Binding Affinities of β-Ionone and Related Flavor Compounds to β-Lactoglobulin: Effects of Chemical Modifications†

Eric Dufour and Tomasz Haertlé*

LEIMA, Institut National de la Recherche Agronomique, B.P. 527, 44026 Nantes Cedex 03, France

The effects of chemical modifications, esterification or reductive alkylation, on binding properties of several terpenes by β-lactoglobulin were examined. As shown by fluorescence quenching, β-lactoglobulin and its derivatives bind β-ionone, but it does not bind α-ionone, neither geraniol nor R(+)- and S(−)-limonene. It is suggested that its binding site, presumably situated within the calyx-shaped protein fold (North, A. C. T. *Int. J. Biol. Macromol.* **1989**, *11*, 56–58), has a narrow specificity to the structure formed by the conjugated double bonds of the β-ionone ring and isoprenoid chain, present in both terpenes—β-ionone and retinol. The complexes of β-ionone with the derivatives of β-lactoglobulin (except N-ethyllysyl-BLG) exhibit lower K_d' values than that of the complex of β-ionone with unmodified β-lactoglobulin. N-Methyllysyl-BLG and EtBLG are binding β-ionone stronger than the other even more extensively modified derivatives as, for example, N-ethyllysyl-BLG and MetBLG. The partial loss of BLG β-barrel structure achieved during extensive esterification producing MetBLG, deduced from the analysis of circular dichroism spectra, could explain its less effective binding to β-ionone.

INTRODUCTION

β-Lactoglobulin, which is found in the milk of several mammal species, is one of the most abundant proteins of the whey. In spite of intense studies the biological role of this protein is not well established.

Recent developments in structural studies of small proteins interacting with hydrophobic ligands have shed new light on the molecule β-lactoglobulin. It has been postulated recently that β-lactoglobulin belongs to the "superfamily" of proteins (Pervaiz and Brew, 1985; Papiz et al., 1986; Godovac-Zimmermann, 1988) involved in the strong interactions with small hydrophobic molecules: retinol and its derivatives, pheromones, biliverdines, pyrazines, etc. Retinol binding protein (Newcomer et al., 1984), bilin binding protein (Huber et al., 1987), insecticyanin (Holden et al., 1987), and β-lactoglobulin (Papiz et al., 1986; Monaco et al., 1987) are the best known proteins of this class. All these proteins share common tridimensional structural pattern: an eight-stranded antiparallel β-barrel flanked on one side by an α-helix constituting a hydrophobic pocket. Crystallographic data (Newcomer et al., 1984; Huber et al., 1987) indicate that the ligands of retinol binding protein and bilin binding protein are bound inside the calyx formed by the β-barrel. It is known that, in vitro, β-lactoglobulin binds tightly one retinol molecule per monomer (Futterman and Heller, 1972). It binds also free fatty acids and triglycerides (Brown, 1984; Diaz de Villegas et al., 1987), aromatic hydrocarbons (Farrell et al., 1987), and alkanone flavors (O'Neill and Kinsella, 1987). The chemical and structural variety of its ligands stimulate a question: how is the ligand binding specificity determined?

The correct understanding of the nature of the factors contributing to β-lactoglobulin binding properties may generate ideas for applications of this abundant whey protein or of its nontoxic derivatives as food additives. For instance, β-lactoglobulin could be engineered (i) to bind and protect a wide range of volatile and unstable flavors during food manufacturing or to release them in a more or less controlled way by a simple treatment or (ii) to trap undesirable compounds.

Modification of β-lactoglobulin by enzymatic or chemical treatments may be one way of inducing the conformational change of this protein. Consequently, it may alter or broaden its binding properties. Esterification of carboxyl groups (Fraenkel-Conrat and Olcott, 1945) or alkylation of lysine residues (Means and Feeney, 1968) is a simple, cheap, and nontoxic (if done properly) chemical modification of proteins.

In the paper we try to investigate by fluorescence the interactive properties of native, alkylated, or esterified β-lactoglobulin with several flavor compounds: R(+)- and S(−)-limonen, geraniol, and α- and β-ionone, which are terpenes isolated from the volatile oils of different plants. All these molecules are structurally related to retinol. On one hand, the monoterpenes constitute a large group of natural products with frequent aromatic properties. Geraniol plays a key role in isoprenoid metabolism and R(+)- and S(−)-limonene are obtained by its cyclization (Croteau, 1986). On the other hand, α- and β-ionone are the degradation products of carotene with an aroma similar to that of violets. According to Weeks (1986), β-carotene, the most widely known tetraterpene in plants, is the direct precursor of β-ionone and retinol.

MATERIALS AND METHODS

Preparation of β-Lactoglobulin and Its Derivatives. All chemicals used were of reagent grade if not stated otherwise. β-Lactoglobulin (BLG) 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 a C_{18} column and polyacrylamide gel electrophoresis, it was more than 95% pure. The methylated and ethylated β-lactoglobulin derivatives were prepared according to the procedure described by Fraenkel-Conrad and Olcott (1945).

† Work presented in this paper has been funded by the Institut National de la Recherche Agronomique in the scope of the project "Study of the hydrophobic interactions of β-lactoglobulin".

* Author to whom correspondence should be addressed.

0021-8561/90/1438-1691$02.50/0 © 1990 American Chemical Society

After the extensive esterification the protein–alcohol suspensions were freeze-dried and the excess of acid catalyzer (HCl) was removed under strong vacuum.

Reductive methylation and ethylation of lysine residues in β-lactoglobulin were performed as described by Cabacungan et al. (1982). Then the *N*-methyllysyl-BLG and the *N*-ethyllysyl-BLG were dialyzed and lyophilized. Determination of unmodified lysine residues in the derivatives by the method of Adler-Nissen (1979) shows that, in *N*-methyllysyl-BLG, 70% of the lysine residues are alkylated. The *N*-ethyllysyl-BLG is more alkylated since 85% of the lysine residues are capped.

Circular Dichroism Spectroscopy. Circular dichroism spectra were measured on a Jobin Yvon Mark III dichrograph, and data were recorded on-line by using an Olivetti personal computer. Used cylindrical cells had a path length of 0.02 cm in the far-ultraviolet spectra (190–260 nm). All the spectra were taken at 20 °C by using β-lactoglobulin concentrations in the range 20–30 μM. Concentrations of β-lactoglobulin were determined spectrophotometrically by using, for the calculations, a molecular absorption coefficient ϵ_{278} = 17 600. The methods of Brahms and Brahms (1980) and Chen and Yang (1971) were assayed to simulate the experimental spectra. Subsequently, we used the method of Brahms, which gave the best fit with available β-lactoglobulin X-ray structural data as described by Papiz et al. (1986) and Monaco et al. (1987).

Fluorescence Spectroscopy. Fluorescence spectra were recorded at 20 °C on a Aminco SLM 4800C spectrofluorometer in the ratio mode (I/I_0 or F). The excitation and emission slits were usually set at 4 nm. We have investigated tryptophan fluorescence emission spectra of β-lactoglobulin (ex 290 nm).

The binding of the small hydrophobic molecules was evaluated by following the quenching of protein fluorescence. For titration of BLG or its derivatives with various ligands the following procedure was used. Two milliliters of protein solution, approximately 10 μM, was placed in a cuvette. To this were injected with a micropipet small increments of 4 μL at a time of the ligand solution in ethanol. As the titration proceeded toward its end, larger amounts of ligand were added. At the end of the various titrations the ethanol concentration did not exceed 5%. The tryptophan emission spectra of BLG dissolved in buffered solutions containing 0% or 5% ethanol are quite similar. Additional measurement of circular dichroism spectra did not reveal any chirality changes up to 15% v/v ethanol concentration. On the basis of these results, it was assumed that ethanol influence is negligible in this concentration range. The experiments were performed in 50 mM acetate, pH 3.0, for BLG and its esterified derivatives, and in 50 mM phosphate, pH 7.1, for alkylated derivatives (and BLG).

To eliminate the possibility of the nonspecific interactions of the studied ionones with tryptophan indoles in BLG, the titrations of *N*-acetyl-L-tryptophanamide solutions with an absorbency at 290 nm equal to that of the protein were used as controls. Apparently, this compound displays a fluorescence typical of the protein, but according to obtained results it is unable to interact with the studied compounds.

Determination of Apparent Dissociation Constants. Differences in fluorescence intensity at 332 nm between the complex and free protein were monitored to determine apparent dissociation constants of various ligands complexed by BLG and its derivatives. It was assumed that the change in the fluorescence depends on the amount of protein–ligand complex, and the apparent dissociation constants were obtained according to the method of Cogan et al. (1976)

$$K_d' = (a/1-a)[B - nP_0(1-a)]$$

which can be rearranged to

$$P_0 a = 1/n[B(a/1-a)] - (K_d'/n) \quad (1)$$

where K_d' is the apparent dissociation constant, n is the number of independent binding sites, and P_0 is the total protein concentration. a is defined as the fraction of free binding sites on the protein molecules. The value of a was calculated for every desired point on the titration curve of fluorescence intensity versus total ligand concentration by using the relation

Table I. Estimation of α-Helix and β-Sheet Content in Native and Modified β-Lactoglobulin Using Brahms Method

β-lactoglobulin derivative	α-helix, %	β-sheet, %	aperiodic segments, %
unmodified BLG, pH 3.0	7	52	41
methyl esterified BLG	15	30	55
ethyl esterified BLG	9	47	44
unmodified BLG, pH 7.1	8	44	48
N-methyllysyl-BLG	5	43	52
N-ethyllysyl-BLG	4	42	54

$$a = (F_0 - F)/(F_0 - F_{min})$$

where F represents the fluorescence intensity at a certain B, F_0 is the fluorescence of the free protein, and F_{min} represents the fluorescence intensity upon saturation of all protein molecules. All the fluorescence values were first corrected for the blank. Plotting $P_0 a$ vs $B(a/(1-a)$ yields a straight line with an intercept of K_d'/n and a slope of $1/n$.

RESULTS AND DISCUSSION

Characterization of β-Lactoglobulin Derivatives. During the esterification, BLG is suspended in anhydrous alcohol with the addition of hydrochloric acid as the reaction catalyzer, and esters of BLG carboxyl groups were prepared by using methanol and ethanol. The apparent isoelectric points of methylated β-lactoglobulin (MetBLG) and ethylated β-lactoglobulin (EtBLG) were 9.6 and 8.0, respectively, compared to 4.8 for unmodified β-lactoglobulin as measured after an equilibration in the presence of mixed bed anionite/cationite AG 501-X8 (Bio-Rad) resin. Esterification of BLG by methanol, as it may be deduced from the isoionic point of the modified protein (9.6 for MetBLG and 8.0 for EtBLG), proceeded much more extensively than esterification with ethanol. In addition, the colorimetric determination of the degree of esterification for MetBLG and EtBLG, as shown by ferric hydroxamate chelate assay (Halpin and Richardson, 1985), displays 90% and 21.5% of modified carboxyl groups, respectively. The esterification of BLG induces changes in the gross charge and in the distribution of electrostatic charge on the protein molecules. Consequently, the solubility of the esterified BLG changes and its esterified derivatives are weakly soluble at neutral pH. At pH 3.0, the methyl esterified BLG is less soluble than the ethyl esterified BLG, suggesting a more random and more hydrophobic structure for the methyl esterified derivative (Mattarella et al., 1983; Mattarella and Richardson, 1983) brought about by higher substitution of protein carboxylates. According to the analysis of the obtained circular dichroism data (Table I), MetBLG has partially randomized structure (30% of β-sheet structure). EtBLG, less derivatized than MetBLG, exhibits a secondary structure quite similar to BLG (52% of β-sheet).

Reaction of alkylation performed in mild conditions (Cabacungan et al., 1982) is highly specific, and only free ϵ- and α-amino groups of the proteins are modified. No significant electrostatic changes can be observed; i.e., the measured isoionic points of *N*-methyllysyl-BLG and *N*-ethyllysyl-BLG are 4.7 and 5.0, respectively. The alkylated derivatives, however, are much less soluble at acidic pH than native BLG. The degree of modification of the amino groups was determined by using 2,4,6-trinitrobenzenesulfonic acid (Adler-Nissen, 1979). Surprisingly, the *N*-ethyllysyl-BLG is more alkylated (85% of modified lysine residues) than the *N*-methyllysyl-BLG (70% of modified lysine residues). Nevertheless, BLG and its alkylated derivatives display similar contents of ordered secondary structures (Table I).

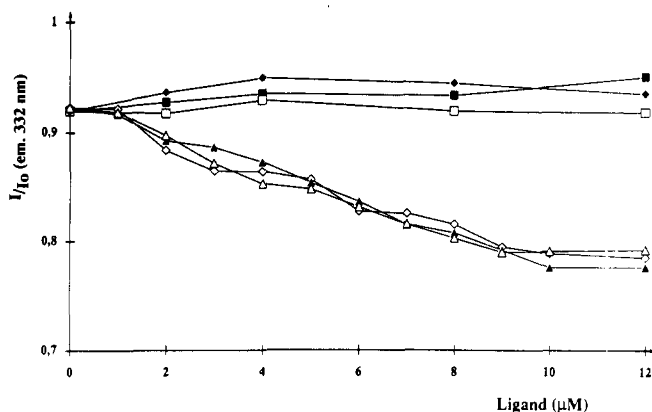

Figure 1. Corrected fluorescence titration curves of β-lactoglobulin and its esterified derivatives with α- and β-ionone. Excitation wavelength; 290 nm; emission wavelength; 332 nm. Binding of α-ionone to β-lactoglobulin, 10.6 μM (■), methyl esterified BLG, 11.2 μM (□), and ethyl esterified BLG, 11 μM (◆). Binding of β-ionone to β-lactoglobulin, 10.6 μM (◇), methyl esterified BLG, 11.2 μM (△), and ethyl esterified BLG, 11 μM (▲).

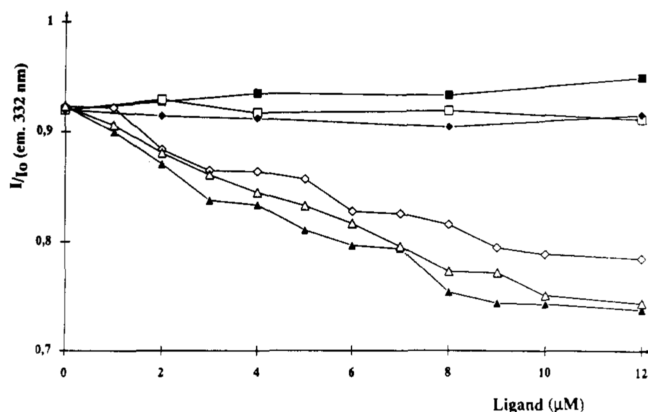

Figure 2. Corrected fluorescence titration curves of β-lactoglobulin and its alkylated derivatives with α- and β-ionone. Excitation wavelength, 290 nm; emission wavelength, 332 nm. Binding of α-ionone to β-lactoglobulin, 10.6 μM (■), N-methyllysyl-BLG, 11.5 μM (□), and N-ethyllysyl-BLG, 7.5 μM (◆). Binding of β-ionone to β-lactoglobulin, 10.6 μM (◇), N-methyllysyl-BLG, 11.5 μM (▲), and N-ethyllysyl-BLG, 7.5 μM (△).

Binding of Flavor Compounds to β-Lactoglobulin and Its Derivatives. The fluorescence emission spectra of BLG or its derivatives were studied as a function of added compounds, and the observed tryptophan fluorescence quenching, due to changes of the polarity in the neighborhood of indoles (Lakowicz, 1983), is indicative of the formation of a complex.

The addition of geraniol or R(+)- and S(−)-limonene to protein solutions does not produce any fluorescence quenching (data not shown), suggesting that these monoterpenes do not bind to BLG or its derivatives or that they do not interfere with BLG tryptophans. In the same way, and this is more surprising, α-ionone binding to BLG or its derivatives cannot be demonstrated by fluorometry (Figures 1 and 2) either. The addition of β-ionone induces a significant quenching of BLG fluorescence. Corrected titration curves for BLG, MetBLG, EtBLG, N-methyllysyl-BLG, and N-ethyllysyl-BLG are shown in Figures 1 and 2, and the maximum fluorescence quenching is obtained at a β-ionone–protein ratio of 1:1. It should be pointed out that the decrease in the fluorescence intensity of the blank N-acetyl-L-tryptophanamide solution is not due, apparently, to the interaction between β-ionone and N-acetyl-L-tryptophanamide, but rather to the inner filter effect as a result of the absorbance of β-ionone at 290 nm.

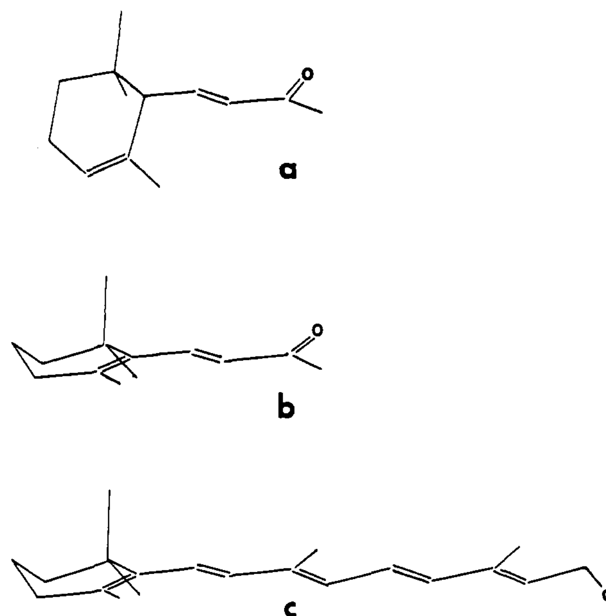

Figure 3. Optimized structures of α-ionone (a), β-ionone (b), and retinol (c). The molecules were built and their geometries minimized with the help of the molecular modeling software Sybyl (Tripos Associates, St. Louis, MO).

It may be concluded on the basis of these results that, from the tested terpenes, BLG and its derivatives bind only β-ionone. β-Ionone and retinol can be derived from β-carotene, and they share the same structure (Figure 3); differing only by the length of the isoprenoid chain. Two ionone isomers, α and β, differ in the placement of the cyclohexenyl double bond, which is conjugated with the one in the isoprenoid chain in the β-isomer. The conjugation of the two double bonds imposes conformational constraints; the β-ionone cycle and all the conjugated double bonds are placed in the same plane as shown by the structure energy minimalization of β-ionone and retinol using the molecular modeling software Sybyl (Figure 3). As seen in Figure 3 prediction of the α-ionone conformation at this energy minimum yields a very different structure. In that way, when their binding to BLG is studied, these two isomers produce totally different results, suggesting that the binding site of BLG, besides its specificity to certain hydrophobic features, is also highly structure specific. It may be assumed that structural constraints brought about by the conjugation of the cyclohexenyl ring double bond through the vicinal C═C double bond system of the isoprenoid tail are essential for the recognition by BLG and a complex formation. As it may be judged from the comparison of the apparent dissociation constants of retinol–BLG (2×10^{-8} M; Fugate and Song, 1980) and β-ionone–BLG (6×10^{-7} M) complexes, the length of the isoprenoid tail seems to be less important in the determination of the binding specificity. BLG and retinol binding protein (Newcomer et al., 1984) display similar tridimensional structures overlapping is more than 95% (Papiz et al., 1986; North, 1989). Newcomer et al. (1984) have shown that retinol is bound in the hydrophobic pocket shaped by an eight-stranded β-barrel.

Thus, in the studied case of BLG one seems to face an intriguing situation of a protein with a narrow specificity to the structural motif formed by the conjugated double bonds of the β-ionone ring and isoprenoid chain. But BLG is known to bind also to structurally different molecules such as free fatty acids and triglycerides (Diaz de Villegas et al., 1987) and alkanone flavors (O'Neill and Kinsella, 1987). The above-mentioned BLG capacity to bind chemically and structurally miscellaneous ligands could

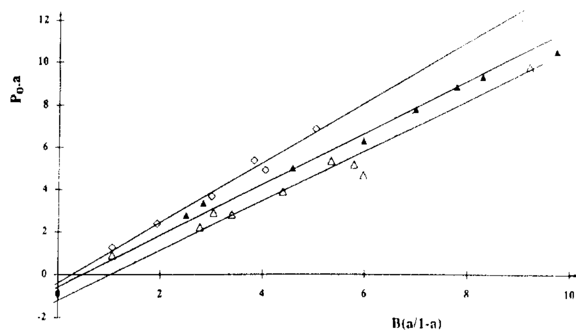

Figure 4. Linear plots of eq 1, P_0a vs $B(a/1-a)$, for the titration of β-lactoglobulin and its esterified derivatives with β-ionone. β-Lactoglobulin (△); methyl esterified BLG (▲); and ethyl esterified BLG (◇).

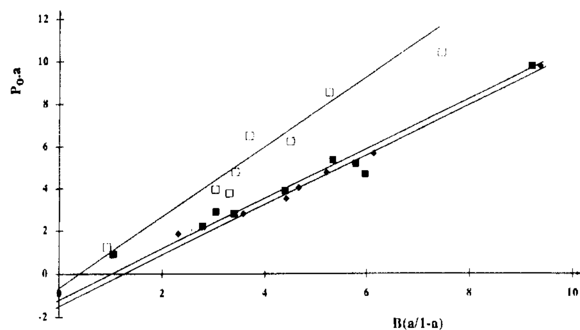

Figure 5. Linear plots of eq 1, P_0a vs $B(a/1-a)$, for the titration of β-lactoglobulin and its alkylated derivatives with β-ionone. β-Lactoglobulin (■); *N*-methyllysyl-BLG (□); and *N*-ethyllysyl-BLG (◆).

Table II. Apparent Dissociation Constants, K_d', and Apparent Molar Ratio of β-Ionone–Protein, *n*, for β-Lactoglobulin and Its Derivatives

β-lactoglobulin derivative	K_d', M	*n*
unmodified BLG	6×10^{-7}	1.08
methyl esterified BLG	3.5×10^{-7}	1.2
ethyl esterified BLG	1.7×10^{-7}	1.3
N-methyllysyl-BLG	3×10^{-7}	1.5
N-ethyllysyl-BLG	7.6×10^{-7}	1.11

suggest that BLG, in addition to the presumable deep central pocket site, may potentially bind these ligands elsewhere—as it has been proposed by Monaco et al. (1987)—in the outer surface site framed by hydrophobic residues.

Determination of the Apparent Binding Constants. The data presented in Figures 1 and 2 for β-ionone binding by BLG were analyzed according to eq 1, and the results are shown in Figures 4 and 5. Values of $(1 - a)$ in the range 0.1–0.9 are used to trace the lines. The apparent binding constants of β-ionone–BLG complexes and the number of binding sites are calculated from the intercept and the slope of the traced lines, respectively. Their numerical values are summarized in Table II. Presented results show differences in apparent dissociation constants, varying between 7.6×10^{-7} M for *N*-ethyllysyl-BLG and 1.7×10^{-7} M for EtBLG.

The results obtained were interpreted in terms of apparent dissociation constants since the exact physical state of free β-ionone is unknown. In fact, the present study can be defined as one more case of interaction in aqueous solution of a protein with slightly polar lipid-like molecules. This partial polar character results in a very low but finite solubility of the ligand, existing in equilibrium with other forms of the unbound ligand, for example, micelles (Cogan et al., 1976).

All the BLG derivatives, except *N*-ethyllysyl-BLG, exhibit lower K_d' than BLG (Table II). In addition, the less derivatized, alkylated or esterified, proteins (*N*-methyllysyl-BLG and EtBLG) bind β-ionone more tightly than more extensively modified derivatives (*N*-ethyllysyl-BLG and MetBLG). MetBLG is the most intensively esterified derivative, as suggested by the measured isoionic point. The partial loss of BLG β-barrel structure in MetBLG, suggested by the interpretation of its circular dichroism spectrum, may be a factor contributing to the increase of the apparent dissociation constant and connected with the decrease of the strength of binding of β-ionone when compared with EtBLG. It should be pointed out, nevertheless, that the chemical modifications in EtBLG may also induce local structural changes undetectable by circular dichroism, which are slightly modifying the conformation of the hydrophobic site(s).

Generally, esterification and alkylation of BLG enhance its binding properties. The apparent dissociation constant of the β-ionone–EtBLG complex is 3.5 times smaller than that of the β-ionone–BLG complex. In addition, our study of the binding of the two ionone isomers demonstrates that BLG, which binds β-ionone but not α-ionone, has a narrow specificity to the pattern formed by the β-ionone ring and the double bond of the isoprenoid chain. The presence of common structural motif in β-ionone and retinol implies that it is likely that both ligands are bound in the same binding site. In the context of this relatively stringent binding specificity toward so chemically similar terpenes, as are α- and β-isomers of ionone, and the binding of other very chemically different ligands by BLG, it may be assumed that this protein molecule displaying so various specificities has also several binding sites which may be placed both inside and outside the β-barrel protein structure. (Note added in proof: After the submission of this paper, we have shown that one BLG molecule binds tightly retinol and heme-CO together. The retinol–heme-CO distance has been estimated, on the basis of fluorescence energy transfer, to be ca. 28 Å.)

ABBREVIATIONS USED

BLG, β-lactoglobulin; EtBLG, ethyl esterified β-lactoglobulin; MetBLG, methyl esterified β-lactoglobulin; *N*-ethyllysyl-BLG, ethyl alkylated β-lactoglobulin; *N*-methyllysyl-BLG, methyl alkylated β-lactoglobulin.

ACKNOWLEDGMENT

We express our gratitude to Dr. F. Toma, Service de Biochimie, CEA de Saclay, for allowing us to use the circular dichrograph.

LITERATURE CITED

Adler-Nissen, J. Determination of the Degree of Hydrolysis of Food Protein Hydrolysates by Trinitrobenzenesulfonic Acid. *J. Agric. Food Chem.* **1979**, *27*, 1256–1262.

Brahms, S.; Brahms, J. Determination of Protein Secondary Structure in Solution by Vacuum Ultraviolet Circular Dichroism. *J. Mol. Biol.* **1980**, *138*, 149–178.

Brown, E. D. Interaction of β-Lactoglobulin and α-Lactalbumin with Lipids: a Review. *J. Dairy Sci.* **1984**, *67*, 713–722.

Cabacungan, J. C.; Ahmed, A. I.; Feeney, R. E. Amines Boranes as Alternative Reducing Agents for Reductive Alkylation of Proteins. *Anal. Biochem.* **1982**, *124*, 272–278.

Chen, Y. H.; Yang, J. T. A New Approach to the Calculation of Secondary Structure of Globular Proteins by Optical Rotatory Dispersion and Circular Dichroism. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 1285–1291.

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.

Croteau, R. Biosynthesis of Cyclic Monoterpenes. In *Biogeneration of Aromas*; Parliment, T. H., Croteau, R., Eds.; ACS Symposium Series 317; American Chemical Society: Washington, DC, 1986; pp 134–156.

Diaz de Villegas, M. C.; Oria, R.; Sala, F. J.; Calvo, M. Lipid Binding by β-Lactoglobulin of Cow Milk. *Milchwissenschaft* **1987**, *42*, 357–358.

Farrell, H. M.; Behe, M. J.; Enyart, J. A. Binding of p-Nitrophenyl Phosphate and other Aromatics Compounds by β-Lactoglobulin. *J. Dairy Sci.* **1987**, *70*, 252–258.

Fraenkel-Conrad, H.; Olcott, H. S. Esterification of Proteins with Alcohols of Low Molecular Weight. *J. Biol. Chem.* **1945**, *161*, 259–268.

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 Serum Albumin, β-Lactoglobulin and the Retinol Binding Protein of Human Plasma. *J. Biol. Chem.* **1972**, *247*, 5168–5172.

Godovac-Zimmermann, J. The Structural Motif of β-Lactoglobulin and Retinol Binding Protein: a Basic Framework for Binding and Transport of Small Hydrophobic Molecules. *Trends Biochem. Sci.* **1988**, *13*, 64–66.

Halpin, M. I.; Richardson, T. Selected Functionality Changes of β-Lactoglobulin upon Esterification of Side Chain Carboxyl Groups. *J. Dairy Sci.* **1985**, *68*, 3189–3198.

Holden, H. M.; Rypniewski, W. R.; Law, J. H.; Rayment, I. The Molecular Structure of Insecticyanin from the Tabacco Horworm *Manduca sexta* L. at 2.6 Å Resolution. *EMBO J.* **1987**, *6*, 1565–1570.

Huber, R.; Schneider, M.; Epp, O.; Mayr, I.; Messerschmidt, A.; Plugarth, J.; Kayser, H. Crystallization, Crystal Structure Analysis and Preliminary Molecular Model of the Bilin Binding Protein from the Insect *Pieris brassicae*. *J. Mol. Biol.* **1987**, *195*, 423–434.

Lakowicz, R. J. Protein Fluorescence. In *Principle of Fluorescence Spectroscopy*; Lakowicz, R. J., Ed.; Plenum Press: New York, 1983; pp 342–381.

Mailliart, P.; Ribadeau-Dumas, B. Preparation of β-Lactoglobulin and β-Lactoglobulin Free Proteins from Whey Retentate by NaCl Salting-out at Low pH. *J. Food Sci.* **1988**, *53*, 743–745.

Mattarella, N. L.; Richardson, T. Physicochemical and Functional Properties of Positively Charged Derivatives of Bovine β-Lactoglobulin. *J. Agric. Food Chem.* **1983**, *31*, 972–978.

Mattarella, N. L.; Creamer, L. K.; Richardson, T. Amidation or Esterification of Bovine β-Lactoglobulin to Form Positively Charged Proteins. *J. Agric. Food Chem.* **1983**, *31*, 968–972.

Means, G. E.; Feeney, R. E. Reductive Alkylation of Amino Groups in Proteins. *Biochemistry* **1968**, *7*, 2192–2201.

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.

Newcomer, M. E.; Jones, T. A.; Aqvist, J.; Sundelin, J.; Eriksson, U.; Rask, L.; Peterson, P. The Three Dimensional Structure of Retinol Binding Protein. *EMBO J.* **1984**, *3*, 1451–1454.

North, A. C. T. Three Dimensional Arrangement of Conserved Amino Acid Residues in a Superfamily of Specific Ligand-Binding Protein. *Int. J. Biol. Macromol.* **1989**, *11*, 56–58.

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

Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The Structure of β-Lactoglobulin and its Similarity to Plasma Retinol Binding Protein. *Nature* **1986**, *324*, 383–385.

Pervaiz, S.; Brew, K. Homology of β-Lactoglobulin, Serum Retinol-Binding Protein and Protein HC. *Science* **1985**, *228*, 335–337.

Weeks, W. W. Carotenoids: a Source of Flavor and Aromas. In *Biogeneration of Aromas*; Parliment, T. H., Croteau, R., Eds.; ACS Symposium Series 317; American Chemical Society: Washington, DC, 1986; pp 157–166.

Received for review November 28, 1989. Revised manuscript received March 29, 1990. Accepted April 10, 1990.

Registry No. β-Ionone, 79-77-6.