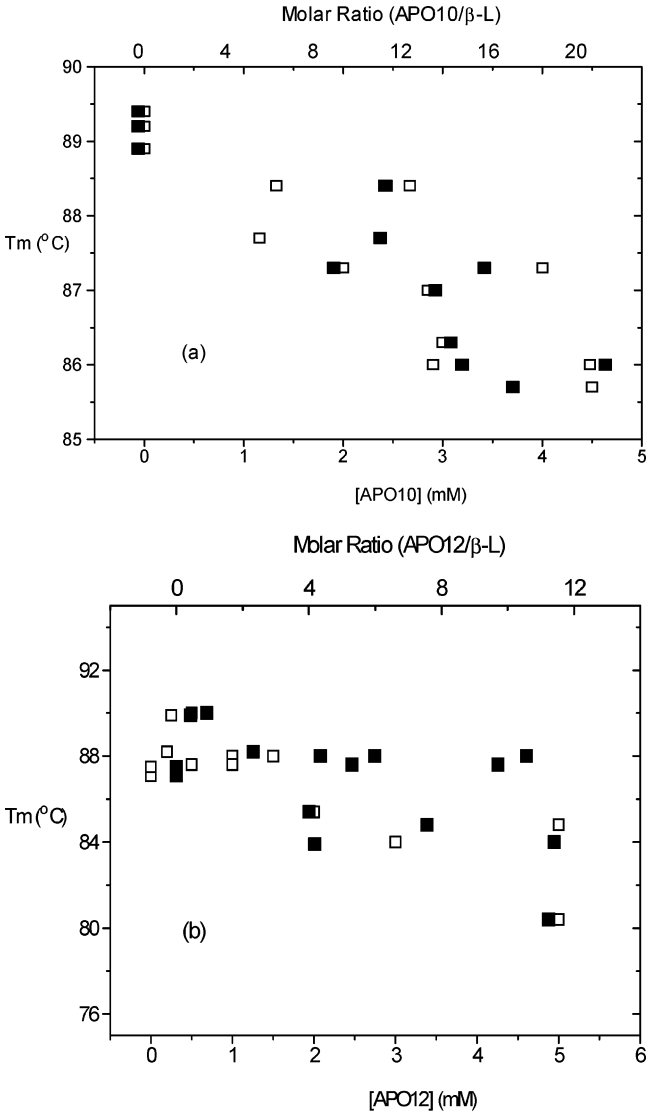

Figure 2. (a) Plot of T_m data for thermal denaturation of β-L versus various concentrations (filled squares) or molar ratios (open squares) of APO10. (b) Plot of T_m data for thermal denaturation of β-L in various concentrations (filled squares) or molar ratios (open squares) of APO12.

structure of the protein is completely disrupted by 5 mM APO10 but the process is reversed by dilution or dialysis.

A summary of DSC experiments performed with various concentrations of APO10 with different molar ratios (MRs) of APO10 to β-L is given in Figure 2a. The thermal transition temperature decreased on addition of APO10. The value of ΔH_{vH} remained nearly constant below 15 mM APO10, but a progressive decrease in the ΔH_c is reflected in the steady decline of the $\Delta H_c/\Delta H_{vH}$ ratio (Figure 3).

DSC studies were made at four different scan rates ranging from 10.7 to 66.5 K/h in the presence and absence of 3 mM APO10. The slope from a plot of T_m versus scan rate was 3 times greater with APO10 (0.06 $\pm$ 0.01 hr) than without (0.02 $\pm$ 0.004 hr), and the ratio of $\Delta H_c/\Delta H_{vH}$ when plotted versus scan rate was 4 times greater with APO10 (0.01 $\pm$ 0.003 hr) than without (0.0026 $\pm$ 0.0004 hr). These results are expected when protein unfolding is followed by an irreversible step.[27]

APO12. DSC experiments performed with various concentrations of APO12 and different molar ratios of APO12 to β-L are summarized in Figure 2b. T_m increased slightly with increasing APO12 concentration up to 0.5 mM and then decreased gradually until a thermal transition was no longer

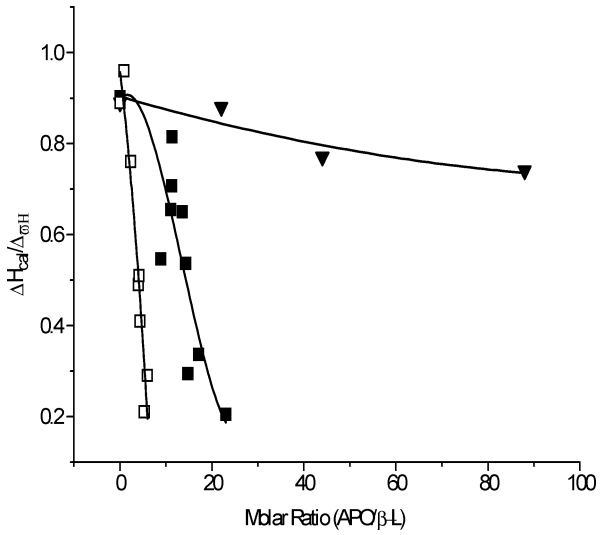

Figure 3. Variation of $\Delta H_{cal}/\Delta H_{vH}$ ratio with molar ratios of APO to β-L that accompanies the thermal denaturation of β-L in the presence of various amounts of APO8 (filled triangles), APO10 (filled squares), and APO12 (open squares) in pH 2.9, 0.2 M glycine buffer. Least-squares lines are drawn through each data set.

observed (with an MR of 11−22 and 5−10 mM APO12 depending upon the protein concentration). The value of ΔH_c decreased for APO12 concentrations beyond the critical micelle concentration (cmc) more rapidly than ΔH_{vH}, and this change was reflected in a decreasing value of the $\Delta H_c/\Delta H_{vH}$ ratio as observed for the APO10−β-L solutions (Figure 3).

APO8. Results from DSC experiments with three different APO8 concentrations are summarized in Table 1. The values of T_m, ΔH_c, and $\Delta H_c/\Delta H_{vH}$ decreased with APO8 concentration as noted for APO10 and APO12. A less abrupt decline of the $\Delta H_c/\Delta H_{vH}$ ratio was observed with increasing surfactant concentrations than for APO10 and APO12 (Figure 3). The thermal transition was completely eliminated in the presence of 15 mM APO8. Relatively dilute protein solutions, 0.113 mM, were used for these studies for comparison with the results from ITC experiments.

ITC Studies. *APO10.* To attempt to describe the binding process of APO10 to β-L at lower temperatures in more detail, several ITC experiments were performed. Protein samples were titrated with 91.6 mM APO10, and the data were corrected with blank titrations of surfactant into buffer.

The heat for the titration into buffer at 25 °C was similar in magnitude to the heat change from the titration into protein (the heat of micelle formation at 25 °C for APO10 is 9.71 kJ/mol[3]), making data analysis uncertain at this temperature. However, reliable results were obtained for titrations at other temperatures where the heat change for the blank titration was much smaller.The enthalpy change for micelle formation is very temperature dependent, and becomes equal to zero at approximately 47.8 °C for APO10.[3] Therefore, ITC titrations were conducted at temperatures near 48 °C with several different protein concentrations to minimize the heat effect of micelle dilution.

The data obtained from four titrations with APO10 are given in Figure 4a. In each case, the curves initially increase and then decrease to zero. The heat for the first few injections are negative (exothermic) below 44.8 °C, but positive above that temperature. Blank titrations of APO10 into buffer were nearly flat due to the small heat of micelle formation at these temperatures, and the heat associated with dilution of the protein solutions was negligible. The experimental data were fitted with a two

TABLE 1: Summary of Thermodynamic Parameters for the Thermal Denaturation of β-L from DSC Studies in the Presence of Various Amounts of APO8 in pH 2.9, 0.2 M Glycine Buffer

β-L (mM)	APO8 (mM)	molar ratio	T_m (°C)	ΔH_c (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_c/\Delta H_{vH}$
0.04−0.4	0	0	88 ± 1	301 ± 33	339 ± 37	0.89
0.225	5	22	86.2	304	356	0.88
0.113	5	44	84.4	234	305	0.77
0.113	10	88	81.1	230	310	0.74
0.113	15	133	*a*	*a*	*a*	*a*

[a] Could not be determined.

TABLE 2: Summary of APO10-β-L Binding Parameters from ITC Titrations of β-L with 91.6 mM APO10 in pH 2.9, 0.2 M Glycine Buffer[a]

temp (°C)	β-L (mM)	n_1	K_1 ($\times 10^4$ M^{-1})	ΔH_1 (kJ/mol)	ΔH_m (kJ/mol)
25.0	0.088	79 ± 1	0.15 ± 3.2	−10.1 ± 0.26	9.71
25.0	0.021	160 ± 1	0.16 ± 1.2	−9.87 ± 0.09	9.71
41.4	0.259	10 ± 0.1	0.17 ± 0.03	−1.17 ± 0.23	1.26
44.7	0.129	16 ± 1	4.3 ± 2	−0.50 ± 0.33	−0.41
45.4[b]	0.119	24 ± 1	2.4 ± 1	0.54 ± 0.04	−0.45
47.85	0.072	16 ± 2	3.6 ± 2	1.59 ± 0.19	−2.05
47.9	0.127	16 ± 2	14 ± 3	2.18 ± 0.19	−2.09
temp (°C)	β-L (mM)	n_2	K_2 ($\times 10^3$ M^{-1})	ΔH_2 (kJ/mol)	$n_2\Delta H_2$ (kJ/mol)
25.0	0.088	101 ± 4	3.6 ± 0.8	−2.19 ± 0.19	−222 ± 0.8
25.0	0.021	159 ± 1	3.2 ± 23	−3.64 ± 0.16	−97.1 ± 0.4
41.4	0.259	31 ± 0.4	3.2 ± 0.3	2.38 ± 0.05	71.5 ± 0.4
45.4[b]	0.119	20 ± 5	0.52 ± 0.08	4.14 ± 0.12	82.8 ± 0.4
44.7	0.129	29 ± 2	4.7 ± 0.9	3.18 ± 0.42	92.0 ± 0.8
44.85	0.072	22 ± 3	3.5 ± 0.6	6.32 ± 1.34	142 ± 4
47.9	0.127	31 ± 3	3.1 ± 0.9	4.14 ± 0.07	128 ± 0.4

[a] Standard errors resulting from fitting the data with the Marquardt algorithm reflect the precision of the data. [b] The solvent for the sample and titrant was 0.15 M NaCl adjusted to pH 7.0.

TABLE 3: Summary of APO12-β-L Binding Parameters from ITC Titrations with 81.2 mM APO12 in pH 2.9, 0.2 M Glycine Buffer[a]

temp (°C)	β-L (mM)	n_1	K_1 ($\times 10^5$ M^{-1})	ΔH_1 (kJ/mol)	ΔH_m (kJ/mol)
32.0	0.112	2 ± 1	640 ± 6400	−1.82 ± 0.14	2.21
36.4	0.265	2 ± 0.1	3.8 ± 2	−0.59 ± 0.05	−0.08
37.9	0.137	5 ± 0.2	4.4 ± 1	−0.09 ± 0.02	−1.78
37.9	0.2245	5 ± 1.5	0.34 ± 6	−0.44 ± 0.08	−1.78
38.0	0.159	8 ± 2	0.19 ± 2	−0.17 ± 0.04	−1.85
temp (°C)	β-L (mM)	n_2	K_2 ($\times 10^3$ M^{-1})	ΔH_2 (kJ/mol)	$n_2\Delta H_2$ (kJ/mol)
32.0	0.112	69 ± 2	9.1 ± 2	−0.50 ± 0.01	−34 ± 0.4
36.4	0.265	32 ± 1	1.4 ± 0.3	0.67 ± 0.03	21 ± 0.4
37.9	0.137	34 ± 0.5	3.9 ± 0.4	0.71 ± 0.02	24 ± 0.4
37.9	0.2245	31 ± 4	1.9 ± 1	0.92 ± 0.21	28 ± 0.8
38.0	0.159	30 ± 3	1.7 ± 0.4	0.96 ± 0.18	29 ± 0.4

[a] Standard errors resulting from fitting the data with the Marquardt algorithm reflect the precision of the data.

independent site binding model, and calculated values of n_1, n_2, K_1, K_2, ΔH_1, and ΔH_2 are given in Table 2. The heat of micelle formation, ΔH_m, at each temperature[3] is also included for comparison with ΔH_1. The results obtained for a titration of β-L at pH 7 in 0.15 M NaCl at 44.4 °C were similar to the results at pH 2.9 in 0.1 M glycine buffer. The protein concentration was also expressed in terms of the molecular mass of the monomer for this experiment.

The values for n_i, K_i, and ΔH_i, given in Table 2 at pH 2.9, were used with eq 1, without eq 2, to generate binding curves for each class of binding sites in the absence of the other, and these results are given in Figure 5. The enthalpy change for the first class of sites is negative below but positive above 44.8 °C, because of micelle dissociation. Three of the four curves representing K_1 approach zero when the total surfactant concentration in the calorimeter cell reaches 2 mM, which is close to the value of the cmc. The initial heat changes associated with the second site, to be identified later as due to APO10-induced protein denaturation, are endothermic, and the curve falls to zero as the MR approaches 80. The total surfactant concentration in the calorimeter cell at the end of the titration was 13.6 mM.

The percentage of native β-L in the presence of various molar ratios of APO10 was calculated from the DSC and ITC data by assuming that the ratio of the enthalpy change at a given concentration of APO10 to the enthalpy change without APO10 was equal to the ratio of native to denatured β-L. The results from these calculations are given in Figure 6. Denaturation occurs at lower values of MR for the DSC studies than for the ITC titrations likely due to the presence of an irreversible step at the higher temperatures. This view is consistent with the results of the DSC studies at different scan rates and reduced ratios of $\Delta H_c/\Delta H_{vH}$ previously noted.

APO12. Several different concentrations of β-L solutions were titrated with 81.2 mM APO12 between 32 and 38 °C. The data obtained for the titration of β-L at 32 and 38 °C are given in Figure 4b, and resemble those for the APO10 titrations. The temperatures selected for these experiments were slightly above

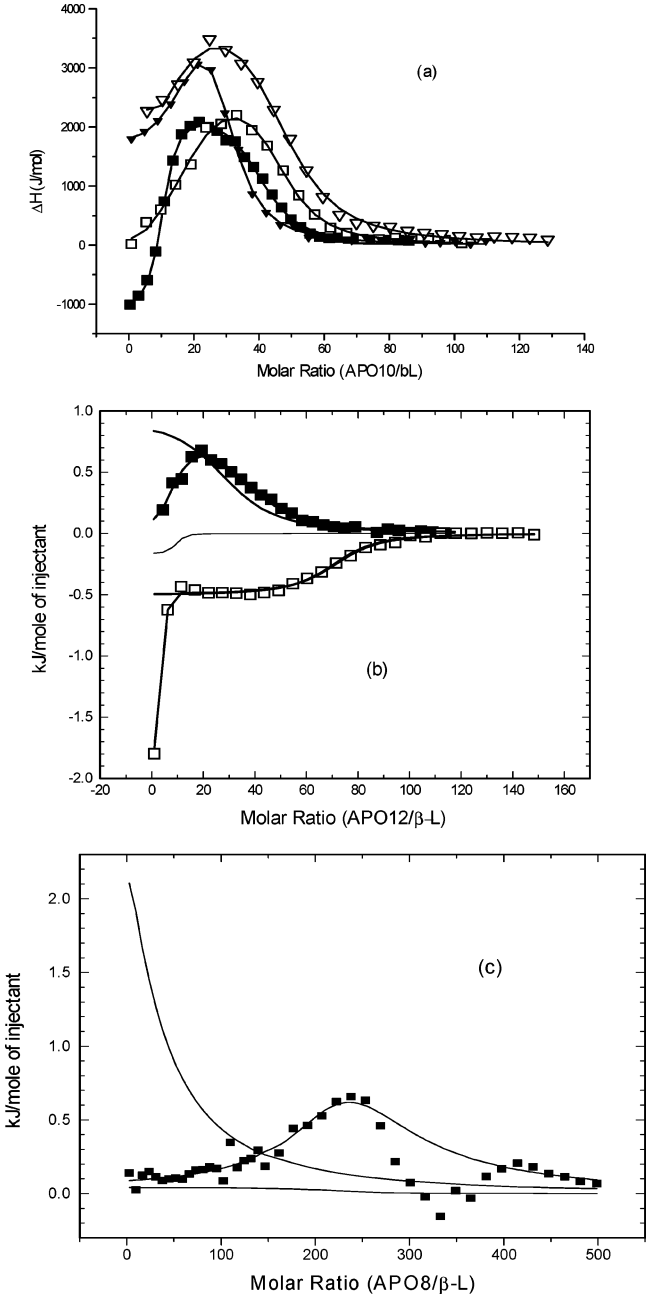

Figure 4. (a) Experimental (symbols) and calculated curves (solid lines) using the data from Table 2 for ITC titrations with APO10 at 41.4 °C (filled squares), 44.7 °C (open squares), 47.85 °C (filled triangles), and 47.9 °C (open triangles). (b) Experimental (symbols) and calculated titration curves (solid lines) obtained from ITC titrations with APO12 at 32 °C (open squares) and 38 °C (filled squares) and calculated curves for the first (dashed line) and second (dotted lines) APO12 binding sites. (c) Experimental (filled squares) and calculated curves (solid line) using the data given in the text for the ITC titration of β-L with APO8 at 60.0 °C, and calculated curves for the first (dashed line) and second (dotted line) binding sites using a single site model as described in the text.

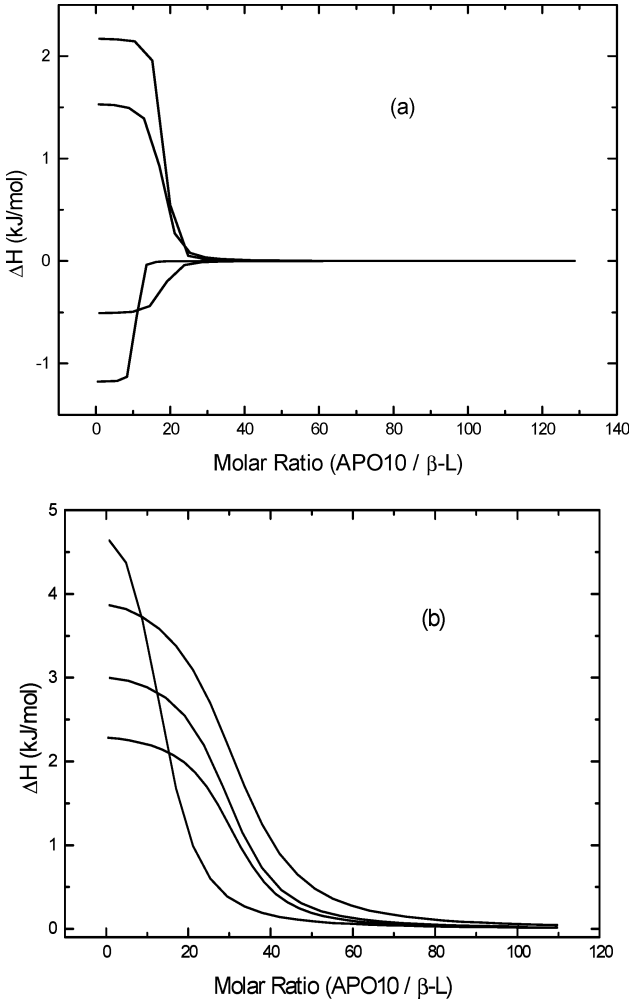

Figure 5. (a) Calculated titration curves obtained from the data in Table 2 using a one site binding model for the first binding site at 41.4 °C (dashed line), 44.7 °C (solid line), 47.85 °C (dash−dotted line), and 47.9 °C (dotted line). (b) Calculated titration curves obtained from the data Table 2 using a one site binding model for the second binding sites at 41.4 °C (dashed line), 44.7 °C (solid line), 47.85 °C (dash−dotted line), and 47.9 °C (dotted line).

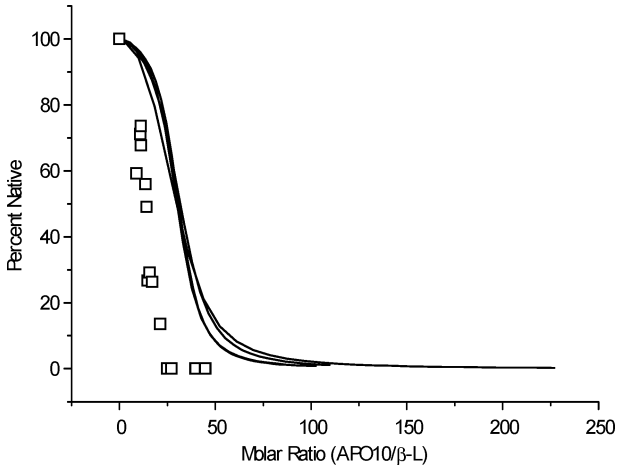

Figure 6. Comparison of denaturation curves, % native versus molar ratio of APO10 to β-L, from DSC (open squares) and ITC at 41.4 °C (dashed line), 44.7 (solid line), 47.85, °C (dash−dotted line), and 47.9 °C (dotted line) experiments for the second binding site using a single site model as described in the text.

and below the temperature at which the heat of micelle formation for APO12 is zero, i.e., 36 °C.[3] The data could be well represented with a two independent site binding model, and the results from this analysis for five titrations are given in Table 3. The enthalpy changes at 32 and 36.4 °C that describe the first binding site are approximately equal to micelle dissociation, but this is not the case for the experiments conducted at 38 °C. The observed enthalpy changes at the higher temperatures are opposite the expected sign and do not exhibit the positive temperature-dependent trend noted for the APO12 titrations at the lower temperatures and all of the APO10

titrations (Table 2). A possible explanation for this behavior may be that the cloud point of 2% APO12 in 0.2 M glycine buffer is lower than in water, 40 °C.[4] The formation of two separate liquid phases was not noted in the syringe, but a small negative enthalpy change above that expected for simple micelle dilution is possible based upon DSC studies with aqueous APO12 solutions.[25] The values obtained for n_2 and K_2 are similar in magnitude to those for APO10 binding to β-L (Table 2), and the $n_2\Delta H_2$ product reflects the expected temperature-dependent trend that accompanies the denaturation of β-L.[20,21] The model represents the data quite well, and the contributions from the second binding site are exothermic at 32 °C and endothermic at 38 °C in agreement with the data given in Figure 4b. A calculated curve for K_1 at 32 °C is not shown since it nearly superimposes on the initial region of the experimental curve.

APO8. An example of the results obtained for the titration of a 0.108 mM β-L solution with 210 mM solution of APO8 at 60 °C is given in Figure 4c. This temperature corresponds to the temperature where the heat of micelle formation of APO8 is expected to be approximately zero.[3] Although the heat of micelle formation is indeed small in 0.2 M glycine buffer, the heat of dilution below the cmc is endothermic and almost comparable in magnitude to the heat generated by reaction with β-L. The two titration curves crossed at an APO8 to β-L molar ratio of about 300, and this prevented the direct use of a two site binding model to describe the APO8−β-L titations. Instead, the normalized concentration data from the experiment without protein was subtracted for data from the titration of the β-L as described for the titration at 25 °C with APO10, and this result is shown as the filled squares in Figure 4c. The solid line represents the curve obtained from the two site model (without the seven data points from the region of the titration near the cmc where the two curves crossed). The values obtained from this analysis for n, K, and ΔH for the first and second sites for were 215 ± 90, (2.3 ± 7.2) × 10^3 M^{-1}, and 42 ± 67 J/mol and 17 ± 180, 150 ± 34 M^{-1}, and 10 ± 66 kJ/mol, respectively. Perhaps of greatest interest is the fact that the product $n_2\Delta H_2$ equals 170 kJ/mol, which is close to a value expected for the denaturation of β-L at 60 °C. The other two curves in Figure 3c result from the use of the values given above to obtain the contributions from each site to the calculated curve.

Reverse Titrations. The results from the ITC titration experiments suggest that APO association with β-L is accompanied by a small binding enthalpy change and a much larger enthalpy change due to denaturation. Therefore, reverse titrations were performed to measure the heat of denaturation directly by placing concentrated solutions of surfactant in the calorimeter cell and injecting β-L solutions into the micellar solution. Examples of the results obtained at different temperatures are given in Figure 7. Blank titrations with buffer as the titrant were also performed at corresponding temperatures for each surfactant. The corrected enthalpy changes were 205 ± 3, 109 ± 6, and 35 ± 0.4 kJ/mol for APO8, APO10, and APO12, respectively. These values and those obtained from injections at other temperatures along with the DSC data are given in Figure 8. A linear fit of the ITC injection data gave values for the slope and intercept of 5.90 ± 0.23 kJ/mol·K and −154 ± 9 kJ/mol, respectively. The value for the slope is within experimental error of the average value previously reported for the heat capacity change from DSC studies for the denaturation of β-L in the absence of urea (5.9 kJ/mol·K[21]). The negative enthalpy changes at low temperatures are consistent with the protein exhibiting cold denaturation.[21]

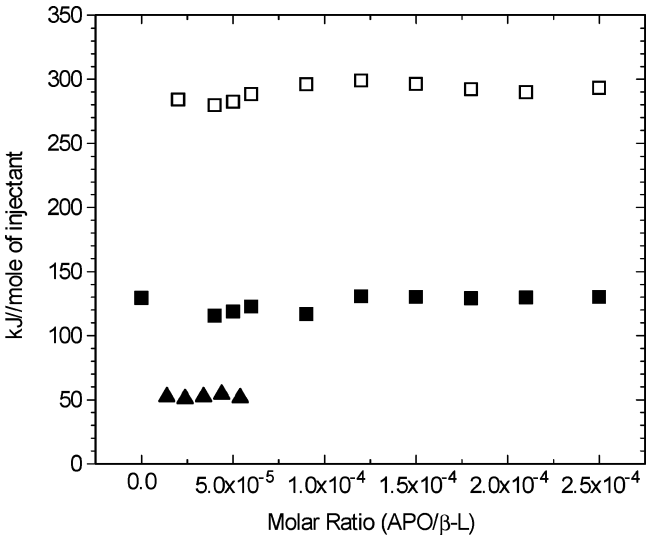

Figure 7. Examples of data obtained from reverse titrations of 0.427 mM β-L into 105 mM APO8 at 60 °C (open squares), 0.427 mM β-L into 91.6 mM APO12 (filled squares), and 0.225 mM β-L into 81.2 mM APO12 (filled triangles). The injection sequence used was four injections of 5 μL followed by six of 10 μL for the APO8 titration, 2 μL followed by four of 5 μL followed by six of 10 μL for the APO10 titration, and 2 μL followed by five of 5 μL with a 100 μL syringe for the APO12 titration.

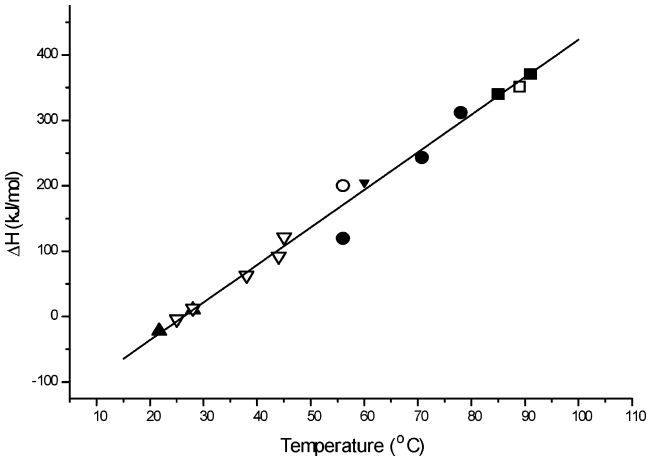

Figure 8. Comparison of enthalpy data obtained for the denaturation of β-L from previous DSC studies[14] with data from reverse ITC titration studies with APO8 (down filled triangle), APO10 (down open triangles), and APO12 (filled triangles). The buffers used for the DSC studies contained 0.1 M KCl (open square), sodium phosphate (filled squares), urea-uncorrected (filled circles), and urea-corrected (open circle). The solid line represents a linear-least-squares fit of the data

Discussion

Thermal denaturation of β-L by DSC was previously[20,21] found to be a two-state transition based on a $\Delta H_c/\Delta H_{vH}$ ratio near 1. Our data in Figure 3 in the absence of surfactants also agrees with the literature. The shapes of the DSC curves, Figure 1, and their quantitative analysis establish the completely reversible nature of APO10-induced denaturation below 61 °C. Thermal denaturation was found to be only partially reversible above 61 °C in either the presence or absence of APO10 from the values of ΔH_c for the first and second scans. The minimum molar ratio of surfactant to β-L required to eliminate the thermal transition for β-L was between 88 and 133, 21 and 25, and 11 and 22 mM for APO8, APO10, and APO12, respectively, depending upon the protein concentration. This trend is opposite the trend in the cmc values of the surfactants, 0.35, 4.0, and 40

mM, respectively.[3] Since hydrophobic bonding is the dominant force for micelle formation of both ionic and nonionic surfactants, the reciprocal nature of these two trends suggests that hydrophobic bonding is also the dominant force for the association of the APO class of surfactants with β-L.

The DSC and ITC results in the absence of APO may be described by the following simple mechanism:

$$\mathrm{N} \leftrightarrow \mathrm{D}; \qquad \Delta H_{\mathrm{N,D}}; \quad \Delta C_p = 5.9 \pm 0.4 \text{ kJ/mol·K} \tag{6}$$

$$\mathrm{D} \Rightarrow \mathrm{D}'; \qquad \Delta H_{\mathrm{D,D}'} = ? \tag{7}$$

where N, D, and D′ correspond to the native and two different denatured states of the protein, respectively. In the presence of APO surfactants, the following mechanism is proposed:

$$\mathrm{N} + n\mathrm{APO} \leftrightarrow \mathrm{N(APO)}_n; \qquad \Delta H_{\mathrm{b}} = 0; \quad \Delta C_{p,\mathrm{b}} = 0 \tag{8}$$

$$\mathrm{N{\cdot}(APO)}_n \leftrightarrow \mathrm{D{\cdot}(APO)}_n; \qquad \Delta H_{\mathrm{D}} = \Delta H_{\mathrm{cal}} = n_2 \Delta H_2; \quad \Delta C_p = 5.9 \pm 0.4 \text{ kJ/mol·K} \tag{9}$$

$$\mathrm{D{\cdot}(APO)}_n + m\mathrm{APO} \Rightarrow \mathrm{D'{\cdot}(APO)}_{n+m}; \qquad \Delta H_{\mathrm{D}'} = 0 \tag{10}$$

It appears that ΔH_{b} and $\Delta C_{p,\mathrm{b}}$ are small since the temperature dependences of ΔH_{cal} obtained from DSC studies or reverse ITC titrations and $n_2\Delta H_2$ obtained from direct ITC studies fall on the same line when plotted versus temperature (Figure 8). The value of $\Delta H_{\mathrm{cal}}/\Delta H_{\mathrm{cal}}$ ratio was lower in the presence than in the absence of APO8, APO10, and APO12 at a fixed scan rate (Figure 3) or with decreasing scan rates in the presence of APO10. These results as well as the partial reversibility of ΔH_{cal} are clear evidence of an irreversible step following thermal denaturation, eqs 7 and 10. However, there were no signs of protein aggregation since the thermal transitions were not sharpened by the presence of any of the three surfactants (only flattened) and the solutions were visibly clear when removed from the DSC cell and cooled to room temperature.

The value of $n_2\Delta H_2$ represents the process described by eqs 8 and 9, whereas the irreversible step described by eq 10 appears to occur above 60 °C since incubation of samples of β-L at 60 °C for 30 min in the presence of 5 mM APO10 did not exhibit evidence of irreversible denaturation when diluted and scanned. Since it is assumed that $\Delta H_{\mathrm{N,D}}$ equals ΔH_{D}, the value of ΔH_{cal} in the presence of the APO surfactants must reflect the decreased concentration of native protein undergoing thermal denaturation. In other words, the relative amounts of the native form, N, and denatured form, $(\mathrm{D{\cdot}(APO)}_n)$, present at each temperature are determined by the equilibrium constants for the overall reaction represented by eqs 8 and 9. The presence of the irreversible step described by eq 10 would be responsible for making the extent of denaturation determined by DSC greater than that determined by ITC in the presence of the same concentration of APO (Figure 3).

A simple mechanism does not exist to account for the numerical values of n_1 and K_1. However the values of ΔH_1 are close to those for micelle dissociation, $-\Delta H_{\mathrm{m}}$, and the enthalpy changes exhibit the same trend with temperature; i.e., $\Delta C_p = 506 \pm 29$ J/mol·K for ΔH_1 versus -515 ± 8 J/mol·K for micelle formation.[3]

It is tempting to speculate that a partially unfolded molten globule-like species is formed as a result of binding the APO surfactants to β-L based upon the similarity of the thermograms observed in this study to those reported for the effects of increasing concentrations of guanidine hydrochloride on the thermal unfolding of apo-α-lactalbumin (reduced ΔH_{cal} but normal ΔH_{vH}).[28] Also, an intermediate was observed during studies of the cold denaturation of β-L, and the weakening of hydrophobic bonds at low temperatures is proposed to be responsible for the formation of this intermediate.[29] However, there is no supporting evidence for the existence of intermediates formed during the heat denaturation of β-L in the absence or presence of urea,[21] which indicates that the protein unfolds as a single cooperative unit. The comparable partial reversibility of the thermal denaturation of β-L in the presence or absence of APO surfactants to that in the presence of urea suggests that thermal denaturation of β-L is highly cooperative in the presence of the APO surfactants as well.

Despite the similarity between the shapes of the ITC curves obtained in this study to the ones reported by Sigh and Kishore[30] for TX-100 binding to two classes of serum albumin sites, totally different mechanisms are apparently responsible for the data. Although our data also required the assumption of at least two classes of binding sites to fit the experimental curves, micelle dilution and protein denaturation were clearly responsible for the observed enthalpy changes. The authors apparently used a TX-100 dilution curve as a blank that was subtracted from the data obtained for the protein. A figure was prepared (Supporting Information) with published values of n_1, n_2, K_1, K_2, ΔH_1, and ΔH_2[30] for the interaction between TX-100 and bovine serum albumin (BSA). The shape of the resulting curve that represents K_2 clearly resembles a binding curve although the negative enthalpy change is not expected.[31] The shape of the curve described by K_1 resembles a TX-100 dilution curve since the breakpoint around MR = 4 is located near the cmc (0.25 mM) at a stoichiometric TX-100 concentration of 0.3 mM. The enthalpy change does not correspond to the heat of micelle dilution due to the way that the data were treated. Further evidence that the first binding reaction for TX-100 to BSA is a micelle dilution reaction rather than a binding reaction is the fact that both ΔH_1[30] and ΔH_{m}[25] extrapolate to the temperature where ΔH is zero (46 ± 0.5 °C), which is a characteristic temperature for surfactants.[32] These similarities between the shape of the curve described by K_1 and a TX-100 dilution curve and the common intercept temperature may be a coincidence, but it is unlikely. The suggestion that it may not be a coincidence justifies the need for further ITC studies similar to the one by Singh and Kishore.[30] The instrumentation that is available today makes it possible to do so. Understanding the basis for these differences will contribute to an understanding of the basis for specificity of ligand-protein binding interactions in general.

Finally, it is interesting that the average value of n_2 for APO10 and APO12 binding to β-L, 30 ± 5, is sufficient for surfactant monomers to cover about 50% of the surface area of β-L. This implies that small clusters of surfactant are associated with β-L in a form that could be generically described as a *submicelle*[33] since the aggregation numbers for micelle formation for APO10 and APO12 are about 130 and 2000, respectively.[4] Therefore, the presence of hydrophobic sites with an affinity for hydrophobic ligands could play a role in the biological function of β-L beyond the established role for the sites in the hydrophobic cavity, such as aiding in digestion by newborn mammals.

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Supporting Information Available: A figure is enclosed that represents the experimental titration curve[30] for the binding

of TX-100 to serum albumin that was previously ascribed to two binding sites. The titration curve was decomposed into two theoretical curves by using the binding constants and enthalpy changes reported by the authors. The shape of one curve (the weaker site) clearly resembles a binding curve. However, the shape of the other curve (the stronger site) closely resembles a TX-100 dilution curve, with a break point occurring at a stoichiometric TX-100 concentration of 0.3 mM (cmc for TX-100 is 0.25−0.28 mM). Further evidence that the curve for the stronger site represents micelle dilution rather than binding is that the enthalpy change for the strong site and ΔH_m extrapolate to zero at the same temperature (46 ± 0.5 °C), which is a characteristic temperature for a given surfactant.[32] The similarity between the shape of the calculated curve for the stronger site and a micelle dilution curve as well as the common intercept temperature may be a coincidence, but it warrants further study. This material is available free of charge via the Internet at http://pubs.acs.org.

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