Surface Hydrophobicity and Aggregation of β-Lactoglobulin Heated near Neutral pH

Anne Laligant, Eliane Dumay, Carmen Casas Valencia,† Jean-Louis Cuq, and Jean-Claude Cheftel*

Laboratoire de Biochimie et Technologie Alimentaires and Ingénierie des Systèmes Macromoléculaires, Centre de Génie et Technologie Alimentaires, Université de Montpellier II (Sciences et Techniques), 34095 Montpellier Cedex 5, France

The surface hydrophobicity of β-lactoglobulin (βLG) was investigated by binding of 8-anilino-1-naphthalenesulfonate (ANS) or retinol (RET). Analysis of ANS–βLG binding at pH 7.5 gave a low number of sites (n = 0.03–0.4) and a high dissociation constant (K_D = 2.0–6.5 × 10^{-5} M), suggesting a low affinity of βLG for ANS. Analysis of RET–βLG binding at pH 7.5 indicated one type of site. n tended to 0.9 when the RET/βLG ratio increased (with a K_D between 0.12 × 10^{-7} and 1.7 × 10^{-7} M). This result is consistent with the existence of one strong hydrophobic binding site for retinol in βLG. The affinity of native βLG for retinol decreased when the pH was lowered from 7.5 to 6.5, probably as a result of the well-known βLG conformational change and the higher proportion of dimers. Heating 1% βLG solutions also decreased affinity for retinol. At pH 6.5, this decrease took place after 3 or 6 min at 75 or 90 °C but required at least 6 min at 90 °C at pH 7.5. However, hydrophobic interaction chromatography indicated a smaller loss of native βLG after heating at pH 6.5 than at pH 7.5. Gel permeation chromatography (at pH 6.0) revealed that progressive heating at pH 7.5 caused the dissociation of βLG dimers into monomers and a subsequent aggregation into oligomers. Heating at pH 6.5 caused the formation of high molecular weight soluble aggregates (labile to SDS). The lesser affinity for retinol probably resulted from these aggregation phenomena.

INTRODUCTION

The biochemical and physicochemical properties of β-lactoglobulin (βLG) have been extensively studied (Tanford, 1970; MacKenzie, 1971; Swaisgood, 1982). The primary, secondary, and tertiary structures of the molecule are known (Green et al., 1979; Eigel et al., 1984; Monaco et al., 1987). βLG undergoes a conformation change in the pH 6.5–7.5 region (Dupont, 1965; MacKenzie and Sawyer, 1967). It binds ligands such as anilinonaphthalenesulfonate (ANS) (Mills and Creamer, 1975; Haque and Kinsella, 1987), fatty acids (Spector and Fletcher, 1970), alkanes or flavor compounds (Mohammazadeh-K, 1969; Jasinski and Kilara, 1985; O'Neill and Kinsella, 1987), and retinol (RET) (Futterman and Heller, 1972; Hemley et al., 1979; Fugate and Song, 1980; Monaco et al., 1987). The method of ligand binding has been used to assess the surface hydrophobicity and the emulsifying properties of some proteins. A few investigators have attempted using ligand binding to determine the changes taking place in βLG upon heating. Hayakawa and Nakai (1985) measured the initial slope of the fluorescence intensity (S_0) vs protein concentration as an index of hydrophobicity, according to Kato and Nakai (1980); after pH 7.0 βLG solutions were heated, at 75–95 °C for 10 min, a decrease in S_0 was observed for the binding of *cis*-parinaric acid, while an increase in S_0 was noted for ANS binding. Haque and Kinsella (1987) reported an increase in the fluorescence intensity of the ANS–βLG conjugate after βLG was heated above 70 °C, at pH 6.8 and in the presence of ethylene glycol bis(β-aminoethyl ether)tetraacetic acid. O'Neill and Kinsella (1988) reported that heating βLG at pH 6.7 and 75 °C for 10–20 min induced the formation of aggregates with several (low affinity) binding sites for 2-nonanone. Jang and Swaisgood (1990) used *trans*-retinal affinity chromatography to assess the effects of heating βLG at 90 °C and pH 2.5. The affinity of βLG for immobilized retinal was found to decrease.

Fluorescent probes are frequently used for binding studies since their analysis is relatively easy and no separation of the bound from the free ligand is required. Nevertheless, few investigators determine the number of binding sites (n) and the corresponding dissociation constant (K_D).

In the present study, the affinity of native βLG for ANS and for retinol was first determined by measuring the binding parameters (n and K_D) at pH 7.5. The changes in the surface hydrophobicity of βLG taking place when a 1% protein solution at pH 7.5 or 6.5 was heated were then assessed by retinol binding at pH 7.5 or 6.5, respectively. In parallel, the content of residual native βLG and the extent of βLG aggregation after heating were determined by hydrophobic interaction chromatography, gel permeation chromatography, and SDS–polyacrylamide gel electrophoresis. The validity and usefulness of the fluorescent ligand binding approach are discussed.

MATERIALS AND METHODS

Materials. β-Lactoglobulin (βLG) (L-2506, batch 52F-8035, noncrystallized) was purchased from Sigma Chemical Co. (St. Louis, MO). βLG concentration was determined at 280 nm, using an $E^{1\%}_{1cm}$ of 9.5 (MacKenzie and Sawyer, 1967). The ammonium salt of 1-anilino-8-naphthalenesulfonic acid (ANS) (A-3125) and *all-trans*-retinol (RET) (R-2750) were also from Sigma. All other chemicals used in this study were of analytical grade.

Heat Processing of Protein Solutions. Aqueous dispersions (1% protein, w/v) of βLG in distilled water were adjusted to pH 6.5 or 7.5 with 0.1 N NaOH or HCl. Ten milliliters of the dispersions was placed in glass tubes (18 mm i.d., 180 mm in height), heated for 3 or 6 min in a water bath at 75 or 90 °C and then rapidly cooled to room temperature with tap water. Samples were then diluted as needed, centrifuged (18800g for 15 min), and filtered on Durapore membranes (0.22 μm; Millipore, Bedford, MA). The resulting clear filtrates were analyzed by liquid

† Present address: Dpto Higiene y Tecnologia de los Alimentos, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain.

0021-8561/91/1439-2147$02.50/0

chromatography or electrophoresis or were subjected to fluorescent ligand binding.

Liquid Chromatography. The quantitation of soluble proteins was carried out by gel permeation chromatography (GPC), using a TSK 3000 column (7.5 mm × 300 mm; Toyo Soda, Tokyo), or by hydrophobic interaction chromatography (HIC) on a phenyl-Superose column (HR 5/5, Pharmacia, Uppsala, Sweden), as already described by Dumay and Cheftel (1989). GPC was performed at a flow rate of 0.5 mL/min, with a 0.06 M phosphate buffer, pH 6.0, containing 0.15 M Na_2SO_4. HIC was performed at a flow rate of 0.5 mL/min, with a linear gradient of ammonium sulfate in 30 min. The starting buffer, 0.05 M Tris-HCl, pH 7.5, 1.27 M in ammonium sulfate, was progressively replaced by 0.05 M Tris-HCl buffer, pH 7.5, 35% (v/v) in acetonitrile. Before HIC, the protein solutions were diluted with 0.05 M Tris-HCl, pH 7.5, containing 1.27 M ammonium sulfate, left overnight at room temperature, and then centrifuged at 18800*g* for 15 min to remove insoluble proteins. All results are the average of two or three chromatographic determinations. For three injections of the same βLG solution, the quantitation of βLG (or βLG variants) was obtained with a coefficient of variation of 2–3% for GPC or 3–4% for HIC.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970), as modified by Shimada and Cheftel (1989). A linear gradient separating gel (8–20% in polyacrylamide) was prepared with an acrylamide/bis(acrylamide) ratio of 22.5. The stacking gel contained 8% polyacrylamide (same ratio). Protein solutions were diluted in 50 mM Tris-HCl buffer, pH 6.8, containing 7.5% glycerol and 2% SDS (w/v) and then heated in boiling water for 4 min.

Binding of Fluorescent Ligands. Determination of the surface hydrophobicity of βLG was carried out using ANS or RET as a fluorescent ligand. The relative fluorescence intensity (FI) of the ligand–protein conjugates was measured on a JY3 spectrofluorometer (Jobin-Yvon, Paris) at room temperature. The wavelengths of excitation (λ_{EXC}) and emission (λ_{EM}) were, respectively, 370 and 480 nm for ANS and 330 and 470 nm for RET, with slit widths of 10 nm. The instrument was calibrated so that 1 mg/L solution of quinine sulfate gave a fluorescence intensity of 45.

Solutions of ANS in sodium phosphate buffer (20 mM, pH 7.5) or of RET in absolute ethanol were prepared daily. In the case of RET binding, both ethanol and the phosphate buffer used for protein dilution were flushed with N_2 to inhibit RET oxidation by air. The concentration of ligand was determined from absorption measurements using the following molar coefficients: 46 000 M^{-1} cm^{-1} at 325 nm for RET in ethanol (Futterman and Heller, 1972) and 4950 M^{-1} cm^{-1} at 350 nm for ANS in 20 mM, pH 7.5, sodium phosphate buffer (Weber and Young, 1964). The FI of each protein–ligand conjugate was measured as a function of both protein and ligand concentrations.

(*1*) *Measurements at Increasing Protein Concentration for Given Ligand Concentrations* (L_0). The protein sample was diluted with 20 mM sodium phosphate buffer, pH 7.5, to reach protein concentrations (P_0) ranging from 3×10^{-5} to 4×10^{-4} M for ANS–βLG experiments and from 10^{-6} to 4×10^{-5} M for RET–βLG. Such protein solutions have no detectable turbidity and give very low FI values. ANS (100 μL) or RET (80 μL) solutions were then brought to a final volume of 3 mL with the various protein solutions at room temperature. After rapid mixing and standing for 1 min, the FI was measured within the next minute.

Binding data were analyzed using either the Scatchard equation (eq 1) (Scatchard, 1949) or the Klotz double-reciprocal equation (eq 2) (Klotz, 1947)

$$\nu/L = n/K_D - \nu/K_D \quad (1)$$

$$1/\nu = 1/n + K_D/(nL) \quad (2)$$

where ν is the moles of ligand bound per mole of total protein, L is the free ligand concentration, P_0 is the total protein concentration, K_D is the apparent dissociation constant, and n is the number of binding sites per molecule of protein. L was assumed to be equal to $L_0[1 - (FI/FI_{MAX})]$ and ν equal to $(L_0/P_0)(FI/FI_{MAX})$, where L_0 is the total ligand concentration and FI_{MAX} is the maximum fluorescence intensity (plateau) when all ligand molecules are supposed to be bound to protein molecules. n and K_D were determined graphically. In the case of Klotz plots, the correlation coefficient (r) was calculated from all experimental values to check the accuracy of the model.

(*2*) *Measurements at Increasing Ligand Concentration for Given Protein Concentrations* (P_0). Protein solutions were prepared in 20 mM sodium phosphate buffer, pH 7.5. Increasing amounts of ligand solution were then added so that the final volume was 3 mL. Controls were prepared with phosphate buffer without protein to determine the FI of the sole ligand. For RET–βLG experiments, the final ethanol concentration was ≤2.7% (v/v) to avoid protein denaturation. Data were analyzed according to the method of Cogan et al. (1976)

$$P_0\alpha = [1/n][L_0\alpha/(1-\alpha)] - [K_D/n] \quad (3)$$

where P_0, L_0, L, K_D and n are as in eqs 1 and 2. α, the fraction of binding sites remaining free, is assumed to be equal to $(FI_{MAX} - FI)/FI_{MAX}$; FI_{MAX} being the fluorescence intensity when all protein molecules are saturated by the ligand. n and K_D were calculated from the linear regression equation.

(*3*) *Method of Wang and Edelman (1971).* In the case of ANS–βLG experiments, it was not possible to reach a plateau for the value of FI by plotting FI vs βLG concentration because turbidity appeared at high protein contents. FI_{MAX} obtained by plotting FI vs ANS concentration was also uncertain because high ANS contents caused quenching and filtering effects (Ward, 1985). Nevertheless, it was possible to calculate FI_{MAX} K_D, and n according to the method of Wang and Edelman (1971) with the following experimental conditions.

In the case of varying ligand concentration, eq 4 was used.

$$1/FI = 1/FI_{MAX} + [(K_D/FI_{MAX})(1/L)] \quad (4)$$

In the present experimental conditions, $L_0 \gg P_0$ and the total (L_0) and free (L) ligand concentrations are practically equal. Therefore, 1/FI can be plotted vs $1/L$ or $1/L_0$ to obtain FI_{MAX} and K_D (L_0, L, P_0, and K_D being as in eqs 1–3).

In the case of varying protein concentration, eq 5 was used

$$L_0/FI = (1/\epsilon) + K_D/[\epsilon(nP_0 - P_L)] \quad (5)$$

where P_L is the concentration of the ligand–protein complex and ϵ is a proportionality factor relating FI to P_L. Since $nP_0 \gg L_0$ and $nP_0 \gg P_L$, eq 5 becomes

$$L_0/FI = (1/\epsilon) + (K_D/\epsilon nP_0) \quad (6)$$

L_0/FI was plotted vs P_0. n was calculated using the value of K_D obtained from eq 4.

In all cases, the βLG concentration was taken as that of the monomer (18 400 Da).

Binding Affinity of Heated β-Lactoglobulin for Retinol. The βLG sample was diluted in 20 mM sodium phosphate buffer, pH 7.5 or 6.5, to reach protein concentrations (P_0) ranging from 1×10^{-6} to 40×10^{-6} M. Retinol concentration was 6×10^{-6} M. The FI of the RET–βLG conjugate was measured at 470 nm (λ_{EM} = 330 nm). n and K_D were determined from the FI_{MAX} values using either Scatchard (1949) or Klotz (1947) plots, as already described.

RESULTS AND DISCUSSION

Binding Affinity of Native β-Lactoglobulin for Anilinonaphthalenesulfonate. The fluorescence intensity (FI) of the ANS–βLG conjugate at pH 7.5 is shown in Figure 1a as a function of βLG concentration for various ANS concentrations. The FI value of the same conjugate at the same pH of 7.5 is shown in Figure 1b as a function of ANS concentration for various βLG concentrations. The FI values were stable with time.

Only the initial part of each curve in Figure 1b should be considered, since quenching effects occur at ANS concentrations $>10^{-4}$ M and since the absorbance of ANS solutions at 370 nm (λ_{EXC}) exceeds 0.2 AU at ANS

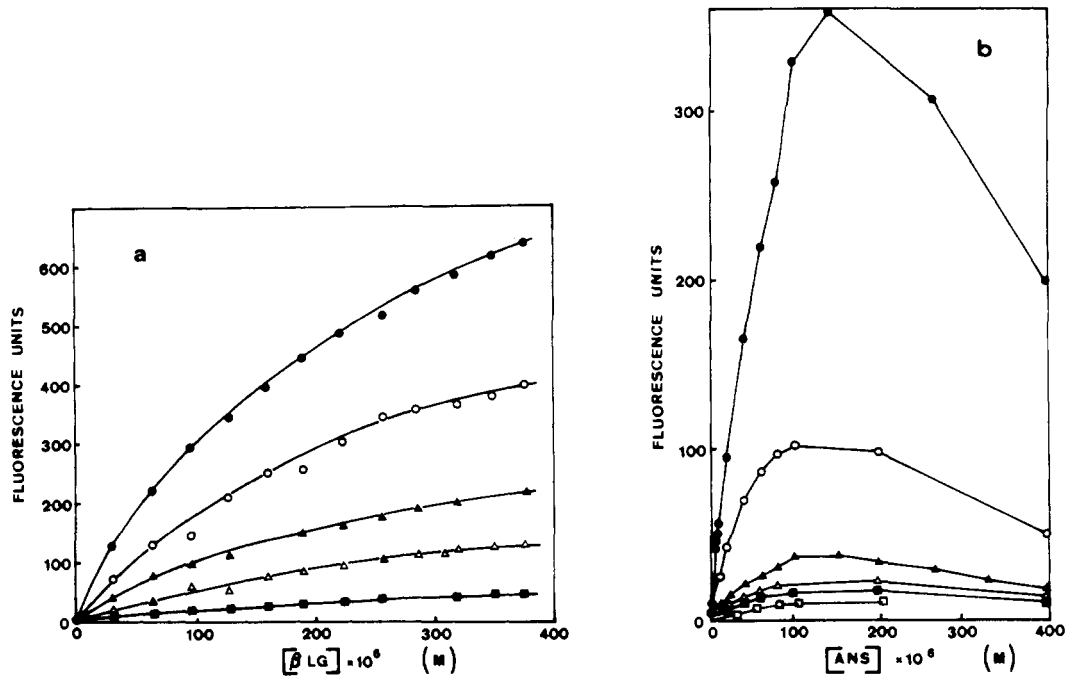

Figure 1. ANS–βLG binding plots at pH 7.5. (a) Fluorescence intensity (FI) of ANS–βLG conjugate as a function of βLG concentration for the following ANS concentrations: (■) 1.5×10^{-6} M; (△) 5×10^{-6} M; (▲) 10×10^{-6} M; (○) 20×10^{-6} M; (●) 40×10^{-6} M. (b) FI of ANS–βLG conjugate as a function of ANS concentration for the following βLG concentrations: (□) 0 M; (■) 0.09×10^{-6} M; (△) 0.87×10^{-6} M; (▲) 2.0×10^{-6} M; (○) 8.9×10^{-6} M; (●) 43×10^{-6} M.

Table I. Binding of 1-Anilino-8-naphthalenesulfonate (ANS) to β-Lactoglobulin (βLG) at pH 7.5: Apparent Dissociation Constant (K_D) and Number of Binding Sites (n)[a]

	βLG concn, M × 10^{-6}			
	0.87	2.0	8.9	43
K_D (M × 10^{-5})	3.7	2.0	2.1	6.5

	ANS concn, M × 10^{-6}				
	1.5	5.0	10	20	40
n	0.09–0.28	0.03–0.10	0.10–0.31	0.07–0.21	0.12–0.37

[a] Binding parameters calculated according to the method of Wang and Edelman (1971). K_D values estimated from Figure 1b. n calculated from Figure 1a, for the K_D values of 2×10^{-5} and 6.5×10^{-5} M.

concentrations $>4 \times 10^{-5}$ M. FI_{MAX} and K_D values were calculated according to the method of Wang and Edelman (eq 4) after the FI of ANS alone was subtracted from the FI values of ANS–βLG conjugates. n values were obtained from Figure 1a using eq 6. Results are given in Table I. High K_D values (2–6.5 × 10^{-5} M) and low n values (<0.4) indicated a low affinity of βLG for ANS. It should be recalled here that the βLG preparation contained about 60% variant A and 40% variant B. Interactions between the sulfonate group of ANS and charged amino acids may also influence ANS binding.

When the same study was carried out with bovine serum albumin, the FI of the ANS–BSA conjugate was 100-fold higher than that with βLG, and the binding parameters (two types of sites; total number of binding sites, four to five; K_D = 1.6–19.1 × 10^{-7} M) (unpublished results) were found to be close to those previously reported (Weber and Young, 1964; Daniel and Weber, 1966; Santos and Spector, 1972).

The binding parameters (n and K_D) of the ANS–βLG conjugate at pH 7.5 have not been previously reported. Mills and Creamer (1975) found 0.78 mol of ANS bound per βLG monomer at pH 6.5 as a result of plotting log FI against ANS concentration. Lovrien and Anderson (1969) suggested one or two binding sites (depending on the method) for the binding of 2,6-methyl ANS to βLG (B variant) at pH 8.0, with high K_D values (2.9×10^{-6} and 5×10^{-5} M). The two methyl groups of methyl-ANS could be responsible for the higher number of binding sites (as compared to the results of Table I).

Other studies on the binding of ligands to βLG cannot be directly compared, because the authors used different ligands [see O'Neill and Kinsella (1987)].

The present study shows that due to the low affinity of βLG for ANS, the binding parameters cannot be calculated from the usual Scatchard, Klotz, or Cogan equations and that it is necessary to select high or low ligand–protein ratios and use Wang–Edelman equations. ANS therefore does not constitute a sensitive or practical probe to study βLG hydrophobicity.

Binding Affinity of Native β-Lactoglobulin for Retinol. The fluorescence intensity of the RET–βLG conjugate at pH 7.5 was measured as a function of βLG concentration at various retinol concentrations (Figure 2a). n and K_D were determined from the FI_{MAX} values using either Scatchard or Klotz plots (Figure 3a,b).

As the retinol concentration increased (0.5–9 × 10^{-6} M), n varied from 0.24 to 0.87 according to Scatchard and from 0.20 to 0.89 according to Klotz (Table II). K_D varied between 0.12×10^{-7} and 1.7×10^{-7} M (Table II). The coefficients of variation determined on three repeats of Scatchard and Klotz plots were 10–15% for n and half a log cycle or less for K_D (Table II). Results in Figure 3a indicate one type of binding site (at pH 7.5).

FI was also measured as a function of retinol concentration for various βLG concentrations at pH 7.5 (Figure 2b). It should be noted that retinol absorbance at 330 nm (λ_{EXC}) reached 0.2–0.3 AU for retinol concentrations $\geq 10^{-5}$ M. Since this absorbance may interfere with fluorescence measurements, RET concentrations below 10^{-5} M were selected. In these conditions, FI_{MAX} values were not obtained at all βLG concentrations. Cogan analysis of curves obtained at βLG concentrations of 4.2×10^{-6} or 6.4

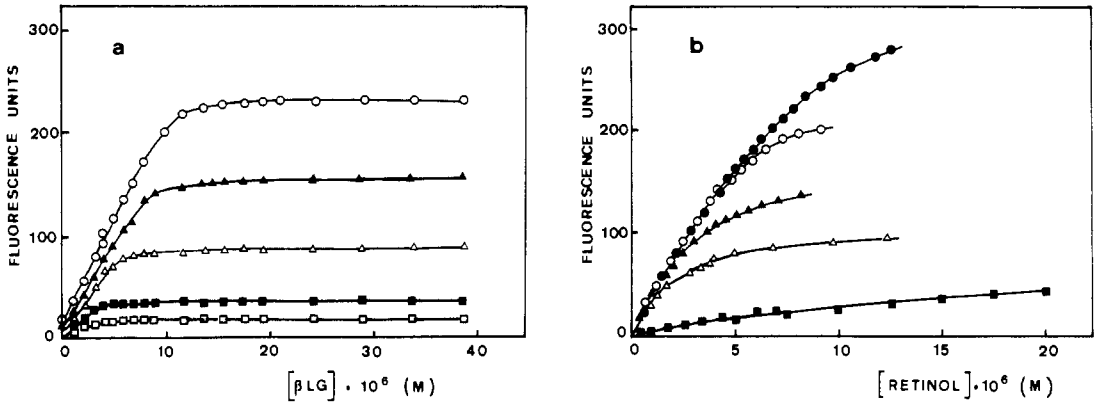

Figure 2. RET–βLG binding plots at pH 7.5. (a) FI of RET–βLG conjugate as a function of βLG concentration for the following RET concentrations: (□) 0.50×10^{-6} M; (■) 0.96×10^{-6} M; (△) 2.4×10^{-6} M; (▲) 5.0×10^{-6} M; (○) 8.1×10^{-6} M. (b) FI of RET–βLG conjugate as a function of RET concentration for the following βLG concentrations: (■) 0 M; (△) 4.2×10^{-6} M; (▲) 6.4×10^{-6} M; (○) 13×10^{-6} M; (●) 22×10^{-6} M.

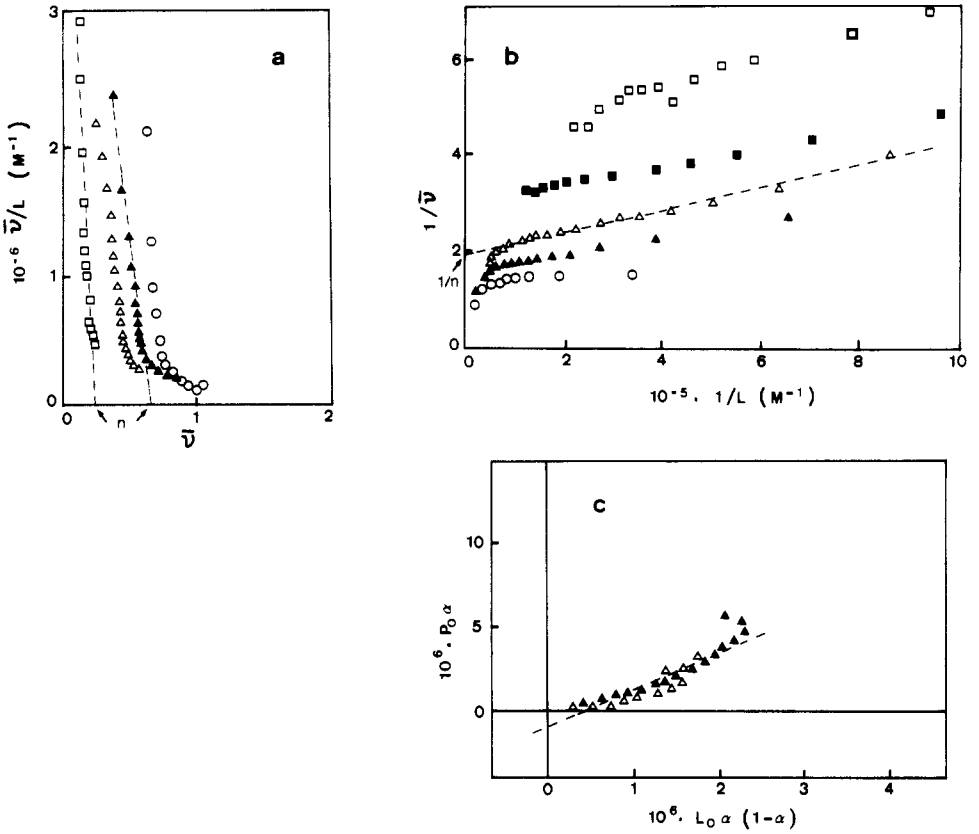

Figure 3. Graphic analysis of RET–βLG binding at pH 7.5. Scatchard (a) and Klotz (b) plots are shown for the following RET concentrations: (□) 0.50×10^{-6} M; (■) 0.96×10^{-6} M; (△) 2.4×10^{-6} M; (▲) 5.0×10^{-6} M; (○) 8.1×10^{-6} M. Cogan plots (c) are shown for the following βLG concentrations: (△) 4.2×10^{-6} M; (▲) 6.4×10^{-6} M.

$\times 10^{-6}$ M gave $n = 0.39$ or 0.4 and $K_D = 6 \times 10^{-7}$ or 5.6×10^{-7} M, respectively. The range of n values was smaller than that in Table II because of the more restricted range of βLG and RET concentrations studied. It was difficult to check lower βLG concentrations without taking into account the FI of free retinol. These results indicated that in the present case calculations according to the Scatchard or Klotz method were preferable to analysis according to the method of Cogan.

Futterman and Heller (1972) incubated βLG (14.3×10^{-6} M) with increasing concentrations of RET ($1–35 \times 10^{-6}$ M) at pH 7.4 and observed a maximum FI for a RET/βLG molar ratio close to 1. Using their data, it was possible to calculate (according to the Cogan method) an n value of 0.63 and a K_D of 5.8×10^{-7} M. These values were not so far from those of Table II. Fugate and Song (1980) determined the stoichiometry of RET–βLG binding at pH 7.5 by fluorescence titration followed by analysis according to the method of Cogan and by radioisotopic labeling followed by analysis according to the method of Scatchard. One mole of RET was reported to bind per monomer of βLG, with a K_D of 2.10^{-8} M (fluorescence), but the calculation steps were not indicated. Jang and Swaisgood (1990) studied the chromatographic elution of βLG through a column packed with immobilized *trans*-retinal beads at pH 7.5. They did not report the number of binding sites but gave a K_D of 3.56×10^{-8} M, which was close to Fugate and Song's value.

In the present study, n is usually <1 and equals 0.9 when the RET/βLG ratio increases and more particularly for retinol concentrations $\geq 6 \times 10^{-6}$ M (Table II). It is likely that n approaches 1 for sufficiently high RET

Table II. Binding of Retinol to β-Lactoglobulin (βLG) at pH 7.5: Number of Binding Sites (n) and Apparent Dissociation Constant (K_D)[a]

	retinol concn, M × 10^{-6}						
	0.50	0.96	2.4	5.0	6.0	8.1	9.0
Analysis According to the Method of Scatchard (1949)							
n	0.24	0.34	0.52	0.65	0.85 (0.10)[b]	0.76	0.87 (0.11)[b]
K_D (M × 10^{-7})	0.12	0.8	1.2	1.3	1.5 (0.41)[b]	0.64	1.4 (0.36)[b]
Analysis According to the Method of Klotz (1947)							
n	0.20	0.34	0.53	0.69	0.83 (0.08)[b]	0.89	0.88 (0.13)[b]
K_D (M × 10^{-7})	0.3	0.7	1.3	1.7	1.2 (0.6)[b]	1.7	1.4 (0.5)[b]
r[c]	0.97	1.0	0.99	0.94	0.90	0.80	0.90

[a] From data of Figures 4a and 5. [b] Average of three independent determinations (and standard deviation). [c] r = correlation coefficient of Klotz plots.

concentrations. This result is consistent with the hypothesis of one strong hydrophobic binding site on βLG (Monaco et al., 1987). It also underlies the necessity of measurements in a wide ligand/protein ratio. These variations in the number of binding sites could be due to the fact that it is difficult to prepare retinol solutions of exactly known concentration. The equilibrium between free and bound retinol may be altered by the following: (1) the formation of retinol micelles in water, even at concentrations as low as 2 μM (Radda and Smith, 1970; Cogan et al., 1976); (2) the partial oxidation of retinol dispersed in aqueous media, although the presence of protein appeared to retard this oxidation (Fisher et al., 1972; Futterman and Heller, 1972; Goodman and Leslie, 1972; Hemley et al., 1979) [The decreasing FI of RET–βLG conjugate observed after 1 min is probably due to RET oxidation. According to Monaco et al. (1987), retinol binds preferentially to the hydrophobic cone close to the surface of βLG and probably remains sensitive to oxidation or to other chemical changes.]; (3) the transformation of retinol into anhydrovitamin A and a hydroxy derivative when retinol was dissolved in ethanol (Hemley et al., 1979; Fugate et Song, 1980).

As a result of this complex equilibrium between free and bound retinol, calculated binding parameters should be considered "apparent". The separation of free and bound retinol by equilibrium dialysis would permit a more precise but less rapid method for binding determination. Nevertheless, the use of retinol as a fluorescent probe remains acceptable provided the ranges of ligand and protein concentrations have been correctly selected and special care has been taken to prevent oxidation phenomena. The binding of such hydrophobic ligands cannot be expected to give the absolute surface hydrophobicity of a protein but may be used to assess relative surface hydrophobicity as the protein conformation changes through thermal or other effects.

Retinol binding to βLG was also studied at pH 6.5, since βLG is known to undergo a conformational change at pH 6.8. Binding parameters were determined for increasing βLG concentrations (1–40 × 10^{-6} M) at a retinol concentration of 6 × 10^{-6} M. Scatchard plots were less linear at pH 6.5 than at pH 7.5 and indicated at least two types of binding sites, instead of one at pH 7.5 (Figure 4a,c). The results for three to four independent determinations are shown in Figure 5. At pH 7.5, n was 0.85 and K_D was 1.5 × 10^{-7} M. At pH 6.5, n_1 (number of primary sites) was 0.60 and n_2 (number of secondary sites) was 0.22. The apparent dissociation constants were, respectively, K_{D1} = 2.9 × 10^{-7} M and K_{D2} = 9.7 × 10^{-7} M (Figure 5). Klotz plots fitted less well at pH 6.5 than at pH 7.5 (Figure 4b,d), and the correlation coefficients were indeed lower at pH 6.5.

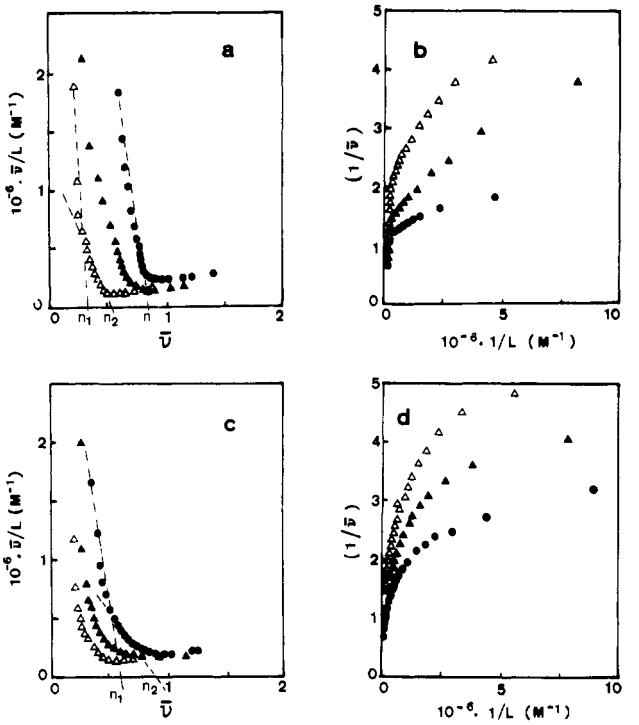

Figure 4. Effect of βLG heating on RET binding, at pH 7.5 (a, b) or at pH 6.5 (c, d). Scatchard (a, c) and Klotz (b, d) plots are shown for nonheated βLG controls (●) or βLG heated at 90 °C for 3 (▲) or 6 (△) min. 1% βLG solutions of pH 6.5 or 7.5 were heated for 3 or 6 min in a water bath at 90 °C.

Above pH 6.8, a proton is released from a buried carboxyl group and the reactivity of the SH_{121} group increases (Tanford, 1970). The βLG dimer dissociation is enhanced at higher pHs and also at lower βLG concentrations and higher temperatures (MacKenzie and Sawyer, 1967). In addition, the βLG A dimer dissociates more easily than that of βLG B. At pH 6.5, the accessibility of the βLG binding site to retinol was probably reduced because the tertiary structure changed. However, the dimer formation may also have masked some hydrophobic amino acids (of the binding site) located in strand I of the molecule. It is known that dimer formation takes place through hydrophobic interactions between amino acids in the strands I of two βLG molecules (Green et al., 1979; Monaco et al., 1987).

Binding Affinity for Retinol of β-Lactoglobulin Heated at pH 6.5 or 7.5. One percent βLG solutions of pH 6.5 or 7.5 were heated at 75 or 90 °C for various periods of time, as indicated under Materials and Methods. Retinol binding was then measured at the corresponding pH values. Binding parameters were determined according to the methods of Scatchard and Klotz for increasing βLG concentrations (1–40 × 10^{-6} M), at a retinol concentration of 6 × 10^{-6} M.

No significant variation of n or K_D was observed after βLG solutions were heated at pH 7.5 and 75 °C for 3 or 6 min (Figure 5). Scatchard and Klotz plots (not shown) obtained from βLG heated at 75 °C were very close to those for the control. After βLG was heated at 90 °C for 3 min, n decreased while K_D increased slightly (Figure 5). After βLG was heated at 90 °C for 6 min, Scatchard plots clearly indicated two types of sites with a second K_D higher than the first and a net decrease of the total number of sites (Figures 4a and 5). These data clearly indicated that heating βLG at 90 °C reduced the hydrophobic binding of retinol.

After 1% βLG solutions were heated at pH 6.5 and 75

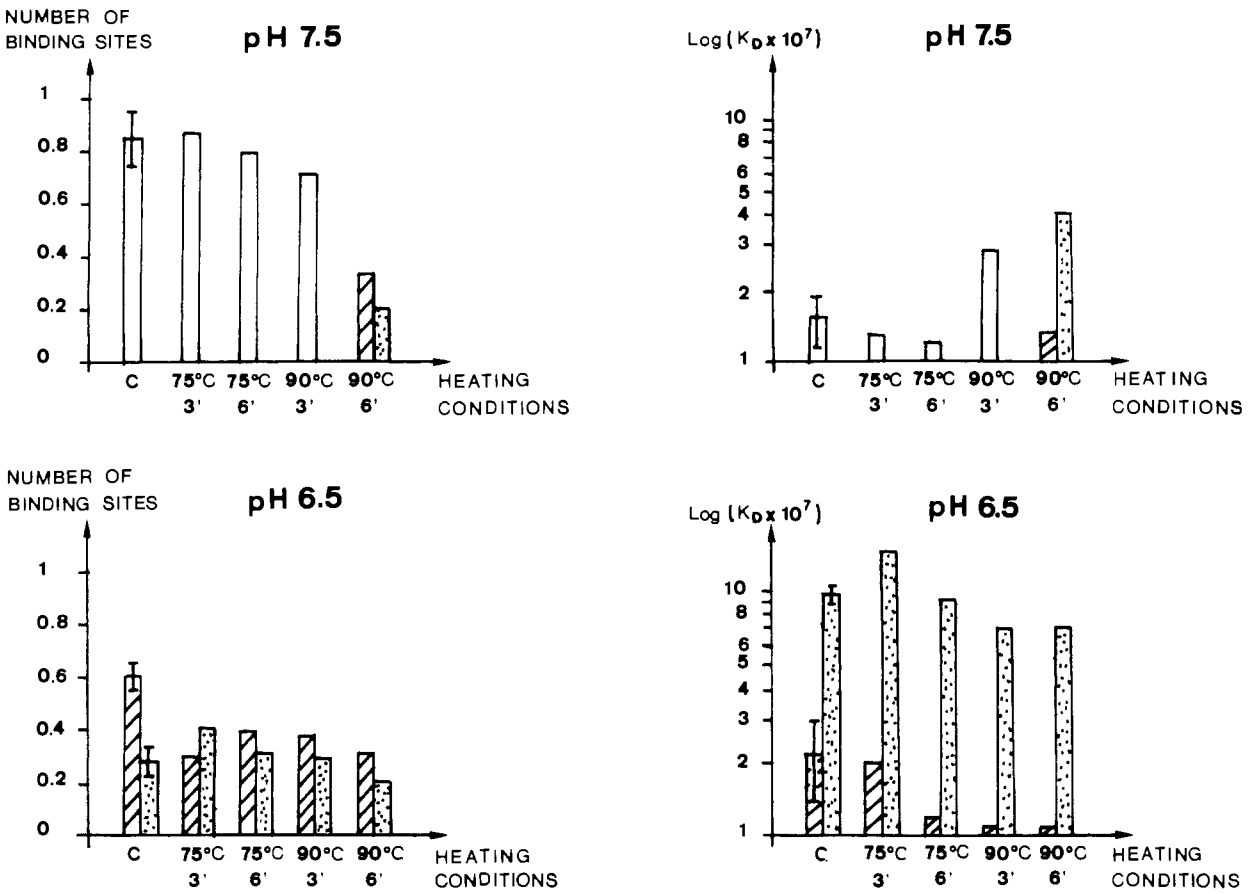

Figure 5. Parameters for the binding of retinol to native (C) or heated βLG at pH 7.5 and 6.5. Data in this figure are calculated from the Scatchard plots in Figure 4. (Open bars) number of sites (n) and dissociation constant (K_D) in the case of one type of sites; (hatched bars) number of primary sites (n_1) and dissociation constant of primary sites (K_{D1}); (dotted bars) number of secondary sites (n_2) and dissociation constant of the secondary sites (K_{D2}). Values for native βLG (C) are the average of three or four determinations (with standard deviation).

or 90 °C for 3 or 6 min, Scatchard plots indicated two types of sites, as already observed for native βLG (Figures 4c). The decrease in total sites ($n_1 + n_2$, i.e., number of primary plus secondary sites) was always significant when heating was carried out at pH 6.5, while, at pH 7.5, it was only observed in the case of the more severe heating conditions (Figure 5). The decrease in K_D values as a function of heating at pH 6.5 probably reflects the fact that only the high-affinity binding sites are now determined as primary sites. In addition, at pH 6.5 the Klotz model was not adequate due to the multiple binding sites. In conclusion, the retinol binding capacity of βLG was more affected by moderate heating at pH 6.5 than at pH 7.5.

Evaluation of Heated β-Lactoglobulin by Chromatography and Electrophoresis. The relative absorbance (ABS_{280}) of heated βLG solutions (1% protein) with regard to βLG control was measured at 280 nm after centrifugation and filtration. There was no marked change except after pH 6.5 solutions were heated at 90 °C for 3 or 6 min, when ABS_{280} decreased by 9% and 11%, respectively, indicating a significant formation of insoluble protein material.

The proportion of residual native βLG in heated βLG solutions was determined by hydrophobic interaction chromatography at pH 7.5 (Figure 6). Heat-denatured βLG was removed by ammonium sulfate precipitation before HIC analysis, as described by Dumay and Cheftel (1989). Results are summarized in Table III.

The loss in native βLG (variants A plus B) increased with the temperature and the heating time and was slightly greater at pH 7.5 than at pH 6.5. At pH 6.5, variant B

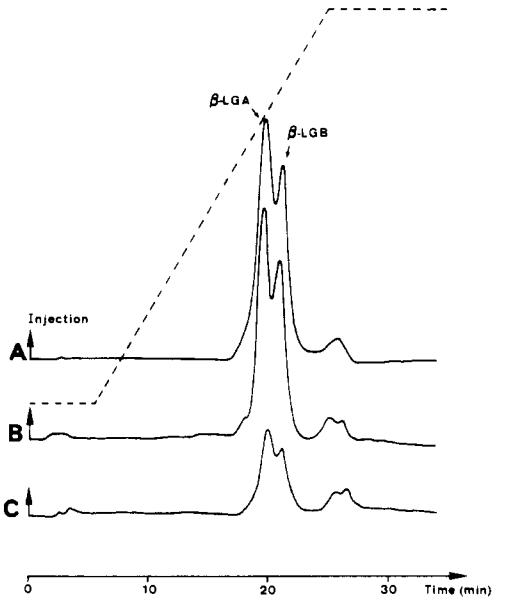

Figure 6. Hydrophobic interaction chromatography of nonheated βLG control (A) or of βLG heated at pH 7.5 for 6 min at 75 (B) or 90 °C (C). Chromatographic conditions were as given under Materials and Methods.

appeared to be more heat sensitive than variant A, as previously noted by Dupont (1965). At pH 7.5, variant A was apparently more heat sensitive than variant B at 75 °C, while the reverse was true at 90 °C (Table III).

The soluble βLG aggregates formed as a result of heating were assessed by gel permeation chromatography at pH

Table III. Proportions[a] of β-Lactoglobulin (βLG) Remaining Soluble after Heat Processing of βLG Solutions at Various pHs and Temperatures[b]

	pH of βLG solutions during heating					
	7.5			6.5		
	variant A, %	variant B, %	total βLG, %	variant A, %	variant B, %	total βLG, %
nonheated controls	100	100	100	100	100	100
75 °C; 3 min	92	100	95	98	95	97
75 °C; 6 min	86	94	88	98	94	96
90 °C; 3 min	70	60	66	77	60	70
90 °C; 6 min	29	26	28	37	22	31

[a] Data represent grams of soluble βLG per 100 g of nonheated soluble βLG (control). The control solution at pH 7.5 contained 1.3 mg/mL of total soluble βLG, 0.83 mg/mL of variant A, and 0.47 mg/mL of variant B. The control solution at pH 6.5 contained 1.43 mg/mL of total soluble βLG, 0.86 mg/mL of variant A, and 0.57 mg/mL of variant B. Average of two or three determinations. [b] Determination by hydrophobic interaction chromatography.

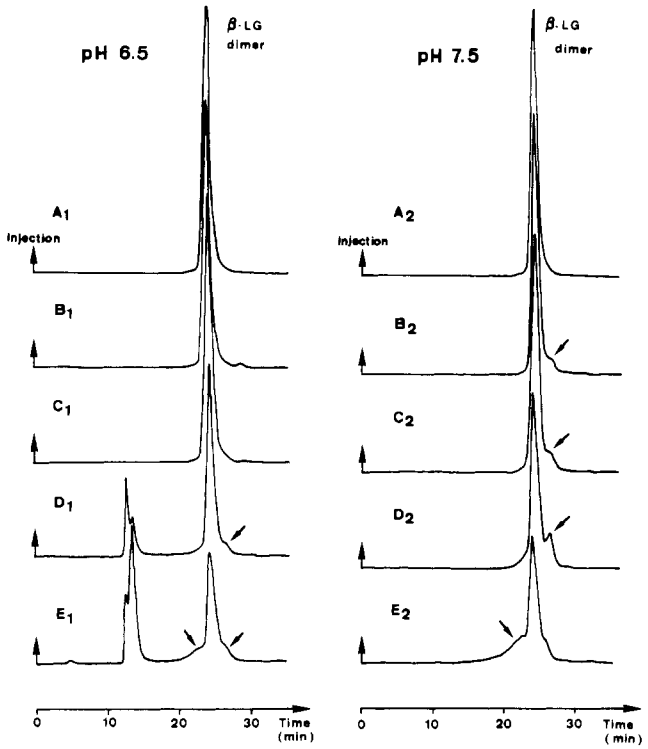

Figure 7. Gel permeation chromatography of βLG heated at pH 6.5 or 7.5. A_1–E_1, pH 6.5; A_2–E_2, pH 7.5. Heating conditions: A_1, A_2, no heating; B_1, B_2, 75 °C, 3 min; C_1, C_2, 75 °C, 6 min; D_1, D_2, 90 °C, 3 min; E_1, E_2, 90 °C, 6 min. Chromatographic conditions were as given under Materials and Methods. Changes in elution patterns are indicated by short arrows.

6.0 (Figure 7). At this pH, βLG is known to exist essentially as dimers. When βLG solutions were heated at pH 6.5 and 75 °C for 3 or 6 min, no new molecular species were formed (Figure $7A_1$–C_1). However, when these solutions were heated at 90 °C for 3 or 6 min, soluble high molecular weight aggregates (600 000–1 000 000) became evident (Figure $7D_1$,E_1). Heating βLG solutions at pH 7.5 and 75 or 90 °C for 3 or 6 min induced the progressive formation of a 18 000 molecular species, different from native βLG (Figure $7B_2$–E_2). This modified βLG does not reassociate into a dimer when the pH is returned to 6.0 for chromatographic analysis. Severe heating conditions (90 °C for 6 min) at pH 7.5 lead to oligomers with molecular weights ranging from 40 000–200 000 (Figure $7E_2$).

SDS–PAGE of βLG solutions (1% protein) heated at

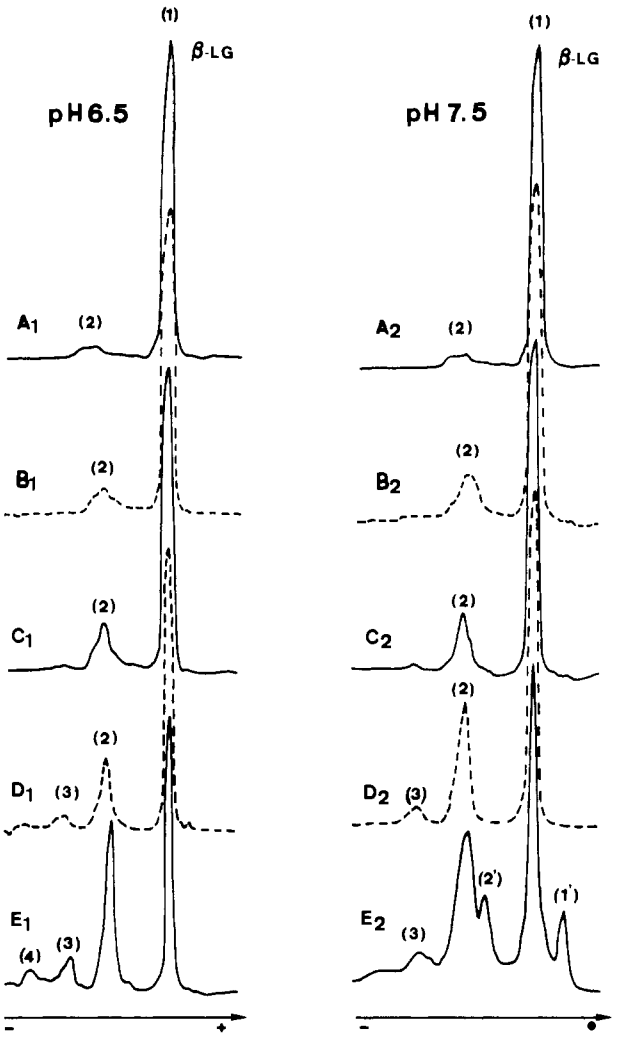

Figure 8. Electrophoretic patterns of βLG heated at pH 6.5 or 7.5. SDS–PAGE: A_1–E_1, pH 6.5; A_2–E_2, pH 7.5. Heating conditions: A_1, A_2, no heating; B_1, B_2, 75 °C, 3 min; C_1, C_2, 75 °C, 6 min; D_1, D_2, 90 °C, 3 min; E_1, E_2, 90 °C, 6 min. Peak numbers: (1) monomer; (2) dimer; (3) trimer; (4) tetra- and pentamer. Conditions of electrophoresis were as given under Materials and Methods.

pH 6.5 or 7.5 for 3 or 6 min indicated an increasing formation of βLG oligomers with heating intensity (Figure 8). These oligomers were not dissociated by SDS. Dimers, trimers, and higher polymers are known to form under such heating conditions through SH/SS interchange reactions (Shimada and Cheftel, 1989). In the case of βLG heated at pH 6.5, the oligomers seen on electrophoregrams may also derive from the partial dissociation by SDS of the higher molecular weight aggregates observed on the gel permeation chromatograms (Figure $8D_1$–E_1). The formation of dimers, trimers, and higher oligomers was more extensive when βLG was heated at pH 7.5 than at pH 6.5 (Figure 8), probably because SH groups were more reactive at higher pH. Two additional protein bands (14 000 and 30 000) became apparent after solutions were heated at pH 7.5 and 90 °C for 6 min. They may correspond to partially hydrolyzed forms of βLG.

Correlation between Ligand Binding and Chromatographic/Electrophoretic Data. Heating 1% βLG solutions at pH 7.5 and temperatures ≥75 °C caused a progressive decrease in the concentration of native βLG (HIC), the formation of a modified βLG monomer (GPC), and the formation of dimers probably through SH/SS interchange reactions (SDS–PAGE). Oligomers were formed at higher heating conditions (90 °C). The affinity

of βLG for retinol decreased mainly after heating at 90 °C. Thus, the formation of modified βLG monomer or disulfide-bound dimers apparently does not hinder retinol binding onto βLG. However, the next step (formation of oligomers) prevents retinol binding. This may be due to steric hindrance and limited access to the hydrophobic cone of βLG.

Heating βLG solution at pH 6.5 in the temperature range 75–90 °C caused a smaller decrease in the concentration of native βLG (HIC) and less formation of modified βLG monomer (GPC) or of S–S-bound βLG dimers (SDS–PAGE). However, high molecular weight aggregates were formed when solutions were heated at pH 6.5 and 90 °C (and not at pH 7.5); 10% of the initial βLG was lost as insoluble aggregates. In parallel, the affinity of βLG for retinol decreased after heating, even at 75 °C.

The decrease in affinity for retinol was greater after solutions were heated at pH 6.5, although less βLG denaturation was observed at pH 6.5 than at pH 7.5.

These data suggest that βLG dimerization through hydrophobic interactions (upon heating at pH 6.5) masked the sites of retinol fixation, while dimerization through SH/SS interchange reactions (upon heating at pH 7.5) had little effect on this property.

Similar retinol binding results were obtained when a commercial whey protein isolate was heated at 90 °C for 6 min (pH 6.5 or 7.5) (unpublished results).

The binding parameters of RET–βLG after βLG was heated in the neutral pH range have not been previously reported. Jang and Swaisgood (1990) studied the extent of denaturation of βLG by affinity chromatography; they found a decrease of βLG binding to immobilized *trans*-retinal, after heating 1% βLG solutions at 90 °C for 5–60 min, at pH 2.5. But it is difficult to compare the effects of their heat processing at pH 2.5 with the present data at pH 6.5 or 7.5.

Haque and Kinsella (1987) studied ANS binding to 1.8% βLG solutions before and after heating at pH 6.8 in the presence of ethylene glycol bis(β-aminoethyl ether)tetraacetic acid (EGTA) to prevent the formation of aggregates; ANS binding increased with the length and temperature of heating above 70 °C.

O'Neill and Kinsella (1988) studied 2-nonanone binding to 1% βLG solutions before and after heating at pH 6.7 and 75 °C for 10 or 20 min. Equilibrium dialysis and double-reciprocal plots were used. They reported the formation of βLG polymers and a simultaneous increase in both n and K_D. It was concluded that the formation of polymers induced a heterogeneity of binding sites and some flavor entrapment.

Bonomi et al. (1988) assessed modifications in the protein constituents of heated milk by way of ANS binding at pH 6.8. Their data suggest that structural changes in the various protein constituents induced interactions between hydrophobic sites and therefore reduced ANS binding.

From these various data, it seems that ligand binding to βLG may decrease when βLG aggregation occurs and when the ligand is not entrapped inside the βLG aggregates.

CONCLUSION

This study shows that the affinity of βLG for 8-anilino-1-naphthalenesulfonate is much smaller than that for retinol. It is possible to measure the binding parameters of retinol and βLG according to the method of Scatchard but special care must be taken to prevent retinol oxidation. The assessment of the surface hydrophobicity of proteins using fluorescent ligands is a delicate method. Since the number of sites depends on the ligand/protein ratio, it is necessary to scan a large range of ligand or protein concentrations. Studies carried out at only one or two ligand or protein concentrations are incomplete and do not provide reliable data.

The surface hydrophobicity of βLG, as measured by retinol binding, was influenced by the pH (6.5 or 7.5) and by a prior thermal treatment of βLG, at the same two pH values. However, the retinol binding parameters (n and K_D) were not always clearly correlated to the intensity of heat processing. A good linear correlation ($R = 0.98$) was observed between the number of retinol binding sites at pH 7.5 and the decreasing proportion of residual native βLG when βLG was progressively heated at pH 7.5. This was not the case at pH 6.5. The marked decrease in retinol binding already observed after mild heating of βLG at pH 6.5 is not easily explained.

βLG contains a high proportion of hydrophobic amino acid side chains, preferentially turned toward the inside of the molecule. Surface hydrophobicity is expected to increase when the molecule unfolds during heating. However, unfolding is immediately followed by protein aggregation, through hydrophobic interactions or through SH/SS interchange reactions (Shimada and Cheftel, 1989). This aggregation is probably responsible for the decreased access of retinol to hydrophobic zones.

In the present case, the determination of surface hydrophobicity by ligand binding is less informative of protein changes during heating than the chromatographic and electrophoretic patterns. The latter precisely indicate the proportion of residual native protein and of small or high molecular weight aggregates.

LITERATURE CITED

Bonomi, F.; Iametti, S.; Pagliarini, E.; Peri, C. A. Spectrofluorimetric Approach to the Estimation of the Surface Hydrophobicity Modifications in Milk Proteins upon Thermal Treatment. *Milchwissenschaft* **1988**, *43*, 281–285.

Cogan, U.; Kopelman, M.; Mokady, S.; Shinitzky, M. Binding Affinities of Retinol and Related Compounds to Retinol Binding Proteins. *Eur. J. Biochem.* **1976**, *65*, 71–78.

Daniel, E.; Weber, G. Cooperative Effects in Binding by Bovine Serum Albumin. I. The binding of 1-Anilino-8-naphthalene Sulfonate. Fluorimetric Titrations. *Biochemistry* **1966**, *5*, 1893–1900.

Dumay, E.; Cheftel, J. C. Heat Treatment of a β-Lactoglobulin Concentrate at Slightly Alkaline pHs. Effects on the Solubility and the Chromatographic Behaviour of β-Lactoglobulin and α-Lactalbumin. *Sci. Aliments* **1989**, *9*, 561–582.

Dupont, M. *Biochim. Biophys. Acta* **1965**, *94*, 573–575.

Eigel, W. N.; Butler, J. E.; Ernstrom, C. A.; Farrell, H. M.; Harwalkar, V. R.; Jenness, R.; Whitney, R. McL. Nomenclature of Proteins of Cow's Milk: Fifth Revision. *J. Dairy Sci.* **1984**, *67*, 1599–1631.

Fisher, D.; Lichti, F. U.; Lucy, J. A. Environmental Effects on the Autoxidation of Retinol. *Biochem. J.* **1972**, *130*, 259–270.

Fugate, R. D.; Song, P.-S. Spectroscopic Characterization of β-Lactoglobulin-Retinol Complex. *Biochim. Biophys. Acta* **1980**, *625*, 28–42.

Futterman, S.; Heller, J. The Enhancement of Fluorescence and the Decreased Susceptibility to Enzymatic Oxidation of Retinol Complexed with Bovine Serum Albumin, β-Lactoglobulin and the Retinol-binding Protein of Human Plasma. *J. Biol. Chem.* **1972**, *247*, 5168–5172.

Goodman, D. S.; Leslie, R. B. Fluorescence Studies of Human Plasma Retinol-Binding Protein and of the Retinol-Binding Protein-Prealbumin Complex. *Biochim. Biophys. Acta* **1972**, *260*, 670–678.

Green, D. W.; Aschaffenburg, R.; Camerman, A.; Coppola, J. C.; Dunnill, P.; Simmons, R. M.; Komorowski, E. S.; Sawyer, L.; Turner, E. M. C.; Woods, K. F. Structure of β-Lactoglobulin at 6Å Resolution. *J. Mol. Biol.* **1979**, *31*, 375–397.

Haque, Z.; Kinsella, J. E. Heat-Induced Changes in the Hydrophobicity of K-casein and β-Lactoglobulin. *Agric. Biol. Chem.* **1987**, *51*, 2245–2247.

Hayakawa, S.; Nakai, S. Relationships of Hydrophobicity and Net Charge to the Solubility of Milk and Soy Proteins. *J. Food Sci.* **1985**, *50*, 486–491.

Hemley, R.; Kohler, B. E.; Siviski, P. Absorption Spectra for the Complexes Formed from Vitamin-A and β-Lactoglobulin. *Biophys. J.* **1979**, *28*, 447–455.

Jang, D. H.; Swaisgood, H. E. Analysis of Ligand Binding and β-Lactoglobulin Denaturation by Chromatography on Immobilized Trans-Retinal. *J. Dairy Sci.* **1990**, *73*, 2067–2074.

Jasinski, E.; Kilara, A. Flavor Binding by Whey Proteins. *Milchwissenschaft* **1985**, *40*, 596–599.

Kato, A.; Nakai, S. Hydrophobicity Determined by a Fluorescence Probe Method and its Correlation with Surface Properties of Proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13–20.

Klotz, I. M. The Effects of Salts and Proteins on the Spectra of Some Dyes and Indicators. *Chem. Rev.* **1947**, *41*, 373–399.

Laemmli, U. K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685.

Lovrien, R.; Anderson, W. Resolution of Binding Sites in β-Lactoglobulin. *Arch. Biochem. Biophys.* **1969**, *131*, 139–144.

MacKenzie, H. A. β-Lactoglobulins. In *Milk Proteins: Chemistry and Molecular Biology*; MacKenzie, H. A., Ed.; Academic Press: New York, 1971; Vol. 2, pp 257–330.

MacKenzie, H. A.; Sawyer, W. H. Effects of pH on β-Lactoglobulins. *Nature* **1967**, *214*, 1101–1104.

Mills, O. E.; Creamer, L. K. A Conformational Change in Bovine β-Lactoglobulin at Low pH. *Biochim. Biophys. Acta* **1975**, *379*, 618–626.

Mohammadzadeh-K, A.; Feeney, R. E.; Smith, L. M. Hydrophobic Binding of Hydrocarbons by Proteins. I. Relationship of Hydrocarbon Structure. *Biochim. Biophys. Acta* **1969**, *194*, 246–255.

Monaco, H. L.; Zanotti, G.; Spadon, P.; Bolognesi, M.; Sawyer, L.; Eliopoulos, E. E. Crystal Structure of the Trigonal Form of Bovine β-Lactoglobulin and of its Complex with Retinol at 2.5 Å Resolution. *J. Mol. Biol.* **1987**, *197*, 695–706.

O'Neill, T.; Kinsella, J. E. Binding of Alkanone Flavors to β-Lactoglobulin: Effects of Conformational and Chemical Modification. *J. Agric. Food Chem.* **1987**, *35*, 770–774.

O'Neill, T.; Kinsella, J. E. Effect of Heat Treatment and Modification on Conformation and Flavor Binding by β-Lactoglobulin. *J. Food Sci.* **1988**, *53*, 906–909.

Radda, G. K.; Smith, D. S. Retinol: A Fluorescent Probe for Membrane Lipids. *FEBS Lett.* **1970**, *9*, 287–289.

Santos, E. C.; Spector, A. A. Effect of Fatty Acids on the Binding of 1-Anilino-8-naphthalene Sulfonate to Bovine Serum Albumin. *Biochemistry* **1972**, *11*, 2299–2302.

Scatchard, G. The Attraction of Proteins for Small Molecules and Ions. *Ann. N.Y. Acad. Sci.* **1949**, *51*, 660–672.

Shimada, K.; Cheftel, J. C. Sulfhydryl Group/Disulfide Bond Interchange Reactions during Heat-Induced Gelation of Whey Protein Isolate. *J. Agric. Food Chem.* **1989**, *37*, 161–168.

Spector, A. A.; Fletcher, J. E. Binding of Long Chain Fatty Acids to β-Lactoglobulin. *Lipids* **1970**, *5*, 403–411.

Swaisgood, H. E. Chemistry of Milk Proteins. In *Developments in Dairy Chemistry*; Fox, P. F., Ed.; Applied Science Publishers: Essex, England, 1982; Vol. 1, pp 1–59.

Tanford, C. Protein Denaturation. *Adv. Protein Chem.* **1970**, *24*, 1–95.

Wang, J. L.; Edelman, G. M. Fluorescent Probes for Conformational States of Proteins. IV. The Pepsinogen-Pepsin Conversion. *J. Biol. Chem.* **1971**, *246*, 1185–1191.

Ward, L. D. Measurement of Ligand Binding to Proteins by Fluorescence Spectroscopy. In *Methods in Enzymology: Enzyme Structure*; Hirs, C. H. W., Timasheff, S. N., Eds.; Academic Press: Orlando, FL, 1985; Vol. 117, pp 400–414.

Weber, G.; Young, L. B. Fragmentation of Bovine Serum Albumin by Pepsin. I. The Origin of the Acid Expansion of the Albumin Molecule. *J. Biol. Chem.* **1964**, *239*, 1415–1423.

Received for review March 20, 1991. Revised manuscript received September 17, 1991. Accepted September 23, 1991.