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Probing the Fatty Acid Binding Site of β-Lactoglobulins

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The interactions of fatty acids with porcine and bovine β-lactoglobulins were measured using tryptophan fluorescence enhancement. In the case of bovine β-lactoglobulin, the apparent binding constants for most of the saturated and unsaturated fatty acids were in the range of 10^{-7} M at neutral *p*H. Bovine β-lactoglobulin displays only one high affinity binding site for palmitate with an apparent dissociation constant of $1 \cdot 10^{-7}$ M. The strength of the binding was decreasing in the following way: palmitate > stearate > myristate > arachidate > laurate. Caprylic and capric acids are not bound at all. The affinity of β-lactoglobulin for palmitate decreased as the *p*H of the incubation medium was lowered and BLG/palmitate complex was not observed at *p*H's lower than 4.5. Surprisingly, chemically modified bovine β-lactoglobulin and porcine β-lactoglobulin did not bind fatty acids in the applied conditions.

KEY WORDS: Fatty acids; binding site; β-lactoglobulin.

1. INTRODUCTION

As far back as the late 1940s, it was reported that β-lactoglobulin (BLG), which is found in the milk of several mammal species, binds fatty acids (Davis and Dubos, 1947) and sodium dodecyl sulphate (Mc Keekin *et al.*, 1949). Developments in the study of BLG binding properties show that it can also bind specifically small hydrophobic ligands such as retinoids (Futterman and Heller, 1972; Dufour and Haertlé, 1991), alkanone flavors (O'Neill and Kinsella, 1987), polyoxyethylene sorbitan monolaurate (Coke *et al.*, 1990), protoporphyrin IX (Dufour *et al.*, 1990), and ellipticine (Dodin *et al.*, 1990). Outcome of structural studies of BLG (Papiz *et al.*, 1986; Monaco *et al.*,1987) suggests that this protein may be classified in the super-family of hydrophobic molecule transporters termed *lipocalins*. Retinol-binding protein (Newcomer *et al.*, 1984), bilin-binding protein (Huber *et al.*, 1987), insecticyanin (Holden *et al.*, 1987), and BLG (Papiz *et al.*, 1986; Monaco *et al.*, 1987) are the best known proteins of this class. All these proteins share a common tridimensional structural pattern: eight stranded antiparallel β-sheet flanked on one side by an α-helix constituting a hydrophobic pocket.

The biological function of BLG still remains unclear. Since it is able to bind fatty acids and to increase the activity of pregastric lipase, it has been recently claimed that ruminant BLG could participate in fat digestion during the neonatal period (Perez *et al.*, 1992). Bovine BLG, purified by non-denaturing methods at neutral *p*H, contains bound fatty acids (Perez *et al.*, 1989); Diaz de Villegas *et al.*, 1987). It also has been postulated that classical isolation procedures of BLG involving exposure to low *p*H or/and drastic modifications of the ionic strength of milk caused conformational changes which decrease the affinity of this protein for fatty acids (Spector and Fletcher, 1970; Perez *et al.*, 1989). The study of thermodynamic stability of BLG at various *p*Hs shows, however, that the temperature of irreversible denaturation increases with a decrease of *p*H from 7.5 to 1.5, suggesting an increase in the net protein stability (Della and Kinsella, 1988). Similar behavior is also observed during BLG unfolding induced by urea at different *p*Hs (Tanford and De, 1961; Takeda and Moriyama, 1989).

The results of experiments aiming at the elucida-

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tion of interactive properties of bovine and porcine BLG, isolated at acid *p*H, with 16 saturated and unsaturated fatty acids varying in aliphatic chain lengths are presented and discussed in this paper.

2. MATERIALS AND METHODS

2.1. Materials

Caprylic acid, capric acid, lauric acid, myristic acid, stearic acid, myristoleic acid, palmitic acid, palmitoleic acid, oleic acid, elaidic acid, linoleic acid, linolelaidic acid, linolenic acid, γ-linolenic acid, arachidic acid, arachidonic acid, and retinol were from Sigma and prostaglandin A_1 was from Fluka. One millimeter solutions in ethanol saturated with N_2 were prepared and stored in the dark to limit possible degradation. β-Lactoglobulin variant B was obtained from homozygote cow's milk following the method of Mailliart and Ribadeau Dumas (1988) and, as judged from high-performance liquid chromatograms on C_{18} column and polyacrylamide gel electrophoresis, it was more than 95% pure. Porcine BLG was purified following the aforementioned method with several changes (Dalgalarrondo *et al.*, 1992). The UV absorbency spectra were recorded on a Cary-1 spectrophotometer (Varian). Concentrations of bovine BLG were determined spectrophotometrically using a molecular absorption coefficient $\varepsilon_{278} = 17{,}600$. Reductive methylation of lysine residues in BLG was performed as described in Cabacungan *et al.* (1982). Methyl esterified BLG (MetBLG) was prepared following the procedure described in Fraenkel-Conratt and Olcott (1945). The main physicochemical characteristics (isoionic point, secondary structure) of BLG derivatives have been reported elsewhere (Dufour and Haertlé, 1990).

2.2 Methods

2.2.1. Fluorescence Spectroscopy

Fluorescence spectra between 300 and 380 nm (excitation: 287 nm) were recorded at 20°C on an Aminco SLM 4800C spectrofluorimeter in the ratio mode. The binding of the fatty acids was measured by following the increase of protein tryptophan fluorescence at 332 nm. The following procedure was used for titration of BLG with fatty acids: 2 ml of BLG solution (or retinol-BLG complex solution), ranging between 10 and 60 μM, were placed in a cuvette and small increments (8 μl) of the ligand solution were injected in the cuvette with a micropipette. The experiments were performed in 50 mM phosphate buffer *p*H 7.0 or 50 mM acetate *p*H 3.0, except with esterified and alkylated BLG when 50 mM phosphate buffer *p*H 6.5 was used. In order to eliminate the dilution of BLG solution by the added ligand solution and tryptophan fluorescence changes induced by alcohol, a blank containing BLG solution titrated with ethanol was monitored as described above. 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, tryptophan fluorescence intensity at 332 nm of free BLG was normalized to 1.

2.2.2. Determination of the Apparent Dissociation Constants

Differences in fluorescence intensity at 332 nm between the complex and free protein (excitation at 287 nm) were monitored according to Cogan *et al.* (1976) in order to determine apparent molar ratio and apparent dissociation constants of BLG/ligand complexes. It was assumed that the change in the fluorescence depends on the amount of protein-ligand complex. When plotting $(P_0 \cdot a)$ vs. $B[a/(1\text{-}a)]$, 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/BLG at saturation; P_0 is the total protein concentration; and B is the total ligand concentration. Here a is defined as the fraction of unoccupied binding sites on the protein molecules. The value of a was calculated for every desired point on the titration curve of fluorescence intensity enhancement using the relationship:

$$a = (F - 1)/(F_{\max-1})$$

where F represents the fluorescence intensity (corrected for the blank) at a certain B and $F_{\max}$ represents the fluorescence intensity upon saturation of BLG molecules.

3. RESULTS AND DISCUSSION

Bovine BLG variant B, called native BLG, was purified by NaCl salting out at low *p*H (Mailliart and Ribadeau Dumas, 1988). Folch's extraction method (Folch *et al.*, 1957) was used in order to delipidate studied protein samples. The analysis of the extract, after methylation of the fatty acids (Morrison and Smith, 1964), by gas chromatography displayed only minute amounts of fatty acids. In addition,

Table I. Apparent Dissociation Constants (K_d') and Apparent Molar Ratio (N) of Saturated Fatty Acid/BLG Complexes

	$K_d'(\times 10^7 M)^a$	N
Native BLG		
Caprylic acid C8:0	—	—
Capric acid C10:0	—	—
Lauric acid C12:0	7.0 ±1.6	0.86 ± 0.05
Myristic acid C14:0	3.3 ± 1.0	0.33 ± 0.01
Palmitic acid C16:0	1.0 ± 0.05	0.93 ± 9.11
Stearic acid C18:0	1.2 ± 0.07	0.89 ± 0.03
Arachidic acid C20:0	4.0 ± 0.03	0.85 ± 0.04
Delipidated BLG		
Myristic acid	1.8 ± 0.05	0.79 ± 0.05
Palmitic acid	1.3 ± 0.3	0.95 ± 0.2

[a]Each value is the average of at least three determinations.

native and "delipidated" BLG showed similar apparent molar ratios (0.93 and 0.95, respectively) and dissociation constants for palmitic acid (Table I). Consequently it was concluded that BLG isolated by salting out at acid pH did not contain lipids. In addition, native BLG is unable to bind fatty acids at acid pH since the titration of BLG in 50 mM acetate buffer pH 3.0 with palmitic acid, myristic acid, or oleic acid does not show any fluorescence enhancement of BLG tryptophans. These data on fatty acid/BLG interactions, as well as the studies of thermodynamic stability of BLG (Della and Kinsella, 1988), indicate that the lowering of the pH from 7.0 to 3.0 induces changes in the tertiary structure of the protein. In addition, BLG exists in various oligomeric states as a function of pH (Timasheff and Townend, 1964; Kessler and Brew, 1970). From the other side, the resistance of BLG to proteolysis by pepsin (Mohan Reddy *et al.*, 1988) demonstrates that the protein structure is relatively compact and stable in aqueous solutions at acid pH.

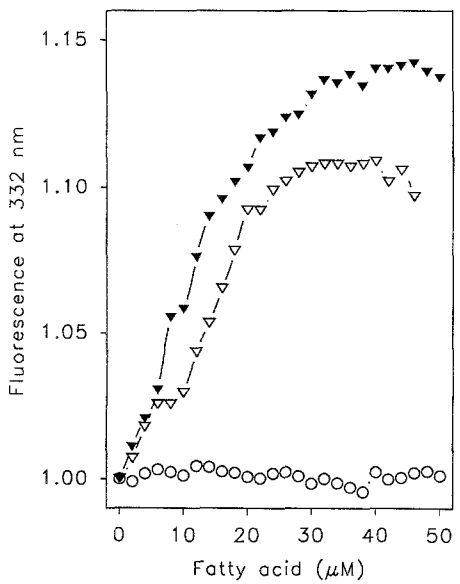

Fig. 1. Corrected BLG tryptophan fluorescence titration curves with caprylic acid (○), palmitic acid (▽), and arachidic acid (▼). During the titrations with caprylic acid, palmitic acid, and arachidic acid, BLG concentrations were 32 μM, 25 μM, and 35 μM, respectively.

BLG fatty acid binding site was studied using fatty acids of different chain length and containing from zero to four double bonds. Figure 1 shows the binding of palmitic and arachidic acids to native BLG at neutral pH. The extent of the observed enhancement of BLG tryptophan fluorescence varies in function of used fatty acid reaching 7% for lauric acid/BLG and 12% for palmitic acid/BLG complexes. In all cases, the maximum fluorescence enhancement of BLG tryptophans is observed for 1:1 stoichiometries, suggesting that BLG has only one high affinity fatty acids binding site. It has been previously reported that bovine BLG has additional low-affinity fatty-acid binding sites, too (Spector and Fletcher, 1970). The apparent binding constants and the apparent molar ratios of saturated and unsaturated fatty acid/native BLG complexes, calculated according to Cogan *et al.* (1976), are presented in Fig. 2 and Tables I and II. Palmitic acid shows the highest affinity ($K_d' = 1 \cdot 10^{-7}$M,$N = 0.93$) for BLG among the studied fatty acids. The apparent dissociation constants of palmitic acid/BLG and oleic acid/BLG complexes reported in this study agree well with the constants published by Perez *et al.* (1992). This may indicate that BLG is not denatured during purification by salting out at low pH.

The addition of caprylic or capric acid to BLG solution does not produce any fluorescence enhancement (Fig. 1), suggesting that these compounds do not bind to the protein in the applied conditions. Lauric acid binds to BLG and the affinity of BLG for saturated fatty acids (Table I) increases gradually from lauric acid ($K_d' = 7 \cdot 10^{-7}$M) to palmitic acid ($K_d' = 7 \cdot 10^{-7}$M). Then the apparent dissociation constants decrease for longer aliphatic chains, as shown in Table I. It seems that BLG fatty acid binding pocket can accommodate the best an aliphatic chain constituted by 16 carbon atoms. Apparently,

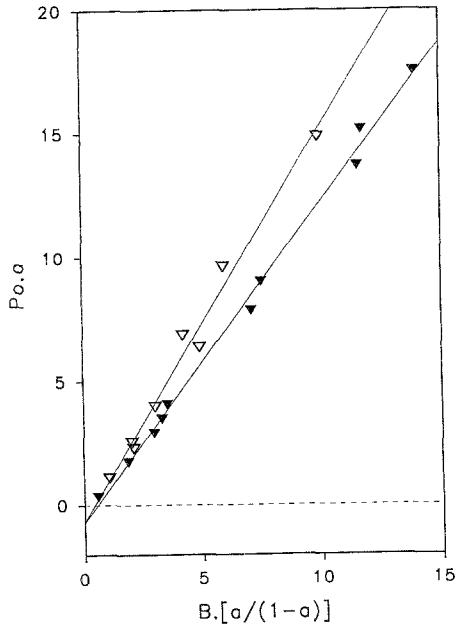

Fig. 2. Graphic representation of $P_0 \cdot a$ vs $B(a/1-a)$ (Cogan *et al.*, 1976) for the titration of BLG with palmitic acid (▽) and arachidic acid (▼).

the nature of fatty acid-BLG interactions is mainly hydrophobic, since the addition of 1 methylene group increases the protein affinity for ligands varying between C_{10}–C_{14} length. It should be emphasized that sodium dodecyl sulphate ($K'_d = 2.3 \cdot 10^{-7}$M) and dodecanyl acetate ($K'_d = 2.6 \cdot 10^{-7}$M) show similar affinities for BLG (E. Lamiot, personal communication). Apparently, however, the binding of fatty acids to BLG requires at least one hydrophobic chain-end since hexadecanedioic acid does not bind to BLG. It should be mentioned that, in contrast to BLG, dicarboxylic acids formed during ω-oxidation of monocarboxylic acids bind to bovine serum albumin (Tonsgard and Meredith, 1991).

As shown in Table II, unsaturated fatty acids bind also to native BLG. In most cases, except for myristoleic acid and arachidonic acid, the unsaturated fatty acids are slightly weaker BLG ligands than the saturated molecules. The apparent dissociation constants of oleic acid/BLG, linoleic acid/BLG, and linolenic acid/BLG complexes are $1.3 \cdot 10^{-7}$M, $1.9 \cdot 10^{-7}$M, and $1.7 \cdot 10^{-7}$M (Table II), respectively, compared to $K'_d = 1.2 \cdot 10^{-7}$M for stearic acid/BLG complex (Table I). BLG binds natural fatty acids (oleic acid, linoleic acid) with slightly higher affinity (Table II) than their *trans* isomers (elaidic acid, linolelaidic acid). Analyzing these results, it is apparent that structural constraints imposed by C=C double bonds in the aliphatic chain of fatty acids only weakly affect their binding to BLG. Surprisingly, prostaglandin A_1, a physiologically important derivative of dihomo γ-linolenic acid, does not bind to BLG in the applied conditions. The binding of various saturated and unsaturated fatty acids by BLG compared with its relatively stringent binding specificity toward the β-isomer of ionones (Dufour and Haertlé, 1990) and retinoids (Dufour and Haertlé, 1991) suggest that these two classes of ligands are bound at two distinct binding sites. The characterization of BLG retinol and fatty acid binding sites may be achieved by fluorescence spectroscopy which is a good technique for such a study, providing the system has fluorophores (Dufour *et al.*, 1990; Dufour and Haertlé, 1991). Retinol fluorescence is weak in solution alone, but it is greatly enhanced upon binding with BLG ($K'_d - 2 \cdot 10^{-8}$M). Its complex with BLG has a fluorescence spectrum (excitation at 342 nm) with a maximum emission at 480 nm (Fugate and Song, 1980). After excitation at 342 nm, palmitic acid/retinol/BLG complex (1/1/1) exhibits a typical fluorescence at 480 nm. In addition, the titration of retinol/BLG complex, as well as BLG, by palmitic acid, shows a typical fluorescence enhancement of tryptophan fluorescence (data not shown). Derived apparent dissociation constants are similar in the two cases: $K'_d = 1 \cdot 10^{-7}$M ($N = 0.93$) and $K'_d = 2.6 \cdot 10^{-7}$M ($N = 0.9$) for palmitic acid bound to BLG and retinol/BLG complex, respectively. These results indicate that

Table II. Apparent Dissociation Constants (K'_d) and Apparent Molar Ratio (N) of Mono-Unsaturated Fatty Acid/BLG Complexes

	$K'_d(\times 10^7\text{M})^a$	N
Myristoleic acid C14:1Δ9	1.6 ± 0.05	0.83 ± 0.06
Palmitoleic acid C16:1Δ9	2.6 ± 0.1	0.82 ± 0.02
Oleic acid C18:1Δ9	1.3 ± 0.05	0.82 ± 0.05
Elaidic acid C18:1Δ9 (*trans*)	1.5 ± 0.02	0.82 ± 0.06
Linoleic acid C18:2Δ9,12	1.9 ± 0.01	0.83 ± 0.08
Linolelaidic acid C18:2Δ9,12 (*trans, trans*)	3.0 ± 0.7	0.86 ± 0.07
Linolenic acid C18:3Δ9,12,15	1.7 ± 0.06	0.91 ± 0.1
γ-Linolenic acid C18:3Δ6,19,12	1.3 ± 0.01	0.93 ± 0.04
Arachidonic acid C20:4Δ5,8,11,14	3.3 ± 0.13	0.83 ± 0.07

[a]Each value is the average of at least three determinations.

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        1                                     20
BLGb :  L-I-V-T-Q-T-M-K-G-L-D-I-Q-K-V-A-G-T-W-Y-S-L-A-M-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-V-Y-V-E-E-
            * *     *     * *   * * * * * * *       * * *   * *   * * * * *   *   * *     * * *
BLGp :  V-E-V-T-P-I-M-T-E-L-D-T-Q-K-V-A-G-T-W-H-T-V-A-M-A-V-S-D-V-S-L-L-D-A-K-S-S-P-L-K-A-Y-V-E-G-

        46                                    65
BLGb :  L-K-P-T-P-E-G-D-L-E-I-L-L-Q-K-W-E-N-G-E-C-A-Q-K-K-I-I-A-E-K-T-K-I-P-A-V-F-K-I-D-A-L-N-E-N-
        * * * * * * * * * * * * * * *   * *     * * *         *   * *   * * * * * * *   * *   * *
BLGp :  L-K-P-T-P-E-G-D-L-E-I-L-L-Q-K-R-E-N-D-K-C-A-Q-E-V-L-L-A-K-K-T-D-I-P-A-V-F-K-I-N-A-L-D-E-N-

        91                                    110
BLGb :  K-V-L-V-L-D-T-D-Y-K-K-Y-L-L-F-C-M-E-N-S-A-E-P-E-Q-S-L-A-C-Q-C-L-V-R-T-P-E-V-D-D-E-A-L-E-K-
                * * * * *       * *   * * * * * *   * *   * *   * *   *   * *   * * * *       * *
BLGp :  Q-L-F-L-L-D-T-D-Y-D-S-H-L-L-L-C-M-E-N-S-A-S-P-E-H-S-L-V-C-Q-S-L-A-R-T-L-E-V-D-D-Q-I-R-E-K-

        136                                                     162
BLGb :  F-D-K-A-L-K-A-L- - -P-M-H-I-R-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I
        *     * * *   *     * *   *           *   * * * * * *
BLGp :  F-E-D-A-L-K-T-L-S-V-P-M-R-I-L- - - -P-A-Q-L-E-E-Q-C-R-V
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Fig. 3. Alignment of bovine (BLGb) and porcine (BLGp) BLG sequences using PC/GENE software. The residue numbers are those of bovine BLG. (*): identical residues.

retinol and palmitic acid are bound to BLG monomer in two different sites.

Alkylated and esterified BLG do not bind palmitic acid at neutral *p*H. However, it has been reported previously that the chemical modifications of BLG enhance the affinity of the protein for retinol (Dufour and Haertlé, 1991). Apparently, the secondary structure changes induced by the chemical modifications (Dufour and Haertlé, 1990), especially in the case of methylated BLG (30% β-sheet instead of 52% for native BLG), disorganize the fatty acid binding site. This observation gives additional evidence that retinol and fatty acids bind to BLG at two nonoverlapping binding sites.

Porcine BLG does not bind palmitic acid at neutral *p*H, whereas it binds retinol with an apparent dissociation constant $K_d' = 1.2 \cdot 10^{-7}$M. At a first sight, the differences in binding specificity's of porcine and bovine BLG are surprising. As shown in Fig. 3, porcine and bovine BLG display 66% of sequence homology. Porcine BLG is monomeric at neutral *p*H, in contrast to ruminant oligomeric BLG (Godovac-Zimmermann, 1988). For example, bovine BLG exists in various oligomeric states in function of *p*H, temperature, and concentration (Timasheff and Townend, 1964). At neutral *p*H, this protein is mainly dimeric, whereas it is monomeric at *p*H 3.0. It was proposed that the strand I [residues 145–150 (Papiz *et al.*, 1986) is involved in the formation of the dimer by making anti parallel interactions with the diad-related strand (Papiz *et al.*, 1986). The interface between monomers also involves stacking of two imidazoles (His-146 residues). Comparison of porcine and bovine BLG sequences shows major differences of their C-termini, when this region is highly conserved in all ruminant BLG sequences (Godovac-Zimmermann, 1988). The deletion of residues 149–152 in porcine BLG, as well as the replacement of His-146 by Arg-146, may explain the monomeric state of porcine BLG. It may also suggest that the fatty acid binding site of bovine BLG is close to the region involved in dimer formation, [i.e., strand I, helix 130–140, and strand A (Papiz *et al.*, 1986]. Prevailing evidence indicates that BLG may bind retinol at the highly conserved area of this protein super-family (Papiz *et al.*, 1986; North, 1989), inside the main hydrophobic pocket. Fatty acids could be bound elsewhere, may be in the external hydrophobic site described by Monaco *et al.* (1987), or at the interface between monomers since both monomerized bovine BLG (acid *p*H) and monomeric porcine BLG do not bind fatty acids.

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