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Interactions of β-Lactoglobulin Variants A and B with Vitamin A. Competitive Binding of Retinoids and Carotenoids

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ABSTRACT: β-Lactoglobulin (β-Lg) is the major whey protein of bovine milk present at a concentration of 2−3 g L^{-1}. Its biological role is still not well-known. However, many studies have suggested that β-Lg may play either nutritional or specific transporter role. The high affinity of β-Lg for retinol and other retinoids was reported. The results of interaction studies of β-Lg with carotenoids, that is, β-carotene, β-cryptoxanthin, and α-carotene, which display similar structures are reported in this study. The affinities of β-Lg for binding of retinoids and carotenoids were compared, providing more information about the binding site(s) of these molecules by β-Lg. Interactions were followed by the measurements of quenching of β-Lg tryptophan fluorescence and retinol fluorescence. The obtained results indicate that carotenoids are bound by β-Lg with high affinity of the order of 10^{-8} M. Measurement of retinol competition with carotenoids for binding by β-Lg suggests that the binding of these two ligands occurs at two different sites of β-Lg.

KEYWORDS: *β-lactoglobulin, retinoids, carotenoids, binding, competition*

INTRODUCTION

β-Lactoglobulin (β-Lg) is the major whey protein of ruminant species, and it is also present in the milk of many, but not all, other mammalian species. It is the major whey protein of cow's milk, present at a concentration of 2−3 g L^{-1}.[1] β-Lg is a small protein with 162 amino acid residues (MW = 18300 Da), soluble in dilute salt solutions. Its amino acid sequence and three-dimensional structure are at the base of its classification in a widely diverse family of lipocalins, with which it shares a common three-dimensional fold: eight stranded antiparallel β-sheets flanked on one side by an α-helix constituting a hydrophobic pocket, where most small hydrophobic ligands bind. Thus, similarly to serum retinol binding protein,[2] β-Lg may act as specific transporter. Besides that, bovine β-Lg has nine genetic variants. The genetic variants A and B are the most common in bovine milk. These variants differ at positions 64 and 118, where Asp and Val in variant A are substituted by Gly and Ala in variant B,[3] respectively.

Despite abundant data accumulated during over half of a century, the biological role of β-Lg is still not well understood. However, its presence in the milk of several mammals has suggested that it may have a nutritional role, supported also by its ability to interact with a great variety of hydrophobic ligands, such as vitamin A,[4−8] fatty acids, and triglycerides.[5,8−13]

Vitamin A was chosen to study ligand interactions with β-Lg because of its importance in a number of physiological functions in various mammal tissues. Vitamin A is essential for the stimulation of growth and proper development of tissues, for normal reproduction, for maintenance of rod vision, and for the differentiation of epithelial tissues.[14,15] Consequently, because humans are unable to synthesize vitamin A de novo, dietary lack of vitamin A leads to many clinical deficiency syndromes. Therefore, "vitamin A requirements" should be fulfilled by consumption of foods from vegetal sources containing provitamin A, β-carotene, β-cryptoxanthin, and α-carotene, or foods from animal sources containing preformed vitamin A, retinol and retinol esters.[16,17]

Consequently, acquiring new information about the interactions of retinoids and carotenoids with β-Lg could answer the question of the unknown biological role of β-Lg and also it could indicate how to exploit the binding of vitamin A by β-Lg to ensure its protection and time-controlled release in the gastrointestinal tract (GIT).

Retinoids share a common structural moiety of β-ionone cycle with an isoprenoid chain. They differ only by the isoprenoid chain: with an alcohol function in retinol, esterified by acetate in retinyl acetate, and with a carboxylic group in retinoic acid. β-Carotene has two β-ionone cycles, joined by an isoprenoid chain.[18]

It was demonstrated that the binding of retinol by β-Lg quenches the fluorescence of Trp 19[4] positioned at the bottom of the calyx.[19] Therefore, Trp fluorescence changes have been used to characterize the binding of retinoids and carotenoids.[11,20,21] The interactions of β-Lg with palmitic acid increase the tryptophanyl fluorescence;[9,22] consequently, the changes in intensity of fluorescence emission of tryptophanyl residues can be used to monitor the environment of these residues in proteins, providing information on the character of local interactions.

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Three potential binding sites have been reported for ligand binding to β-Lg: the internal cavity of the β-barrel, the surface hydrophobic pocket in a groove between the α-helix and the β-barrel, and the outer surface near Trp 19−Arg 124.[23,24] Hence, the aim of this work was, first, to compare the affinity of selected retinoids and carotenoids for β-Lg, using fluorescence spectroscopy, and then to investigate the competitive behavior of retinol and of carotenoids to understand if these two types of ligands bind to two different sites or to the same unique binding site on β-Lg.

MATERIALS AND METHODS

Materials. To prevent oxidation and isomerization, *all-trans* isomers of retinol, retinal, β-carotene, β-cryptoxanthin, α-carotene, and palmitic acid (Sigma Chemical, Saint Quentin Fallavier, France) were dissolved in ethanol in the dark to give 0.2−0.4 mM stock solutions. All chemicals used were of reagent grade. β-Lg variants A and B were obtained from homozygote cow's milk following the method of Mailliart and Ribadeau Dumas,[25] and as judged from high-performance liquid chromatograms, polyacrylamide gel electrophoresis, and mass spectroscopy, it was >95% pure. Circular dichroism (CD) was used to confirm the native structure of the β-Lg. The concentration of β-Lg was determined spectrophotometrically by using for the calculation a molecular absorption coefficient ε_{278} = 17600 M^{-1} cm^{-1}. The following molecular absorption coefficients were used to calculate ligand concentrations: retinol, ε_{325} = 46000 M^{-1} cm^{-1}; retinal, ε_{383} = 42880 M^{-1} cm^{-1}; β-carotene, ε_{450} = 140656 M^{-1} cm^{-1}; α-carotene, ε_{444} = 150323 M^{-1} cm^{-1}; β-cryptoxanthin, ε_{452} = 106491 M^{-1} cm^{-1}.

β-Lg Titration with Different Ligands. Fluorescence spectra were recorded at 20 °C on a Hitachi F4500 spectrofluorometer (Tokyo, Japan) in the ratio mode. The excitation and emission slits were set at 5 nm. The binding of retinoids and carotenoids was measured by following the fluorescence quenching of protein tryptophan (at 332 nm) and/or retinoid fluorescence at 480 nm. The binding of palmitic acid was measured by following the increase of tryptophan β-Lg (Trp β-Lg) fluorescence at 332 nm. The changes of the Trp β-Lg fluorescence, induced by hydrophobic ligands, are due to energy transfer between the excited indole (tryptophan) ring and the ligands, changes of polarity in the neighborhood of the tryptophanyl residues, or both effects.[26] Hence, the higher the fluorescence of the tryptophan, the lower the binding in the case of retinoids and carotenoids. In contrast, with palmitic acid binding the observed phenomenon is the reverse. In fact, the binding of this molecule induces an increase of the Trp β-Lg fluorescence at 332 nm. It was observed that the binding of palmitic acid and of other fatty acids induced an enhancement of tryptophan fluorescence.[27] This is probably due to the decrease of the polarity in the neighborhood of tryptophan indole, enhancing their emission yield.

The following procedure was used for titration of β-Lg with retinol, retinal, β-carotene, β-cryptoxanthin, and α-carotene: 2.5 mL of protein solution, varying between 2 and 4 μM, was placed in a cuvette and a small increment of 6 μL of the ligand solution in ethanol (retinol, retinal, and palmitic acid) or in tetrahydrofuran (THF) (β-carotene, β-cryptoxanthin, and α-carotene) was injected with a micropipet to reach molar ratios of 1.7 in the case of retinoids and 1 in the case of carotenoids. Ethanol or THF added with the ligand during titration never exceeded 4% (v/v). The experiments were performed in 50 mM phosphate buffer, pH 7.[18,27] Fluorometric measurements of the controls containing *N*-acetyl-L-tryptophanamide, with an absorbency at 287 nm equal to that of protein, titrated with these ligands were performed, and the results of all titrations were analyzed according to the method of Cogan et al.[27] The decrease in fluorescence emission intensity of *N*-acetyl-L-tryptophanamide solution during the titration is not due to the interaction between ligand and tryptophanamide, but it results only from the inner filter effect as a consequence of ligand absorbance at 287 nm. All fluorescence intensities were corrected for the blank containing 4% of ethanol or THF. The fluorescence changes of the blank were subtracted from each considered titration point.

Competition for the β-Lg Binding Site between Retinol and Other Ligands. The same conditions and concentrations as described above were used to study ligand competition. The competition for β-Lg binding site was studied by adding retinol to β-Lg saturated before with each of the following ligands: palmitic acid, retinal, β-carotene, β-cryptoxanthin, or α-carotene. Then, the reversed titration was made by adding one of the above-mentioned ligands to the β-Lg−retinol complex. The binding of these ligands was measured by following the fluorescence emission quenching of two β-Lg tryptophans (332 nm) and the change of fluorescence emission intensity of retinol (480 nm).

Determination of the Apparent Dissociation Constants. After correction for the control according to the method of Cogan et al.,[27] differences in fluorescence emission intensity at 332 nm between the complex and free protein (excitation at 287 nm) were monitored to measure the apparent dissociation constants of β-Lg with various ligands, that is, retinol, retinal, palmitic acid, β-carotene, β-cryptoxanthin, and α-carotene. It was assumed that the change in fluorescence intensity depends on the amount of protein−ligand complex.

The mass law equation[27] $K'_d = (nP_0 \times B)/PB$ was used to derive a convenient work equation of apparent dissociation constants. When $(P_0\alpha)$ versus $B[\alpha/(1-\alpha)]$ is plotted, 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 and n (in mol/mol) is the number of independent and equivalent sites per protein at saturation, all of which possess the same binding affinity. P_0 is the total protein concentration, and B is the total ligand concentration. α is defined as the fraction of unoccupied binding sites on the protein molecules. The value of α was calculated for every desired point on the titration curve of fluorescence intensity enhancement using the relationship

$$\alpha = (F_{max} - F)/(F_{max} - F_0)$$

where F represents the fluorescence intensity (corrected for the blank) at a certain B, F_{max} represents the fluorescence intensity upon saturation of β-Lg molecules, and F_0 is the initial fluorescence intensity in the absence of ligand.

All experiments described above were carried out three times with three different samples.

RESULTS AND DISCUSSION

Titration of β-Lg with Different Ligands. The properties of β-Lg binding site(s) were studied using retinoids and carotenoids as ligands. Figure 1 shows the binding of retinol, retinal, β-carotene, β-cryptoxanthin, and α-carotene to β-Lg at neutral pH. The titration patterns were quite different between retinoids and carotenoids. Additionally, they reached various fluorescence quenching intensities upon saturation with the ligand. The extent of the observed quenching of Trp β-Lg fluorescence varies according to the applied retinoid and carotenoid compound. It depends also on the β-Lg variant studied (A or B). The observed quenching reaches, at equimolar ratios, for variant B 44% with retinol and retinal and around 20% in the case of α-carotene and β-carotene binding (Table 1). This could be explained by the structural differences between the studied ligands. In fact, carotenoids have two β-ionone cycles joined by an isoprenoid chain, whereas retinoids possess only one β-ionone cycle, which makes it less hydrophobic than carotenoids. This results in easier retinoid accessibility to β-Lg. Indeed, Dufour et al.[18] have shown that β-carotene binding induces smaller Trp quenching than binding of retinol. However, no data concerning β-Lg interactions with β-cryptoxanthin and α-carotene are available.

The apparent binding constants of ligand−β-Lg complexes and the number of independent and equivalent sites per β-Lg, n, at saturation (Table 1; Figure 2) were calculated as detailed

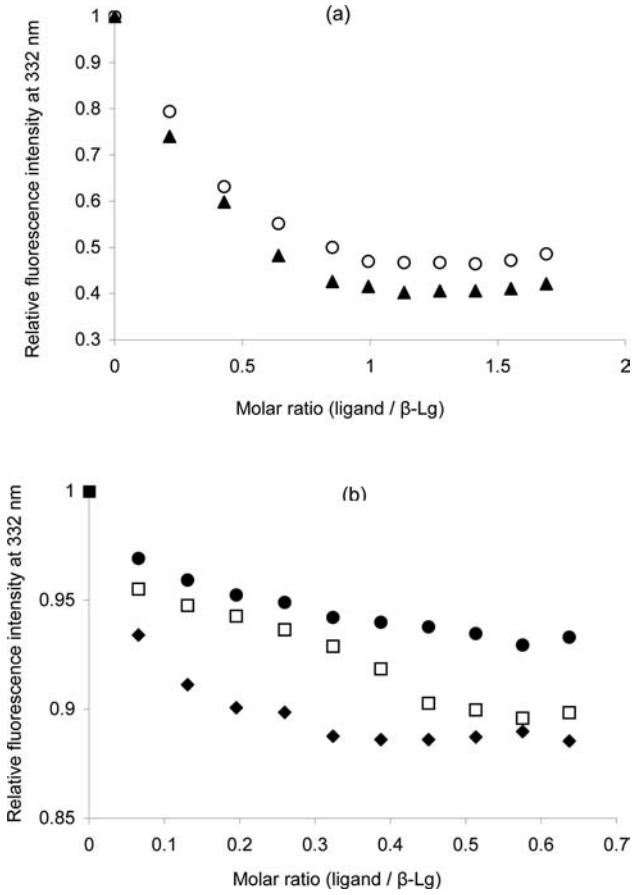

Figure 1. Corrected β-Lg tryptophan relative fluorescence emission intensity titration plots with (a) retinoids (○, retinal; ▲, retinol) and (b) carotenoids (◆, β-carotene; □, β-cryptoxanthin; ●, α-carotene). A representative plot is shown; number of repeats = 3.

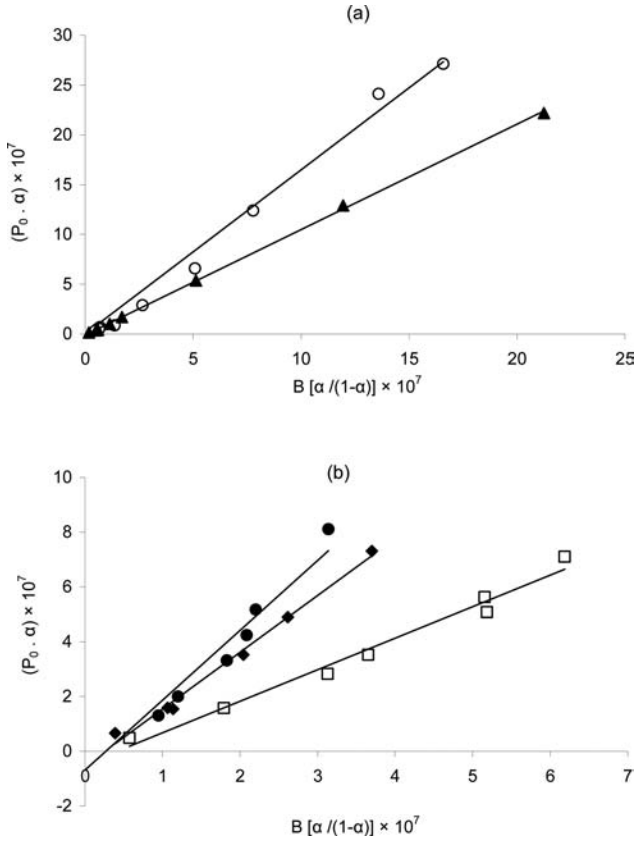

Figure 2. Linear least-squares plots of equation ($P_0\alpha$) versus B [$\alpha/(1-\alpha)$] × 10^7 for the titration of β-Lg with (a) retinal (○) and retinol (▲) and (b) α-carotene (●), β-carotene (◆), and β-cryptoxanthin (□). A representative curve is shown; number of repeats = 3.

under Materials and Methods.[27] The n values reported in Table 1 are not integer due to the reliability of the Cogan model, and the error values represent only the fitting errors. β-Carotene and retinol show the highest affinities for β-Lg variant B ($K'_{d(\beta\text{-carotene})}$ = 1.23 × 10^{-8} M, $K'_{d(retinol)}$ = 4 × 10^{-8} M) among the studied ligands. However, all dissociation constants varied between 2.07 × 10^{-8} and 8.57 × 10^{-8} M. In addition, small differences were observed also between β-Lg variants A and B. In fact, as previously mentioned, these two variants differ only by two amino acids, and only the amino acid at position 118 is located at the entrance of the hydrophobic site. At this position, Val in variant A is substituted by Ala in variant B, which is a less

Table 1. Apparent Dissociation Constants (K'_d) and Apparent Molar Ratios of Ligand/Protein (n) for β-Lactoglobulin Calculated Using the Cogan Method[a]

		β-Lg A		β-Lg B	
ligand (retinoid/carotenoid)		n	K'_d (×10^{-8} M)	n	K'_d (×10^{-8} M)
retinol	results	0.62 ± 0.05	7.13 ± 4.40	0.73 ± 0.17	4.05 ± 0.70
	extinction Trp (%)	53		44	
retinal	results	0.65 ± 0.13	6.10 ± 2.20	0.51 ± 0.07	6.30 ± 1.90
	extinction Trp (%)	63		44	
β-carotene	results	0.51 ± 0.03	2.07 ± 0.40	0.52 ± 0.10	1.23 ± 0.10
	extinction Trp (%)	19		19	
α-carotene	results	1.23 ± 0.50	4.50 ± 1.20	0.96 ± 0.20	8.57 ± 2.80
	extinction Trp (%)	10		21	
β-cryptoxanthin	results	0.88 ± 0.01	2.20 ± 0.30	0.85 ± 0.02	4.00 ± 1.70
	extinction Trp (%)	7		11	

[a]Results represent the average ± SD (three samples).

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bulky amino acid and could therefore allow better interactions. These results agree with the dissociation constant found in the literature, with K'_d for interaction of β-Lg with retinol of 4.4 × 10^{-8} M[11,18,21,26] and K'_d for interaction of β-Lg with retinal of 5.5 × 10^{-8} M.[11] However, the previously reported K'_d for interaction of β-Lg with β-carotene of 35 × 10^{-8} M[18] is quite different from our results. This could be attributed to the different solvent used. In our experiment β-carotene was dissolved in THF, which is more hydrophobic than ethanol, and this could be a cause of differences of the affinity of β-carotene for the hydrophobic site. Cogan et al.[27] found that human retinol binding protein binds retinol with affinities (K'_d = 19 × 10^{-8} M) similar to what was obtained in our study.

Competition of Ligands for β-Lg Binding Site. *Competition between Retinol and Palmitic Acid.* Quenching of tryptophan fluorescence and variation of retinol fluorescence (emission) intensity were measured to follow the binding of retinol to β-Lg−ligand complex and the binding of ligand to β-Lg−retinol complex. First, an experiment of competition between retinol and palmitic acid was performed to compare binding of retinol with binding of carotenoids, given that retinol is known for its high affinity for the hydrophobic pocket of β-Lg.[12] Titration of β-Lg−retinol complex with palmitic acid shows an increase of Trp β-Lg fluorescence intensity (at 332 nm) and a parallel gradual decrease of retinol fluorescence emission intensity at 480 nm (Figure 3a). Trp β-Lg fluorescence intensity enhancement and retinol fluorescence quenching suggest that retinol and palmitic acid compete for the same binding site and that the addition of palmitic acid to the β-Lg−retinol complex induced gradual release of retinol (decrease of fluorescence at 480 nm) and the binding of palmitic acid in the hydrophobic pocket of β-Lg (increase of the Trp fluorescence of β-Lg at 332 nm), as described under Materials and Methods. The data available in the literature are contradictory: some authors report that palmitic acid competes with retinol for the same site,[4,12,28] but others suggest that these two ligands bind at different sites.[5] To solve this contradiction the reversed titration experiment was also performed. Addition of retinol to the β-Lg−palmitic acid complex induced a quenching of tryptophan fluorescence and at the same time an increase of retinol fluorescence (Figure 3b), indicating the gradual palmitic acid release and retinol binding. This behavior would strengthen the hypothesis that both ligands would bind in the same site.

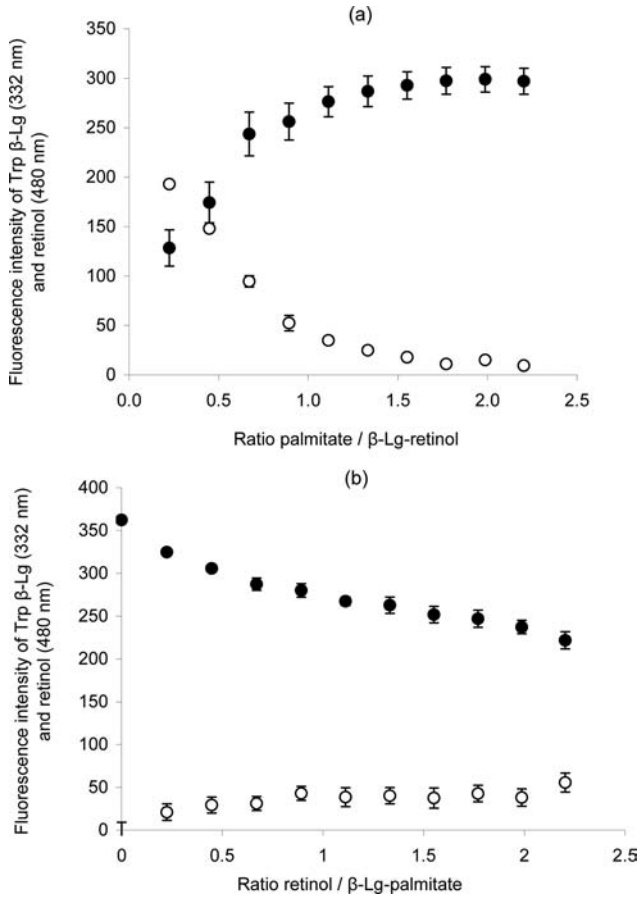

Figure 3. Corrected fluorescence emission intensity titration: (a) addition of palmitic acid to retinol−β-Lg complex; (b) addition of retinol to palmitic acid−β-Lg complex; (●) tryptophan fluorescence at 332 nm; (○) retinol fluorescence at 480 nm. Number of repeats = 3.

Retinol/Carotenoid Competition. Competition between retinol and carotenoids for the β-Lg binding site was not studied before. Hence, these experiments were performed to determine if carotenoids also bind to the same site of retinol or not. For this purpose, two different titrations were performed, and the same previous interpretations of decrease and increase of retinol and Trp β-Lg fluorescence emission were carried out. First, complexes of β-Lg−carotenoids were prepared, and retinol was added gradually to each. Second, a β-Lg−retinol complex was prepared, and each carotenoid ligand was gradually added to this complex, forming β-Lg−β-carotene or −β-cryptoxanthin or −α-carotene. After that, the tryptophan (332 nm) and retinol (480 nm) fluorescence intensities were measured, and the obtained results show a different behavior from what was observed in the case of retinol/palmitic acid competition (Figures 4 and 5).

During the addition of retinol to the β-Lg−carotenoid complex, an increase of retinol fluorescence (480 nm) was observed in the case of all carotenoids (Figure 4a). The rate of fluorescence increase differs between carotenoids, retinal, and palmitic acid: the fluorescence intensity of retinol at 480 nm of 80 and 50 with retinal and palmitic acid, respectively, and of 140−180 with carotenoids (Figure 4a). Titration patterns of retinol binding in the presence of β-carotene, β-cryptoxanthin, and α-carotene in the β-Lg−carotenoid complex were similar to that of control (uncomplexed β-Lg), which suggested that retinol could bind to the interior cavity of β-Lg in the presence of carotenoids. Otherwise, smaller fluorescence intensity changes were observed during titrations with retinal and palmitic acid, which suggested that these ligands were bound to the same binding site as retinol and, consequently, the added retinol could not totally bind to β-Lg; therefore, the smallest retinol fluorescence enhancement (at 480 nm) was observed as compared with the control. Then, for the same experiment, β-Lg Trp fluorescence was measured at 332 nm. Figure 4b shows a decrease in Trp β-Lg fluorescence in the presence of all ligands. A less pronounced decrease is observed with retinal and palmitic acid, which reflects the competition for the same binding site as reported above. Besides, binding plots obtained with carotenoids follow the same variations as the control plot, which is in agreement with a binding of retinol and carotenoids to different sites as suggested previously.

To strengthen these assumptions, experiments of competition by adding different ligands to β-Lg−retinol complex were realized (Figure 5). The measurement of retinol fluorescence

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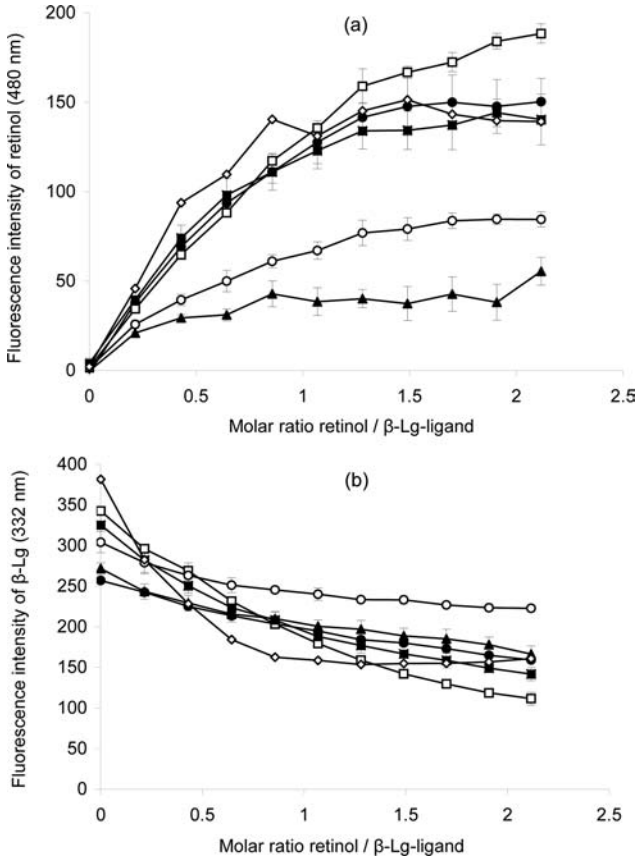

Figure 4. Change of (a) retinol fluorescence emission intensity at 480 nm and (b) β-Lg fluorescence intensity at 332 nm as a function of addition of retinol to β-Lg–α-carotene (●), to β-Lg–β-carotene (■), to β-Lg–β-cryptoxanthin (□), to β-Lg–retinal (○), and to β-Lg–palmitic acid (▲) complexes. Number of repeats = 3. (◇) Control is unbound β-Lg.

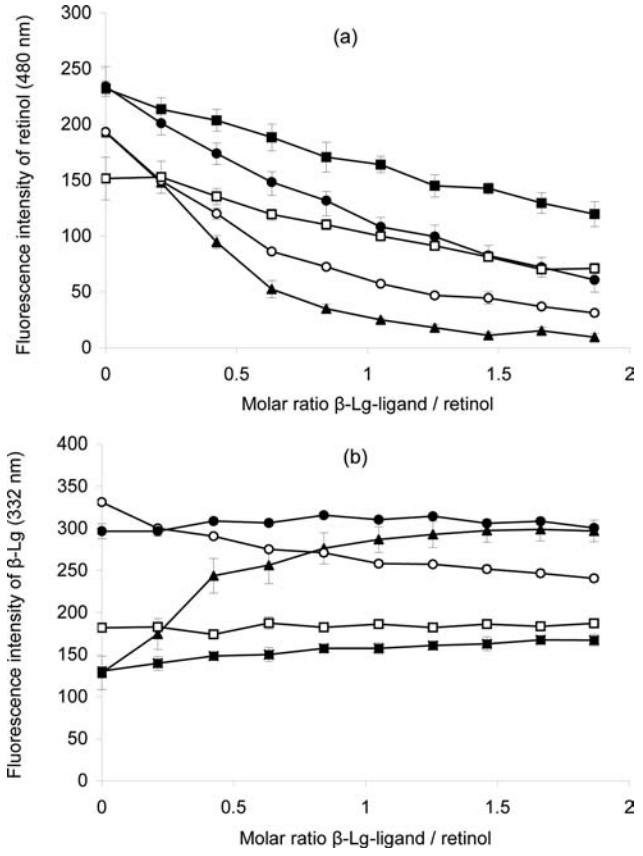

Figure 5. Change of (a) retinol fluorescence intensity at 480 nm and (b) β-Lg fluorescence intensity at 332 nm as a function of addition of α-carotene (●), β-carotene (■), β-cryptoxanthin (□), retinal (○), and palmitic acid (▲) to the β-Lg–retinol complex. Number of repeats = 3.

intensity at 480 nm showed a decrease of fluorescence in the case of all studied ligands. However, a more pronounced decrease was observed by adding retinal and palmitic acid (25 and 10, respectively) as compared with an average of 80 with carotenoids (Figure 5a). This fluorescence decrease suggests that during competitive titrations with palmitic acid and retinal more retinol was released from β-Lg, which explains the pronounced decrease of fluorescence emission at 480 nm. Consequently, palmitic acid and retinal compete with retinol for the same binding site on/in β-Lg. In contrast, carotenoids induced smaller fluorescence decrease. This small decrease of retinol fluorescence at 480 nm suggests that the majority of β-ionone ring of retinol is still located near the β-Lg tryptophan and, thus, retinol is less released. The more pronounced decrease of fluorescence at 480 nm in the case of palmitic acid and retinal suggests smaller energy transfer between the β-ionone ring of retinol and the β-Lg tryptophan and, thus, greater retinol release.

In the same experiment, β-Lg Trp fluorescence was measured at 332 nm (Figure 5b). Almost no variation was observed during titrations with carotenoids, contrary to a fluorescence increase with palmitic acid and a small fluorescence decrease with retinal. The constant fluorescence intensity of Trp β-Lg in the presence of carotenoids suggests that the retinol–β-Lg complex is stable. The fluorescence increase and decrease in the presence of palmitic acid and retinal, respectively, suggest the gradual release of retinol, as described in the previous section. This implies that only these two ligands compete with retinol. Thus, the observed fluorescence variations confirm the binding of retinal and palmitic acid after retinol release. Besides, the stability of fluorescence intensity observed during the addition of carotenoids (Figure 5b) indicates that no retinol release is occurring in their cases. Consequently, there is no competition between retinol and carotenoids for the same β-Lg binding site. Previous studies suggested the binding of *cis*-parinaric acid and retinol to β-Lg at two different sites.[18,20] Additionally, protoporphyrin IX and retinol were shown to bind at different sites.[5]

In conclusion, the obtained results indicate the absence of differences between variants A and B of β-Lg in their interactions with retinoids and carotenoids. They show also that despite the high hydrophobicity of β-carotene, it has an important affinity for β-Lg. The obtained results confirm the hypothesis of binding of retinol and palmitic acid at the same interior cavity and also suggest that retinal competes with retinol for the same binding site contrary to carotenoids, which have different binding sites.

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