# Interaction of Alkylsulfonate Ligands with β-Lactoglobulin AB from Bovine Milk

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The interaction of alkyl sulfonate ligands (AL) with bovine β-lactoglobulin AB was measured using Trp fluorescence enhancement. One binding site per protein molecule was observed. The location of this site was related with the dimer formation and could be coincident with the fatty acids and SDS binding site. The apparent binding constants for AL were in the range of $10^{-6}$ M, at pH 6.8. At pH 3.0 no binding was observed by this fluorescence method. The strength of the interaction was decreasing in the following way: AL16 > AL12 > AL14 ≫ AL10. Other sites on the monomer were evidenced by the protective action of the AL toward the urea unfolding of the protein.

**Keywords:** *β-Lactoglobulin; hydrophobic ligands; fluorescence; unfolding*

## INTRODUCTION

β-lactoglobulin (β-LG) is the major protein in the whey of ruminant milk. This protein may be classified in the super family of the hydrophobic molecule transporters termed *lipocalins*. These small globular proteins share a common three-dimensional structural pattern consisting of eight strands of antiparallel β sheet twisted into a cone-shaped barrel which constitutes a hydrophobic pocket.

β-LG is a globular protein with a monomer molecular weight of about 18 300 and exists in various oligomeric states as function of pH, temperature, concentration, ionic strength, and genetic variant. Below pH 3.5 and near pH 7.0 β-LG solutions have to be considered as a mixture of monomers and dimers. The equilibrium between the monomeric and dimeric form of β-LG is shifted to the monomeric form when the ionic strength is decreased or the temperature is increased (5−76 °C) (Aymard et al., 1996; Renard et al., 1998).

β-LG is known to bind retinoids, fatty acids and SDS (Pérez and Calvo, 1995). Binding of retinoids to β-LG causes a quenching of fluorescence of tryptophan (Trp) (Fugate and Song, 1980; Cho et al., 1994; Dufour et al., 1994; Wang et al., 1997); however, the interaction between β-LG with palmitate and SDS enhances the Trp fluorescence (Pérez et al., 1989; Frapin et al., 1993). Consequently, the changes of fluorescence intensity has been used to study the binding of these ligands to the protein. While the binding constants has been calculated, the region of the protein involved in the interaction still remains unclear. β-LG has two potential sites for binding hydrophobic ligands: one in the calyx formed by the β-barrel (Papiz et al., 1986) and the other in an external hydrophobic pocket between the α-helix and the β-barrel (Monaco et al., 1987). The sites for binding of palmitate and retinoids on β-LG have been the subject of several investigations, but the results have not been clear. It has been suggested that retinol binds in the external pocket (Monaco et al., 1987; Puyol et al., 1991); however, most of the experimental evidence points to the calyx as the binding site (Cho et al., 1994; Narayan and Berliner, 1997). Conflicting conclusions also have been drawn regarding the binding site for palmitate. Some investigators have suggested that these two ligands bind in the same site (Puyol et al., 1991; Creamer, 1995), but others (Frapin et al., 1993; Dufour et al., 1994; Wang et al., 1998) have reported evidence indicating different binding sites for retinol versus those for fatty acids and other aliphatic compounds such as SDS. Frapin et al. (1993), using fluorescence measurements, have proposed that fatty acids could be bound at the interface between monomers since both monomerized bovine β-LG (acid pH) and monomeric porcine β-LG do not bind fatty acids.

Wang et al. (1999) have reported that retinol binds in the central calyx and palmitate binds in the surface pocket near the dimer contact region. Qin et al. (1998) and Wu et al. (1999) have shown that β-LG can be cocrystallized with palmitic acid within its central cavity. This fact may be considered to be strong evidence for the central cavity being the major binding site in solution as well in the crystal for the hydrophobic ligands. Some authors also reported that the binding of palmitate and SDS increases the stability of the protein toward urea unfolding (Creamer, 1995; Narayan and Berliner, 1997).

In this work we used a group of alkylsulfonate ligands (AL), characterized by a similar sulfonate head and a progressively longer hydrocarbon tail, to elucidate the interactive properties of β-LG. This study may generate ideas for applications of this protein, produced in large amounts by the dairy industry, as a carrier of a wide range of hydrophobic molecules.

## EXPERIMENTAL PROCEDURES

**Materials.** Bovine β-LG AB and the AL were purchased from Sigma-Aldrich. The AL used were the sodium salts of 1-decanesulfonic acid (AL10), 1-dodecanesulfonic acid (AL12), 1-tetradecanesulfonic acid (AL14), and 1-hexadecanesulfonic acid (AL16). All other chemicals were analytical reagent grade.

**Binding of AL to β-LG.** The binding of AL was measured by following the increase of protein Trp fluorescence at 337

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nm (excitation at 295 nm) in a Jasco FP-770 spectrofluorometer. The following procedure was used for titration of native β-LG with AL: 2.5 mL of 20 μM β-LG solution was placed in a cuvette, and small increments of the ligand solution diluted in ethanol were injected in the cuvette with a micropipet. The experiments were performed in 50 mM phosphate buffer (pH 6.8) or 50 mM acetate buffer (pH 3.0). To subtract the Trp fluorescence changes induced by alcohol, a blank containing β-LG solution titrated with ethanol was used as control. The fluorescence intensity changes of the blank were subtracted from fluorescence intensity measurements of the ligand/protein complexes for every considered titration point. In all cases, before correction for the blank, β-LG tryptophan initial fluorescence was normalized at $F = 1$. The binding of AL12 and AL14 was performed at two temperatures: 25 and 40 °C. The effect of urea on the AD16-β-LG interaction was also studied in this work.

**Determination of the Apparent Binding Constants.** The numeric values of binding parameters reported in this work were calculated according to the procedure of Cogan et al. (1976). By plotting ($P_o\alpha$) vs $B[\alpha/(1-\alpha)]$, a straight line is obtained with an intercept of $K'_d/n$ and a slope of $1/n$, where $K'_d$ is the apparent dissociation constant, $n$ is the apparent molar ratio of ligand/β-LG at saturation, $P_o$ is the total protein concentration, and $B$ is the total ligand concentration. α is defined as the fraction of unoccupied binding sites on the protein molecules.

The value of α was calculated for every point on the titration curve using the following equation:

$$\alpha = \frac{F_{max} - F}{F_{max} - F_o}$$

where $F$ represents the fluorescence intensity at a certain concentration of the ligand, $F_{max}$ is the fluorescence intensity upon saturation of all the protein molecules, and $F_o$ represents the initial fluorescence intensity.

**Equilibrium Unfolding of β-LG.** A series of urea solutions were made from weighed quantities of urea in phosphate or acetate buffer. A 2.5 mL aliquot of the various urea solutions was placed directly into the spectrofluorimeter cell and an aliquot of β-LG solution was added to give a final protein concentration of 20 μM. The emission spectrum (excitation at 295 nm) of each solution was run about 10 min after protein addition, and then it was scanned across the peak and $\lambda_{max}$ measured. $\lambda_{max}$ changed with urea concentration in a simple sigmoidal fashion in accordance with the loss of the protein native structure (Creamer, 1995). The urea concentration ($C^*$) at which the midpoint transition occurred was determined, and the $\lambda_{max}$ at this concentration was named as $\lambda^*_{max}$. Aliquots of AL solution were then added to the β-LG−urea mixture of concentration $C^*$. About 2 h after each addition, the spectrum of each mixture was redetermined in order to obtain $\lambda_{max}$. The differences between these values and $\lambda^*_{max}$ were calculated ($\Delta\lambda_{max}$).

## RESULTS

Figure 1A shows the binding isotherms of different AL to β-LG at 25 °C, and pH 6.8. The apparent dissociation constants ($K'_d$) and apparent molar ratios ($n$) for AD/β-LG complexes, obtained by fitting of Cogan's plots (Figure 1B), are shown in Table 1. The values of $K'_d$ obtained were of the same order of the values reported by several authors for amphiphilic ligands with longer aliphatic chains such as SDS and fatty acids (Frapin et al., 1993; Lamiot et al., 1994). $n$ values ranged from 0.70 to 1.70, suggesting the presence of two binding sites on the β-LG dimer. AL16 shows the highest affinity (lowest $K'_d$) for β-LG among the studied ligands. At pH 3.0, no binding was detected by fluorescent measurements for any AL.

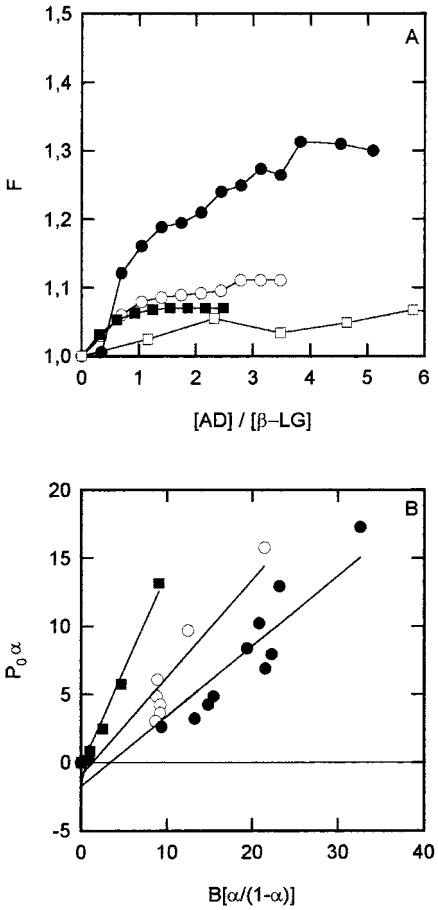


**Figure 1.** (A) Corrected β-LG tryptophan fluorescence emission titration curves with AL10 (□), AL12 (○), AL14 (●), and AL16 (■). (B) Graphic presentation (Cogan et al., 1976) of $P_o\alpha$ vs $B[\alpha/(1-\alpha)]$ of β-LG titration with AL12 (○), AL14 (●), and AL16 (■). Protein concentration: 20 μM. Temperature: 25 °C, pH = 6.8.

**Table 1. Apparent Dissociation Constants ($K'_d$) and Apparent Molar Ratios ($n$) for AD/β-LG Complexes**

| compd | $T$ (°C) | $K'_d$[a] × $10^6$ (M) | $n$[a] |
|---|---|---|---|
| AD10 | 25 | n.d.[b] | n.d. |
| AD12 | 25 | 1.89 ± 0.92 | 1.27 ± 0.14 |
| AD12 | 40 | 0.72 ± 0.42 | 0.91 ± 0.05 |
| AD14 | 25 | 4.97 ± 1.67 | 1.70 ± 0.20 |
| AD14 | 40 | 0.52 ± 0.63 | 0.47 ± 0.12 |
| AD16 | 25 | 0.25 ± 0.16 | 0.70 ± 0.03 |

[a] Each value is the average of at least three determinations. [b] Not determined.

The effect of temperature on the binding of AL12 and AL14 is shown in Figure 2. Both $n$ and $K'_d$ values (Table 1) decreased when the binding was assayed at 40 °C.

The effect of urea concentration on the $\lambda_{max}$ of the emission spectra of the β-LG in the presence and abscence of AL at pH 6.8 is shown for AL16 in Figure 3. Similar results were obtained with the other ligands and at pH 3.0. Although $\lambda_{max}$ was nearly constant in the range of urea concentrations 0−3 M, an increase of about 15−20% in fluorescence intensity was observed in the same range. In Figure 3 the variation of the apparent association constant ($K'_a = 1/K'_d$) with urea concentrations is also included for the binding at pH 6.8. No interaction was found for values of urea concentrations higher than 3 M. The obtained results indicate that AL stabilizes the structure of β-LG toward urea unfolding. In Figure 4 the $\Delta\lambda_{max}$ were plotted vs

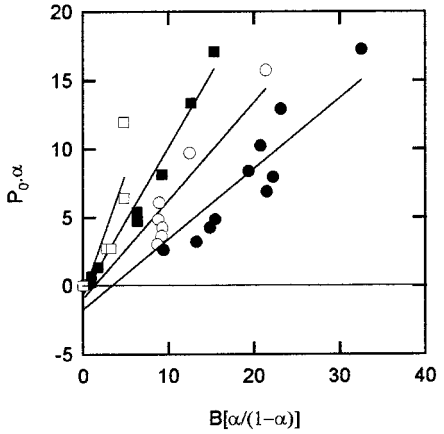


**Figure 2.** Cogan plot of $\beta$-LG titration with AL12 at 25 °C (○), and at 40 °C (■); with AL14 at 25 °C (●), and at 40 °C (□). Protein concentration: 20 $\mu$M, pH = 6.8.

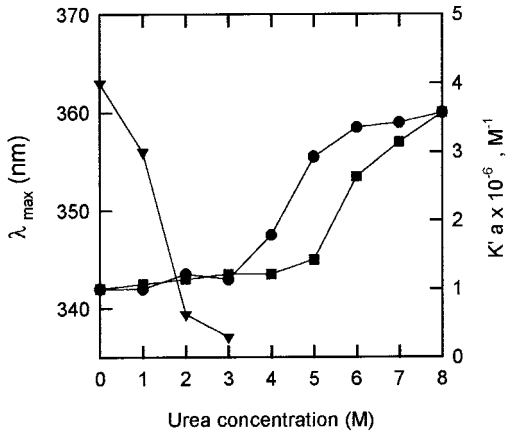


**Figure 3.** Effect of urea concentration on the $\lambda_{max}$ of $\beta$-LG intrinsic fluorescent emission in the absence (●) and presence (■) of AL16. (▼) Relationship between urea concentration and binding affinity of $\beta$-LG for AL16. Protein concentration: 20 $\mu$M. AL16 concentration: 73 $\mu$M. Temperature: 25 °C, pH = 6.8.

AL concentration for the different AL used, at pH 3.0 and 6.8. Less effective was the AL10; meanwhile, AL12, AL14, and AL16 had a similar action.

## DISCUSSION

The present results show that AL, as well as fatty acids and SDS, enhances the Trp fluorescence at pH 6.8. In the binding study, the lowest apparent dissociation constant was obtained for AL16 (Table 1). It seems that the $\beta$-LG binding site for AL can accommodate an aliphatic chain constituted by 16 carbon atoms. Similar results were reported by Frapin et al. (1993) for fatty acids, suggesting that AL and fatty acids interact with the same binding site on $\beta$-LG. However, although these authors did not find $\beta$-LG fluorescence enhancement for fatty acids of aliphatic chains ≤10 carbon atoms, we did observed a weak signal for AL10 (Figure 1). The decrease of $K'_d$ values with increasing temperature point to a major contribution of hydrophobic interactions in the binding.

On the other hand, the variation of $n$ with the temperature could be related to the monomer−dimer equilibrium of $\beta$-LG at pH 6.8. In that sense the results of Aymard et al. (1996) showed that increasing the temperature from 25 to 40 °C produced a decrease of

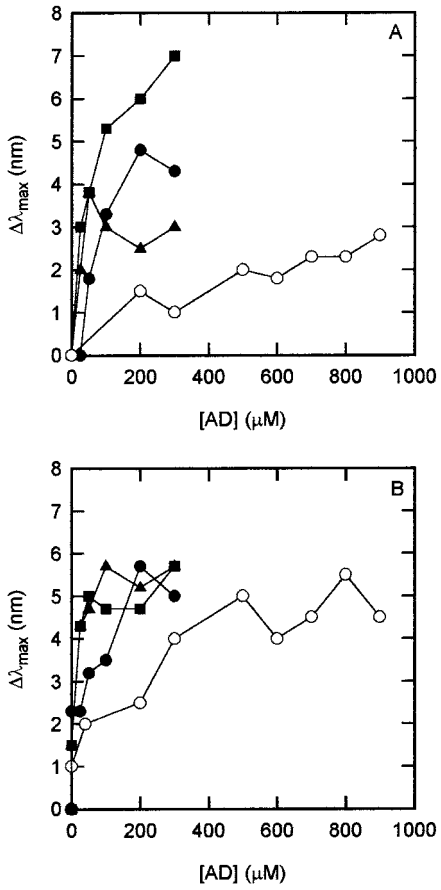


**Figure 4.** $\Delta\lambda_{max}$ vs AL concentration. (A) At pH 3.0. (B) At pH 6.8. (○) AL10, (●) AL12, (■) AL14, (▲) AL16. Protein concentration: 20 $\mu$M. Temperature: 25 °C.

the dimeric fraction. Therefore, the decrease of $n$ in the same temperature range suggests that there are binding sites related to the monomer−monomer interaction. This result was consistent with the Wang (1998) analysis from a different approach.

Several authors related the spectrophotometric changes observed for $\beta$-LG at urea concentration about 4.5 M to the loss of $\beta$-sheet structure, while at urea concentration up to 3 M, only the complete dimer dissociation takes place (O'Neill and Kinsella, 1987; Creamer, 1995). O'Neill and Kinsella (1987) indicated that the effect of urea on the interaction of hydrophobic ligands to $\beta$-LG in the range 0−3 M urea is caused primarily by the effect of urea on the conformation of the protein and not by reduction of the driving force of the binding. Renard et al. (1998) have demonstrated that the association in dimers reduces the intrinsic Trp fluorescence of $\beta$-LG; meanwhile, the position of $\lambda_{max}$ remains constant. Our results (Figure 3) showed that $\lambda_{max}$ was nearly constant at urea concentrations in the range 0−3 M with an increase of about 15−20% in fluorescence intensity in the same range. This fact supports the hypothesis that the initial effect of urea was the dimer dissociation. Therefore, the loss of binding observed by the fluorescence method at urea concentrations near 3−4 M, where the central calyx is mainly conserved, was also consistent with AL binding sites out of the $\beta$-barrel and related to the dimer existence (Frapin et al., 1993).

The recent results obtained by Wu et al. (1999) showing the cocrystallization at pH 7.5 of $\beta$-LG with palmitic acid within its central cavity appears opposite

to this hypothesis. The failure of their experiments at pH 6.5 was attributed to the occlusion of the binding site, via a conformational change, at pH values below 7.0. It is tempting to speculate that the inner site becomes accessible at high pH, whereas at lower pH only the sites related to the dimer existence are available.

Increased β-LG stability has been observed in the presence of palmitate and SDS (Creamer, 1995). Higher stability in the presence of these ligands may result from a required dissociation prior to unfolding. Binding to sites in both monomers has been invoked as the cause of the protective effect. A similar analysis may be applied to this protective action of AL in β-LG unfolding by urea at pH 6.8 (Figure 4B), but this was not the case for pH 3.0 (Figure 4A), where the β-LG is already in its monomeric form. Therefore, our results suggest that the protective action of AL was exerted by the fraction of AL bound to the monomer, not related to the dimer existence. This is a fraction not detectable by fluorescence enhancement but evidenced by others methods. Wang et al. (1998), using an ultrafiltration method, determined that 2 mol of palmitate are bound per mol of dimer with a higher affinity, and a larger number of palmitate molecules (>20) are bound per mole of monomer with a lower affinity.

The efficiency of AL in stabilizing the native structure of β-LG was also related to the length of the hydrocarbon tail. This behavior could indicate that the increased stability of the protein to the unfolding may be attributed to the strengthening of the hydrophobic interactions and to the exclusion of water from the binding sites by the hydrophobic ligands.

## ABBREVIATIONS USED

β-LG, beta-lactoglobulin; AL, alkyl sulfonate ligands; AL10, 1-decanesulfonic acid; AL12, 1-dodecanesulfonic acid; AL14, 1-tetradecanesulfonic acid; AL16, 1-hexadecanesulfonic acid; SDS, sodium dodecyl sulfate; Trp, tryptophan.

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