Insect Sex Pheromone Binding by Bovine β-Lactoglobulin

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The binding of 24 derivatives of *Cydia pomonella* sex pheromone (dodecanol framework with no, one, or two conjugated double bonds at different positions of the aliphatic chain) and sodium dodecyl sulfate (SDS) to unmodified, acylated, alkylated, and esterified β-lactoglobulin (BLG) was studied by fluorescence spectrophotometry. Their apparent dissociation constants were in the range $0.2–0.9 \times 10^{-6}$ M (apparent molar ratio ca. 1). Dodecyl acetate and SDS displayed the highest affinity for BLG, when (*E,E*)-6,8-dodecadienyl acetate and (*E,E*)-7,9-dodecadienyl acetate did not bind to BLG in the applied conditions. The apparent dissociation constants of derivatives unconstrained by conjugated double bonds did not differ. The BLG binding affinity of other studied pheromone derivatives depends on (i) the number of double bonds, (ii) their position in the aliphatic chain, and (iii) their isomerisation (*Z* or *E*). Since, neither (*E*)-3-dodecenyl acetate nor (*Z,E*)-8,10-dodecadienyl acetate nor retinol binding constants changed during their simultaneous binding by BLG, it was concluded that they bind in different binding sites on the BLG molecule. Surprisingly, the chemical modifications that increased the hydrophobicity of BLG prevented its binding of the apolar ligand pheromone molecules.

INTRODUCTION

β-Lactoglobulin (BLG), which is found in the milk of several mammal species, is one of the most abundant proteins of bovine milk whey. Structural studies of BLG (Papiz et al., 1986; Godovac-Zimmerman, 1988) suggest that this protein may be classified in the superfamily of the small globular proteins involved in hydrophobic molecule transport termed lipocalins. Retinol binding protein (Newcomer et al., 1984), bilin binding protein (Huber et al., 1987), insecticyanin (Holden et al., 1987), and BLG are the best known proteins of this class. All of 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. In the 1940s, it was demonstrated that BLG may bind various fatty acids (Davis and Dubos, 1947) and their derivatives such as sodium dodecyl sulfate (SDS) (McKeekin et al., 1949). Developments in the studies of BLG binding properties show that it can also bind various small hydrophobic ligands such as retinol (Futterman and Heller, 1972), alkanone flavors (O'Neill and Kinsella, 1987), surfactants (Coke et al., 1990), porphyrins (Dufour et al., 1990), and ellipticine (Dodin et al., 1990) with considerable specificity. According to recent observations, retinol/BLG stoichiometry may be doubled when the BLG is in the "molten globule state" what may be induced, to some extent, by change of the medium polarity (Dufour and Haertlé, 1990a). In addition, it was shown that retinol and protoporphyrin IX are bound in two different BLG binding sites (Dufour et al., 1990). The correct understanding of the nature of the factors contributing to BLG binding properties may generate ideas for applications of this abundant whey protein or of its derivatives. Modification of BLG by enzymatic or chemical treatments may be one of the ways to modify or broaden the binding properties (Dufour and Haertlé, 1990b, 1991). BLG, which is produced in large amounts by the dairy industry, could be engineered to bind and protect a wide range of hydrophobic molecules.

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Insect sex pheromones display a large structural diversity, and they are largely used as efficient insect lures (Schneider, 1992). The study of pheromone/BLG interactions may give information on structural constraints involved in ligand binding by the protein. BLG could protect and retain these volatile and unstable hydrophobic molecules used in field protection (Audemart et al., 1979). Codling moth (*Cydia pomonella*) is a worldwide pest foraging on apples, and the major component of its pheromonal effluvia [(*E,E*)-8,10-dodecadien-1-ol] is named codlemone (Roelofs et al., 1971). It is part of a family of homologous compounds with C_{12} aliphatic chains (Einhorn et al., 1984). Several of these compounds lure the *Lepidoptera* males and are incorporated in the composition of synthetic attractants (Causse et al., 1988; Howell et al., 1992).

The results of experiments aiming at the elucidation of interactive properties of native and modified BLG with 24 molecules derived from *C. pomonella* sex pheromone are presented and discussed in this paper.

MATERIALS AND METHODS

Materials. Sodium dodecyl sulfate (Serva, France), lauryl acetate (Sigma, France), and dodecanol (Sigma, France) were used without further purification. Codlemone and its derivatives were a kind gift of Dr. J. Einhorn (INRA-Versailles, France). Stock solutions (1 M) of pheromones in ethanol were prepared and stored deep-frozen to limit possible degradation. Mass spectrometry confirmed the purity of these compounds. All other chemicals used were of reagent grade. β-Lactoglobulin (BLG) variant B was obtained from homozygous cow's milk according to the method of Mailliart and Ribadeau Dumas (1988), and as judged from high-performance liquid chromatograms on a C_{18} column and polyacrylamide gel electrophoresis, it was more than 95% pure. Interactions of BLG with fatty acids seem to be independent of the use or absence of extra delipidation procedures during purification of the protein (Frapin et al., 1993). It is suggested that the standard purification procedure frees the protein from fatty acid ligands. The UV absorbency spectra were recorded on a Cary 1 spectrophotometer (Varian). Concentrations of BLG were determined spectrophotometrically by using a molecular absorption coefficient $\epsilon_{278} = 17\,600$. Reductive methylation and ethylation of BLG lysine residues were performed according to the method of Cabacungan et al. (1982).

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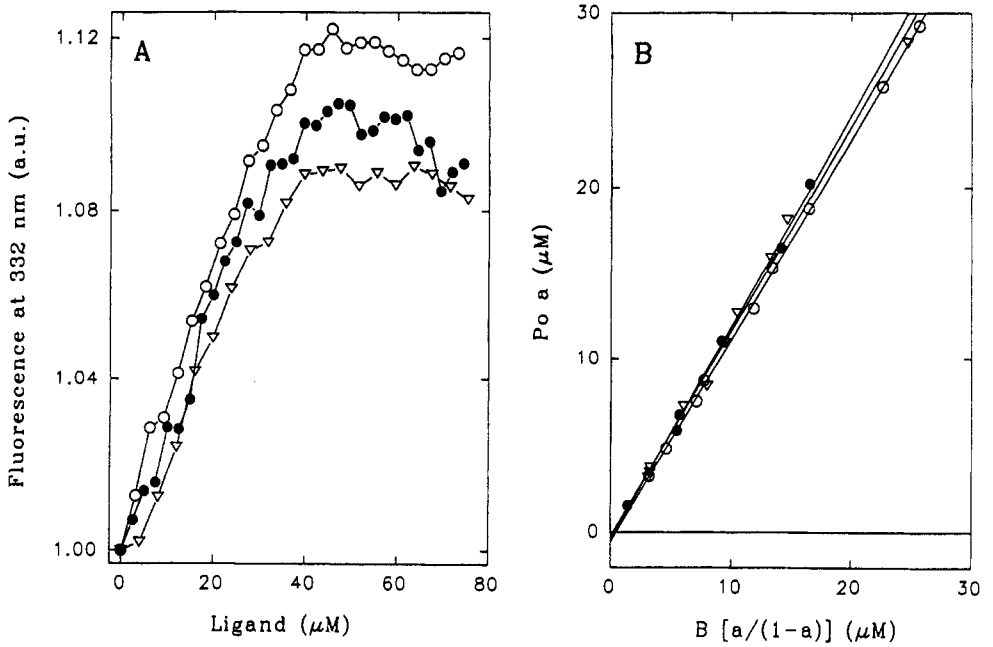

Figure 1. (A) Corrected BLG tryptophan fluorescence emission titration curves with dodecanol (▽), dodecyl acetate (●), and sodium dodecyl sulfate (○). (B) Graphic presentation (Cogan et al., 1976) of P_0a vs $B[a/(1-a)]$ of BLG titration with dodecanol (▽), dodecyl acetate (●), and sodium dodecyl sulfate (○). Protein concentration was 39.7 μM.

Acylation with stearic anhydride was done as described by Creuzenet et al. (1992). The yields of alkylation and acylation, determined by measuring the decrease in free amino group with o-phthaldialdehyde (Church et al., 1983), are 95, 85, and 40%, respectively, for formyl-, acetyl-, and stearyl-modified BLG. The methylated BLG derivative (MetBLG) was prepared by following the procedure described by Fraenkel-Conrad and Olcott (1945). The colorimetric determination of the degree of esterification for MetBLG, as shown by ferric hydroxamate chelate assay (Halpin and Richardson, 1985; Bertrand-Harb et al., 1991), shows that 90% of capped carboxyl groups were modified. The main physicochemical characteristics (isoionic point, secondary structure) of these derivatives have been reported elsewhere (Dufour and Haertlé, 1990b; Creuzenet et al., 1992).

Fluorescence Spectroscopy. Fluorescence emission spectra between 300 and 380 nm (excitation 287 nm) were recorded at 20 °C on an Aminco SLM 4800C spectrofluorometer in the ratio mode. The binding of the small hydrophobic molecules was measured by following the increase of protein tryptophan fluorescence at 332 nm. The following procedure was used for titration of native or modified BLG with SDS, codlemone, and its derivatives: 2 mL of BLG solution (or retinol/BLG complex solution), varying between 10 and 40 μM, was placed in a cuvette, and small increments of 8 μL of the ligand solution extemporaneously diluted in ethanol were injected in the cuvette with a micropipet. The experiments were performed in 50 mM phosphate (pH 7.0) except for MetBLG, where acetate buffer (pH 3.0) was used. To subtract the excessive effects of dilution of BLG by the added ligand solution and tryptophan fluorescence changes induced by alcohol, a blank containing BLG 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, BLG tryptophan initial fluorescence was normalized at $F_{332nm} = 1$.

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 the procedure of Cogan et al. (1976) 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. By plotting (P_0a) vs $B[a/(1-a)]$, 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. 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.

Table 1. Apparent Dissociation Constants (K'_d) and Apparent Molar Ratios (n) of Saturated Compound/BLG Complexes

compound	K'_d[a] (μM)	n[a]
SDS	0.23 ± 0.03	0.92 ± 0.04
dodecanol	0.34 ± 0.08	0.90 ± 0.08
dodecyl acetate	0.26 ± 0.04	0.85 ± 0.01

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

RESULTS

Figure 1A shows corrected BLG titration curves with SDS, dodecyl acetate, and dodecanol. The observed increases of BLG tryptophan fluorescence emission intensities were in the same range, yielding 8.5, 9.5, and 10.9% for dodecanol, dodecyl acetate, and SDS, respectively. The fluorescence emission intensity plateaus for a ligand/protein ratio of 1/1. Dodecanol/BLG showed K'_d = 0.34 μM and n = 0.90. Dodecyl acetate/BLG (K'_d = 0.26 μM; n = 0.85) and SDS/BLG (K'_d = 0.23 μM; n = 0.92) complexes display quite similar apparent dissociation constants, and the number of ligand binding sites *per* BLG monomer is close to 1 (Table 1). Table 2 presents the dependence of apparent dissociation constants on the position of the double bond in the aliphatic chains of dodecanol and dodecyl acetate derivatives with single double bonds and on their isomerization (*Z* or *E*). There are small differences between dodecanol and dodecyl acetate derivatives unsaturated in the same position depending on double-bond isomerization. The smallest dissociation constants are observed in the case of the double bond in position 3 [K'_d = 0.35 and 0.28 μM for (*E*)-3-

Table 2. Apparent Dissociation Constants (K'_d) and Apparent Molar Ratios (n) for Monounsaturated Compound/BLG Complexes

compound	K'_d[a] (μM)	n[a]
(*E*)-3-dodecen-1-ol	0.35 ± 0.01	0.86 ± 0.06
(*E*)-7-dodecen-1-ol	0.44 ± 0.01	0.86 ± 0.02
(*E*)-8-dodecen-1-ol	0.54 ± 0.03	0.88 ± 0.10
(*E*)-9-dodecen-1-ol	0.64 ± 0.03	0.91 ± 0.05
(*Z*)-7-dodecen-1-ol	0.61 ± 0.09	0.89 ± 0.04
(*Z*)-8-dodecen-1-ol	0.60 ± 0.04	0.92 ± 0.08
(*Z*)-9-dodecen-1-ol	0.56 ± 0.05	0.93 ± 0.01
(*E*)-3-dodecenyl acetate	0.28 ± 0.01	0.99 ± 0.05
(*E*)-7-dodecenyl acetate	0.41 ± 0.07	0.89 ± 0.07
(*E*)-8-dodecenyl acetate	0.45 ± 0.03	0.86 ± 0.05
(*E*)-9-dodecenyl acetate	0.57 ± 0.06	0.87 ± 0.10
(*E*)-10-dodecenyl acetate	0.48 ± 0.02	0.88 ± 0.07
(*Z*)-7-dodecenyl acetate	0.59 ± 0.06	0.91 ± 0.07
(*Z*)-8-dodecenyl acetate	0.60 ± 0.04	0.85 ± 0.02
(*Z*)-9-dodecenyl acetate	0.46 ± 0.03	0.86 ± 0.10

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

Table 3. Apparent Dissociation Constants (K'_d) and Apparent Molar Ratios (n) of Complexes between Conjugated Dienes and BLG

compound	K'_d[a] (μM)	n[a]
(*E,E*)-8,10-dodecadien-1-ol	0.92 ± 0.06	0.85 ± 0.07
(*E,Z*)-8,10-dodecadien-1-ol	0.70 ± 0.03	0.93 ± 0.04
(*Z,E*)-8,10-dodecadien-1-ol	0.76 ± 0.02	0.93 ± 0.01
(*E,E*)-6,8-dodecadienyl acetate	ni[b]	ni
(*E,E*)-7,9-dodecadienyl acetate	ni	ni
(*E,Z*)-7,9-dodecadienyl acetate	0.85 ± 0.10	0.80 ± 0.06
(*E,E*)-8,10-dodecadienyl acetate	0.64 ± 0.08	0.86 ± 0.04
(*Z,E*)-8,10-dodecadienyl acetate	0.91 ± 0.02	0.84 ± 0.01

[a] Each value is the average of at least three determinations. [b] ni, no interaction detected in the conditions used.

dodecen-1-ol and (*E*)-3-dodecenyl acetate, respectively]. Higher values were observed in the case of *Z* isomers in position 7 or 8 and in the case of *E* isomers in position 9. Lower affinity was observed for (*E*)-9-dodecen-1-ol (K'_d = 0.64 μM) (Table 2).

Results of BLG titration with codlemone and its derivatives with two conjugated double bonds are presented in Table 3. All of the *E* and *Z* isomers of the studied compounds bind to BLG. The highest BLG affinity was observed for (*E,Z*)-8,10-dodecadien-1-ol (K'_d = 0.70 μM), and the lowest was seen for (*Z,E*)-8,10-dodecadienyl acetate (K'_d = 0.91 μM). Two *E,E* isomer compounds also bind to BLG. They are codlemone [(*E,E*)-8,10-dodecadien-1-ol] and its acetate derivative. No BLG tryptophan fluorescence enhancement was observed in the case of (*E,E*)-6,8-dodecadienyl acetate or (*E,E*)-7,9-dodecadienyl acetate. Esterified, alkylated, and acylated BLG does not bind (*E*)-3-dodecenyl acetate and (*E,Z*)-7,9-dodecadienyl acetate in the applied conditions.

It is known that BLG binds tightly one retinol molecule per monomer *in vitro* ($K'_d = 2 \times 10^{-8}$ M; Fugate and Song, 1980). The addition of (*E*)-3-dodecenyl acetate and (*Z,E*)-8,10-dodecadienyl acetate to retinol/BLG (1/1) solution induces a typical tryptophan fluorescence enhancement. The apparent dissociation constants determined for (*Z,E*)-8,10-dodecadienyl acetate/BLG, (*Z,E*)-8,10-dodecadienyl acetate/retinol/BLG, (*E*)-3-dodecenyl acetate/BLG, and (*E*)-3-dodecenyl acetate/retinol/BLG complexes are 0.91, 0.90, 0.28, and 0.21 μM, respectively. These data may indicate simultaneous binding of these two types of ligands (retinoids and studied pheromones) at two different binding sites on or in monomeric BLG molecule.

DISCUSSION

The BLG binding of small hydrophobic ligands has been previously studied (Davis and Dubos, 1947; Futterman and Heller, 1972; Coke et al., 1990; Dufour et al., 1990). The structural and chemical variety of known BLG ligands as well as its relatively stringent binding specificity toward β-ionone (Dufour and Haertlé, 1990b) raises a question: how is the ligand binding specificity determined? Apparently, in the case of the studied compounds, the position of double bonds, isomer type, and aliphatic chain length (Spector and Fletcher, 1970; Diaz de Villegas et al., 1987; Perez et al., 1989) are important factors in the binding of these ligands to BLG. Investigation of the binding of 24 related compounds derived from codlemone by this protein allows an approach to the understanding of some of its potential binding specificity.

BLG binds fatty acids [K'_d = 0.24 μM for palmitate/BLG complex (Perez et al., 1992)] and SDS which share a common structural pattern of aliphatic compounds and differ only in their polar termini. It has been claimed (Jones and Wilkinson, 1976) that the binding of SDS to BLG is driven essentially by ionic interactions. In contrast, Hillquist-Damon and Kresheck (1982) have suggested that, in addition to the formation of an ion pairing, the SDS/BLG interaction must involve hydrophobic interactions too.

Obtained apparent dissociation constants for SDS/BLG, dodecanol/BLG, and dodecyl acetate/BLG complexes (Table 1) are very similar and indicate that this kind of ligand/BLG interaction has mainly hydrophobic character. BLG complexes with dodecen-1-ol or dodecenyl acetate with double bonds in the same positions show similar apparent dissociation constants too (Tables 1 and 2). The character of the head of these saturated or monounsaturated compounds, displaying weak constraints in their aliphatic chains, has little effect on their binding to BLG. The observed differences may be explained essentially by the smaller or bigger bulkiness and hydrophobicity of the termini of these ligands. Surprisingly, the apparent dissociation constants of (*Z,E*)-8,10-dodecadien-1-ol, codlemone, and their acetate derivatives vary in function of their extremities (Table 3).

When the compounds with one double bond are analyzed, it is seen that the (*E*)-3-dodecenyl acetate/BLG complex displays the highest affinity when BLG binds (*Z*)-7-dodecen-1-ol, (*Z*)-8-dodecen-1-ol, and (*E*)-9-dodecen-1-ol with slightly weaker affinity (Table 2). Structural constraints imposed by C=C double bonds at positions 7, 8, and 9 are decreasing the BLG specificity for these ligands. In the case of the compounds with an *E* isomer of a double bond at positions 3, 7, 8, and 9, the farther the C=C double bond is situated from the polar end, the smaller is the BLG affinity for such a ligand. This suggests that BLG interactions with these ligands are more stringent around carbons C7, C8, and C9 than at carbon C3. This implies also that the hydrophobic terminal part of the ligand is more essential to the BLG recognition than the opposite alcohol or ester end of the molecule. It is likely that the polar end of these compounds protrudes outside the binding site. Interestingly, it seems that the biological receptors react to some extent to the stimulation conferred also by the polar fragments (ends) of these molecules since codling moth males are responding the most to the stimulation with (*E*)-9-dodecen-1-ol, (*E*)-10-dodecen-1-ol, and their acetate derivatives and with (*Z*)-10-dodecen-1-ol and its acetate derivative (Audemart et al., 1979).

Higher K'_d values observed in the case of dodecadiene derivative/BLG complexes suggest that structural con-

straints imposed by conjugated double bonds decrease their recognition by BLG and complex formation. As in the above discussed case of dodecene derivatives, it appears that, in dodecadiene compounds, placement of the double bonds in positions 7, 8, and 9 has the most unfavorable effect on the binding of these compounds by BLG (Tables 2 and 3). In the applied conditions, (*E*,*E*)-6,8-dodecadienyl acetate and (*E*,*E*)-7,9-dodecadienyl acetate do not bind to BLG, while (*E*,*Z*)-7,9-dodecadienyl acetate does. It implies that structural constraints imposed by *E*,*E* conjugated double bonds at positions 6,8 and 7,9 disturb efficiently the binding of these compounds to BLG. Data obtained with the conjugated dienes indicate that the *E*,*Z* and *Z*,*E* conjugation in positions 6,8 and 7,9 is less restrictive for binding than *E*,*E*. These results could suggest that the pheromone methylene groups at positions 7, 8, and 9 may be in close contact with the amino acids forming the binding pocket. Apparently, the binding of codlemone and its derivatives to BLG is driven by the hydrophobic interactions and also depends on (i) the number of double bonds in the chain, (ii) their position, and (iii) their isomerization (*Z* or *E*). An alternative explanation would point toward general change of shape of these ligands.

It is shown that (*E*)-3-dodecenyl acetate (Table 2) and (*Z*,*E*)-8,10-dodecadienyl acetate (Table 3) bind to BLG, as well as to retinol/BLG complexes (discussed before) with similar apparent dissociation constants. Retinol fluorescence is very weak in aqueous solutions, but it is greatly enhanced when vitamin A is bound to BLG ($K'_d = 2 \times 10^{-8}$ M). Its complex with BLG exhibits a typical fluorescence emission spectrum (excitation at 342 nm) with a maximum at 480 nm (Fugate and Song, 1980). On one hand, after excitation at 342 nm, (*E*)-3-dodecenyl acetate/retinol/BLG complex (1/1/1) exhibits a typical fluorescence at 480 nm, and, on the other hand, the addition of (*E*)-3-dodecenyl acetate [or of (*Z*,*E*)-8,10-dodecadienyl acetate] to retinol/BLG complex induces an increase of tryptophan fluorescence intensity which plateaus for pheromone/complex (1/1). These results indicate that retinol and (*E*)-3-dodecenyl acetate [and (*Z*,*E*)-8,10-dodecadienyl acetate] are bound to the BLG monomer at two different sites. In the applied conditions, esterified, acylated, and alkylated BLG does not bind codlemone and its derivatives at all. Previous studies have shown that esterification and alkylation generally enhance the binding affinity of retinol and β-ionone to the modified protein (Dufour and Haertlé, 1990b, 1991). The structural changes induced by the chemical modifications (Dufour and Haertlé, 1990b), especially in the case of methylated BLG, do not disassemble the structure of the retinol binding site. Apparently, modification of BLG structure by chemical treatments disassembles the pheromone binding site. This observation gives additional evidence that retinol and pheromones bind to BLG at two nonoverlapping binding sites.

It has been shown previously that BLG monomer binds retinol and protoporphyrin IX at two different binding sites. Obviously, BLG, like serum albumin, may bind a large variety of hydrophobic molecules. Studies of BLG binding of various hydrophobic ligands may have an interesting outcome as the transport and protection of relatively unstable compounds like these pheromones are concerned.

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