ELSEVIER

Biochemical Pharmacology 64 (2002) 1651–1660

Biochemical Pharmacology

Retinoic acid binding properties of the lipocalin member β-lactoglobulin studied by circular dichroism, electronic absorption spectroscopy and molecular modeling methods

Ferenc Zsila*, Zsolt Bikádi, Miklós Simonyi

Department of Molecular Pharmacology, Institute of Chemistry, Chemical Research Center, P.O. Box 17, Budapest 1525, Hungary

Received 5 June 2002; accepted 24 July 2002

Abstract

Interaction between the Vitamin A derivative all-*trans* retinoic acid and the lipocalin member bovine β-lactoglobulin (BLG) was studied by circular dichroism (CD) and electronic absorption spectroscopy at different pH values. In neutral and alkaline solutions achiral retinoic acid forms a non-covalent complex with the protein as indicated by the appearance of a negative Cotton effect around 347 nm associated to the narrowed and red shifted π–π* absorption band of the ligand. The induced optical activity is attributed to the helical distortion of the conjugated chain caused by the chiral protein binding environment. As the disappearing CD activity showed in the course of CD–pH titration experiment, retinoic acid molecules dissociate from BLG upon acidification but this release is completely reversible as proved by the reconstitution of the CD and absorption spectra after setting the pH back to neutral. This unique behavior of the complex is explained by the conformational change of BLG (Tanford transition) which involves a movement of the EF loop at the entrance of the central cavity from open to closed conformation in the course of pH lowering. From these results it was inferred that retinoic acid binds within the hydrophobic calyx of the β-barrel.

Keywords: All-*trans* retinoic acid; Bovine β-lactoglobulin; Induced chirality; Ligand binding; Circular dichroism; Tanford transition

1. Introduction

All-*trans* retinoic acid (Fig. 1), a member of the retinoid family, plays an important role in morphogenesis [1,2] differentiation [3] and homeostasis during embryonal and postnatal life [4,5]. This Vitamin A derivative affects retinoid receptors and retinoid target genes in both normal and malignant tissues [6,7] and causes differentiation and apoptosis of immature malignant cells e.g. leukemic promyelocytes. The influence of all-*trans* retinoic acid and its 9-*cis* and 13-*cis* isomers on cell maturation has resulted in its use in a number of clinical disorders including certain human leukemias [8,9] several skin cancers, psoriasis [10] and acne [11]. Therefore, it has a great potential as a pharmaceutical agent. Owing to their polyenic structure, the hydrophobic retinoids are hardly soluble and chemically unstable in water which makes their manipulation difficult. In the biological fluids, however, specific proteins can be found which are able to bind and carry these hydrophobic compounds [12]. Constituting more than 40 proteins, the so-called lipocalins bind and transport a variety of physiologically important endogenous and exogenous ligands such as retinol, pheromones, prostaglandins, carotenoids etc. [13]. Lipocalin crystal structures are highly conserved and comprise a single eight-stranded continuously hydrogen-bonded anti-parallel β-barrel, which encloses an internal ligand-binding site [14]. The lipocalin member β-lactoglobulin (BLG) found in large quantities in the milk of several mammals is a globular protein with a molecular weight of 18,362 for genetic variant A and 18,276 for B, corresponding to 162 amino acids [15]. The two genetic variants differ in two amino acid residues; Asp 64 and Val 118 in variant A are replaced by Gly and Ala in variant B, respectively. Its structure is represented by a β-barrel, with an added α-helix lying on

* Corresponding author. Fax: +36-1-325-7750.
E-mail address: zsferi@chemres.hu (F. Zsila).

Abbreviations: AM1, Austin method 1; BLG, bovine β-lactoglobulin; CD, circular dichroism; CE, Cotton effect; FWHM, full-width at half-maximum; INDO/S, intermediate neglect of differential overlap for spectroscopy; QM/MM, quantum mechanical/molecular mechanical; UFF, universal force field.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01350-3

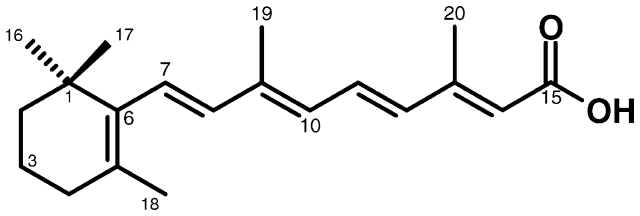

Fig. 1. Chemical structure of all-*trans* retinoic acid.

the surface. BLG from ruminant species is dimeric around neutral pH but between pH 2 and 3 it tends to dissociate into monomers due to electrostatic repulsion between the subunits [16]. Despite of intensive research, biological function of this protein has not yet been satisfactorily resolved. A remarkable property of BLG is its ability to bind *in vitro* several hydrophobic molecules such as retinoids, fatty acids, Vitamin D, cholesterol etc. Several studies suggest that there are at least two binding sites for hydrophobic molecules in the BLG monomer; one in the internal cavity and the other on the outer surface located between the β-barrel and the α-helix. In the case of long-chain fatty acids recent X-ray crystallographic studies proved these molecules to accommodate within the central cavity [17,18]. However, the exact binding location and nature of retinoids is a matter of debate. Monaco *et al.* deduced from X-ray diffraction analyses of the retinol–BLG complex that retinol is bound by the superficial binding pocket of the protein [19]. Chen *et al.*, using proteolysis techniques, found that the central core of the β-barrel, with the α-helix removed, still bound to immobilized retinal in a manner similar to the intact protein [20]. Furthermore, site-directed mutagenesis in the putative interior and surface pockets proposed that retinol binds in the conserved interior cavity rather than the surface pocket [21]. Contrary to this, it was concluded from results of fluorescence energy transfer measurements that retinol and retinoic acid bind to the surface pocket rather than the interior cavity [22].

BLG is an attractive candidate for protein engineering and modification of its structure *via* chemical or enzymatic way opens possibilities to alter its binding properties [23,24]. Complexation of retinoids with BLG alleviating the solubility problems can increase the bioavailability of retinoids and might be a potential method to enhance their therapeutic effects. Therefore, in an attempt to better understand retinoid binding properties of BLG we have used the circular dichroism (CD) spectroscopy as an assay to investigate retinoic acid binding properties of BLG. Although CD spectroscopy is a very informative method for probing and determining ligand–biopolymer interactions its possibilities are largely unexploited in the field of ligand binding of lipocalins [25]. To date, only the work of Fugate and Song has been published applying CD, UV and fluorescence measurements to characterize the retinol–BLG complex [26]. Their findings were as follows: BLG dimer binds two retinol molecules with a binding constant (K_d) of 2×10^{-8} M calculated from fluorescence titration data. The complex exhibited a negative, induced CD band associated to the red shifted vibrationally resolved absorption band of retinol. The induced optical activity was attributed to an excitonic coupling between the retinol and a tryptophan residue at or near the binding site. Retinol binding of BLG did not show pH dependence as inferred from fluorescence spectroscopy measurement.

Since retinoic acid is an optically inactive molecule, the induced CD band found in our experiments clearly indicated that this ligand binds to the asymmetric protein environment in neutral and alkaline solutions as well. Due to the accommodation of retionic acid to its binding site its conjugated chain becomes helically twisted which is responsible for the observed CD activity. Furthermore, we demonstrated and discussed the pH-dependent binding of retinoic acid to BLG which is evidence for binding in the central cavity.

2. Materials and methods

Cow milk β-lactoglobulin (mixture of A and B variants, approximately 90% purity grade) was obtained from Sigma and used as supplied (catalog no. L-3908, lot no. 119H7008).

All-*trans* retinoic acid was purchased from Aldrich and was used without further purification. All other chemicals used were of analytical grade. Buffer solution used to prepare β-lactoglobulin solutions was: 0.01 M phosphate buffer at pH 7.0.

2.1. Preparation of BLG solution

For spectroscopic sample preparation, BLG was dissolved in a buffer solution. Its concentration was determined by absorbance at 278 nm with an ε of 17,600 M^{-1} cm^{-1} [23].

2.2. Preparation of ligand solution

Retinoic acid stock solution was freshly prepared using absolute ethanol which had been purged with nitrogen gas. The concentration of retinoic acid was determined spectrophotometrically by its molar extinction coefficient (ε) at 350 nm (45,000 M^{-1} cm^{-1}) [23].

2.3. Titration of BLG with retionic acid

The following procedure was used for titration of BLG with all-*trans* retinoic acid at pH 7.0: 2 mL of 4.5×10^{-5} or 2.35×10^{-5} M protein solution was placed in a rectangular cuvette with 1 cm optical path-length and small amounts of the ligand stock solution were added with an automatic pipette in 8 μL aliquots to achieve the values

of ligand/protein molar ratios from 0.065 to 0.91 and 0.5 to 1.36, respectively. Ethanol added with the ligand never exceeded 6% (v/v).

2.4. CD–pH titration of the retinoic acid–BLG complex

An amount of 0.5 mL ethanolic ligand solution ($c = 1.15 \times 10^{-3}$ M) was added to 14 mL of a 4.78×10^{-5} M BLG solution prepared in double distilled water. After measuring its pH (6.97) 2 mL was placed in a rectangular cuvette with 1 cm optical path-length and the CD and absorption spectra were registered. Then volumes (μL) from 0.1 M HCl solution were pipetted consecutively to achieve pH 6.66, 6.40, 6.18, 5.80, 5.18, 4.47 and 3.22, respectively, and the spectra were measured at each pH values. Finally, the opalescent solution (pH 3.22) was reneutralized by 1 M NaOH (pH 7.03, clear solution).

For pH measurements a digital pH meter (Radelkis, Budapest) with a combined glass electrode was used.

2.5. Circular dichroism and UV–VIS absorption measurements

CD and UV–VIS spectra were recorded between 250 and 450 nm on a Jasco J-715 spectropolarimeter at $25 \pm 0.2^{\circ}$ in a rectangular cuvette with 1 cm path-length. Temperature control was provided by a Peltier thermostat equipped with magnetic stirring. All spectra were accumulated three times with a bandwidth of 1.0 nm and a resolution of 0.2 nm at a scan speed of 50 nm min^{-1}. Induced CD is defined as the CD of retinoic acid–BLG mixture minus the CD of BLG alone at the same wavelengths and is expressed as ellipticity in millidegrees.

2.6. Molecular modeling and semiempirical quantum mechanical calculations

Initial optimization of retinoic acid was carried out by the Gaussian 98W program using AM1 semiempirical quantum chemical method. Docking of the resulted structure to the inner cavity of β-lactoglobulin was performed by the FlexiDock package of Sybyl 6.6 molecular modeling program (Tripos Inc., St. Louis, MO) on a Silicon Graphics Octane workstation under Irix 6.5 operation system. The three-dimensional coordinates of β-lactoglobulin complexed with palmitic acid were obtained from the Protein Data Bank (entry PDB code 1B0O). Then the resulting complex was energy minimized with Gaussian 98W program by QM/MM method within which AM1 method was applied to the ligand atoms and UFF molecular mechanics force field to the protein using tight convergence criteria.

To obtain the rotatory strength of the chiral conformer of retinoic acid in the complex, INDO/S quantum mechanical calculation was taken employing the BDZDO/MCDSPD program package [27].

3. Results

3.1. Electronic absorption spectrum of all-trans retinoic acid

The room temperature electronic absorption spectrum of all-*trans* retinoic acid taken in ethanol is shown in Fig. 2. Between 210 and 450 nm only a single, intense, smooth absorption band can be seen centered at 345.6 nm with a weak tailing on the higher energy side. It is assigned to a π–π^* type, electronic dipole allowed but magnetically forbidden excitation having a transition moment oriented along the long axis of the conjugated chain. The bandwidth at half-maximum of the absorption band is 5817 cm^{-1} (FWHM value). It should be noted that the complete lack of vibronic structure and band broadening in the absorption spectra of retinal and retinoic acid derivatives even at low temperatures are attributed to the presence of the cyclohexene ring. Open chain polyenals and carotenoids having carboxylic end groups show distinct vibrational fine structure in their principal absorption bands [28,29]. Nonbonding interactions arising from steric crowd of methyl groups on the β-ionone ring and the C_7, C_8 hydrogens of the polyene chain cause twisting around the C_6–C_7 bond, which produces a large number of conformers that have slightly shifted vibronic excitations respective to each other; so their superposition leads to a broad, vibrationally unresolved band in the absorption spectrum [30–32]. Consequently, any impact which, more or less, restricts this conformational variability would decrease the absorption bandwidth. Because retinoic acid lacks molecular chirality its CD spectrum gives a zero line (not shown).

3.2. Circular dichroism spectrum of BLG in buffer solution

The near-UV CD spectrum of BLG (Fig. 3) shows a negative band with prominent fine structure due to the presence of chirally perturbed aromatic residues [33]. Since the absorption of tryptophans (Trp 19 and 61) occurs at longer wavelength and is dominant relative to that of tyrosines (Tyr 20, 42, 99 and 102) and phenylalanines (Phe 82, 105, 136 and 151) the negative peaks observed at 293.2 and 286.2 nm can be accounted for the asymmetrically perturbed indole chromophores while the CD contribution below 280 nm is mainly due to the chiral environment of phenylalanine and tyrosine residues [34,35].

3.3. Circular dichroism and electronic absorption spectra of retinoic acid–BLG complex at neutral pH

All-*trans* retinoic acid was added progressively to the 4.5×10^{-5} M BLG solution (pH 7.0) to achieve ligand/protein ratios from 0.065 to 0.91. Upon ligand addition a negative CD band appears centered about 347 nm and its intensity increases with the ligand concentration up to

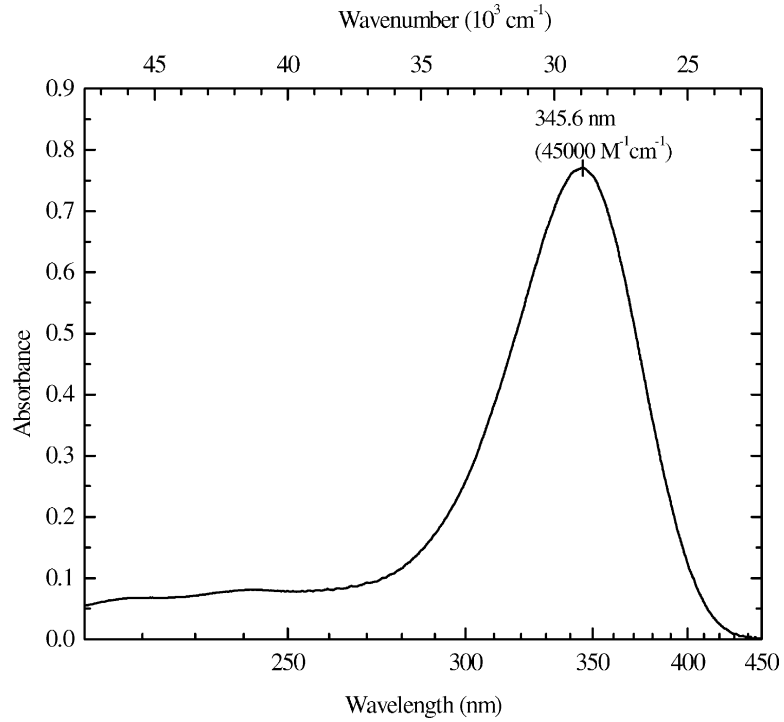

Fig. 2. Ultraviolet–visible absorption spectrum of all-*trans* retinoic acid in ethanol at room temperature ($l = 1$ cm; $c = 1.7 \times 10^{-5}$ M).

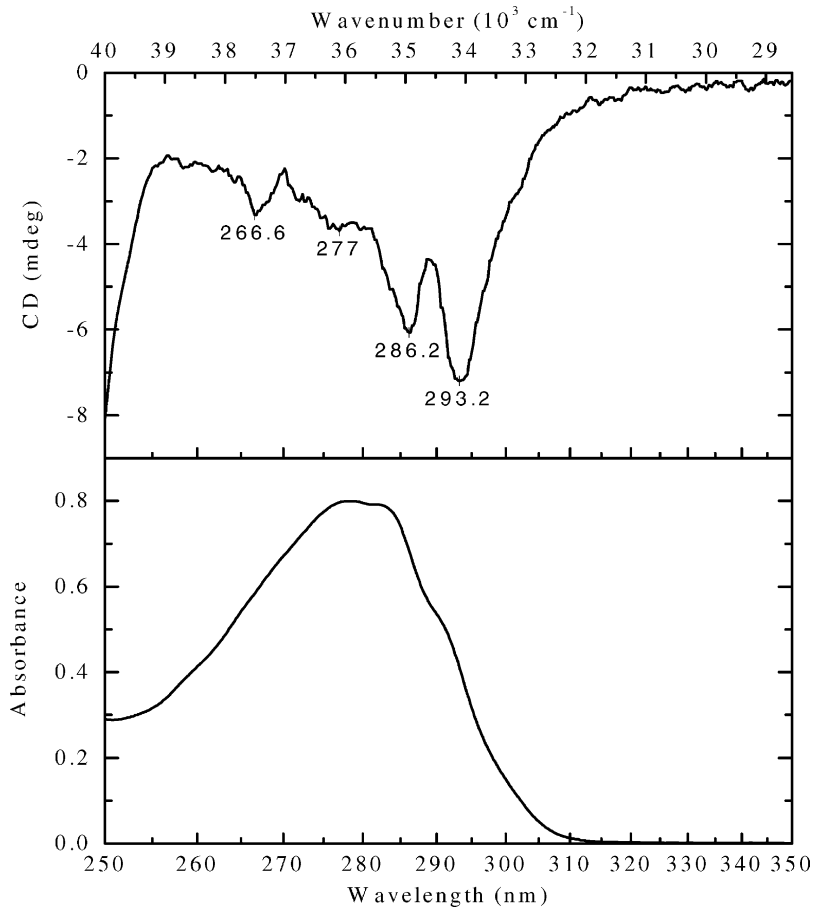

Fig. 3. Circular dichroism and absorption spectra of BLG between 250 and 350 nm in 0.01 M phosphate buffer at pH 7.0 ($l = 1$ cm; $c = 4.5 \times 10^{-5}$ M; $t = 25°$).

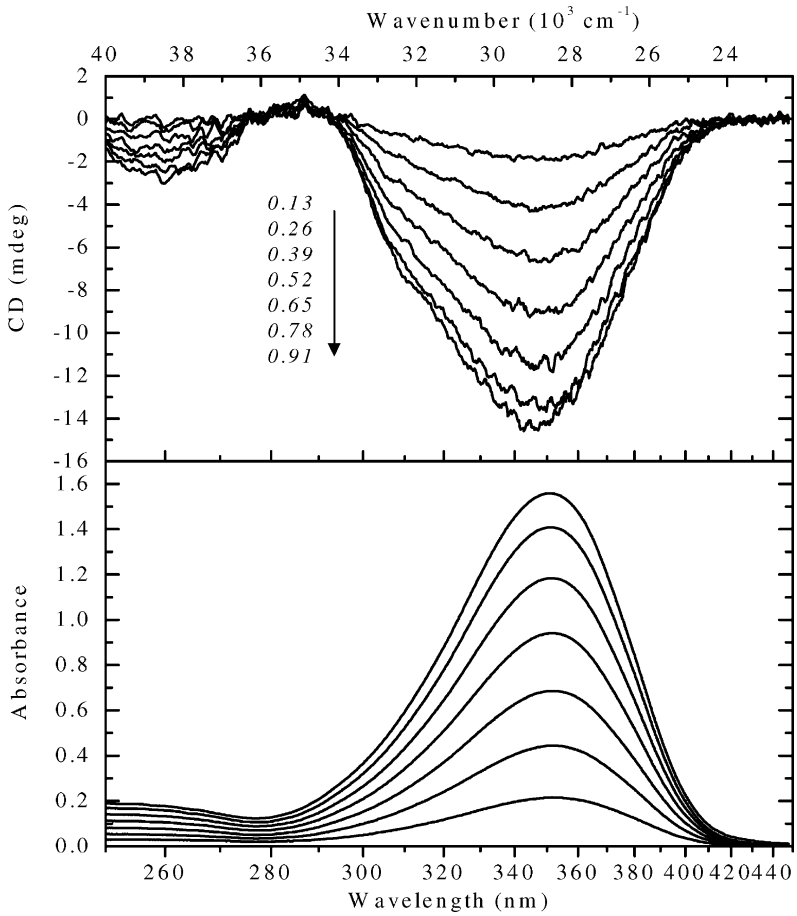

Fig. 4. Induced circular dichroism and UV–VIS spectra of retinoic acid bound to BLG. The concentration of BLG was 4.5×10^{-5} M; the ligand was added as aliquots of ethanolic stock solution ($l = 1$ cm; $t = 25°$). For clarity, only selected curves are shown. Italicized numbers denote the increasing ligand/protein ratio.

−14.5 millidegree (Fig. 4). The shape of this induced Cotton effect (CE) is similar to the corresponding absorption band but the wavelength positions of their maxima do not match each other since the CD band is always blue shifted by ∼3 nm relative to the absorption maxima. In order to gain further information from the CD spectral data, the optical rotatory strength (R) of the induced CD band was calculated at 0.52 ligand/protein ratio using the equation: $R = 2.296 \times 10^{-39} \int_{\sigma_2}^{\sigma_1} (\Delta\varepsilon(\sigma)/\sigma)\, d\sigma$ where $\Delta\varepsilon$ is the molar dichroic absorption coefficient (M^{-1} cm^{-1}) and σ is the wave number. The integration was performed between 34199.7 and 23474.2 cm^{-1} (292.4 and 426 nm) and the rotatory strength was found to be -5.43×10^{-39} cgs unit or −0.586 Debye–Bohr magneton.

It is worth mentioning that the absolute value of this rotatory strength is similar to those that were obtained from CD spectra of retinol bound to BLG (−0.347 Debye–Bohr magneton, [36]) and of all-*trans* retinoic acid bound to human retinol binding protein (+0.77 Debye–Bohr magneton, [37]). Furthermore, the magnitude of the rotatory strength and the molar dichroic absorption coefficient ($\Delta\varepsilon = -12$ M^{-1} cm^{-1} at 347 nm) of the retinoic acid–BLG complex suggest that the induced optical activity results not only from mixing of electronic and magnetic transitions of the amino acid residues and the polyene chromophore; other significant contribution to the induced chirality comes from the chiral conformation of the ligand caused by steric interactions with the binding site.

The UV band of retinoic acid is red shifted by ∼6 nm (345.6 nm → 351–352 nm, $\Delta\nu \approx 500$ cm^{-1}) in the presence of BLG relative to the spectrum taken in ethanol. This shift is accompanied by the alteration of the absorption bandwidth (Fig. 5). At first, the FWHM values (full-width at half-maximum) gradually decrease showing a minimum (≈5300 cm^{-1}) between 0.55 and 0.60 ligand/protein ratios (Fig. 5) and then, upon further ligand addition, begin to increase but remain significantly smaller in the course of the whole titration than the corresponding bandwidth value obtained in the ethanolic solution (5817 cm^{-1}). Therefore, it can be concluded that the BLG-bound percent of retinoic acid is largest between 0.55 and 0.60 ligand/protein ratios.

3.4. The pH dependent response of circular dichroism and electronic absorption spectra of retinoic acid–BLG complex

In distilled water (pH 6.97) the retinoic acid–BLG complex shows very similar CD and absorption spectral behavior to that found in the pH 7.0 buffer. Adding 0.1 M

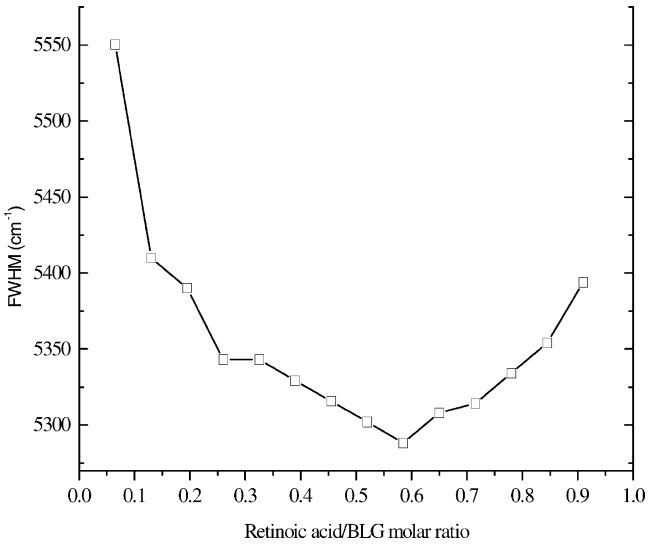

Fig. 5. The absorption bandwidth values (FWHM = full-width at half-maximum) plotted in the function of retinoic acid–BLG molar ratios.

HCl to this solution (pH is shifted to ≈2) significant, time-dependent spectral changes occur; the negative CD band begins to lose intensity parallel with the flattening and red shift of the absorption band. After 10 min, the CD band completely disappears and the solution becomes strongly opalescent. These alterations clearly indicate the dissociation of the complex and the aggregation of free retionic acid molecules [38]. Reversing the order of the ligand addition–acidification we obtain the same spectroscopic results. The solution will promptly be opalescent and no induced CD band can be seen; instead a noisy, featureless curve can be measured showing no change with time (Fig. 6). However, setting the pH to ≈9 by adding small volume of 1 M NaOH the turbid solution becomes instantly clear and the negative CD band appears again together with the blue shift and bandwidth narrowing of the absorption curve (Fig. 6) suggesting the reconstitution of the retinoic acid–BLG complex. In order to gain better insight into the pH dependence of the CD and absorption spectra, a pH titration experiment was performed. In Fig. 7 CD and absorption curves of retinoic acid–BLG solution obtained between pH 6.97 and 3.22 (ligand/protein ratio is 0.87) are shown. Magnitude of the negative CD band is significantly decreases already at pH 6.66 and practically disappears below pH 6 while the solution became opalescent. Below pH 6.18 only a weak, negative band is present around 407 nm in the CD spectra which corresponds to the red shifted (384 nm) and broadened absorption band. At the

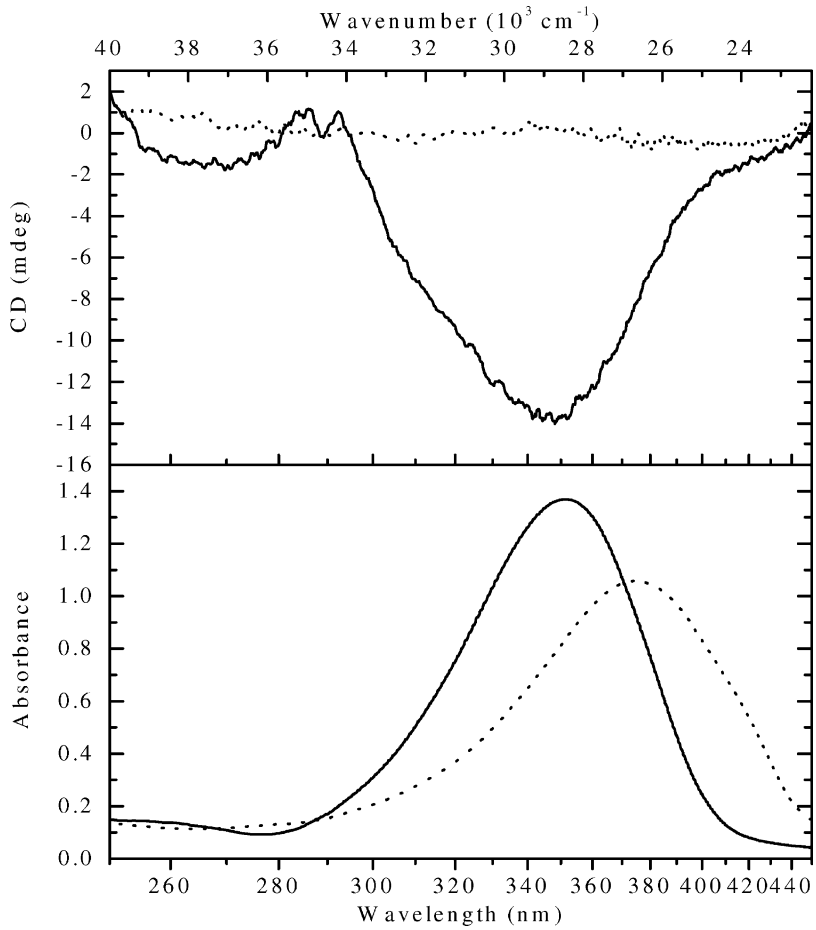

Fig. 6. CD and absorption spectra of retinoic acid–BLG mixture. Dotted line: acidified BLG solution (pH ≈ 2) after addition of 100 μL retinoic acid ($c_{BLG} = 5.0 \times 10^{-5}$ M; $c_{ligand} = 3.35 \times 10^{-5}$ M). Solid line: spectra of the former solution at pH ≈9.

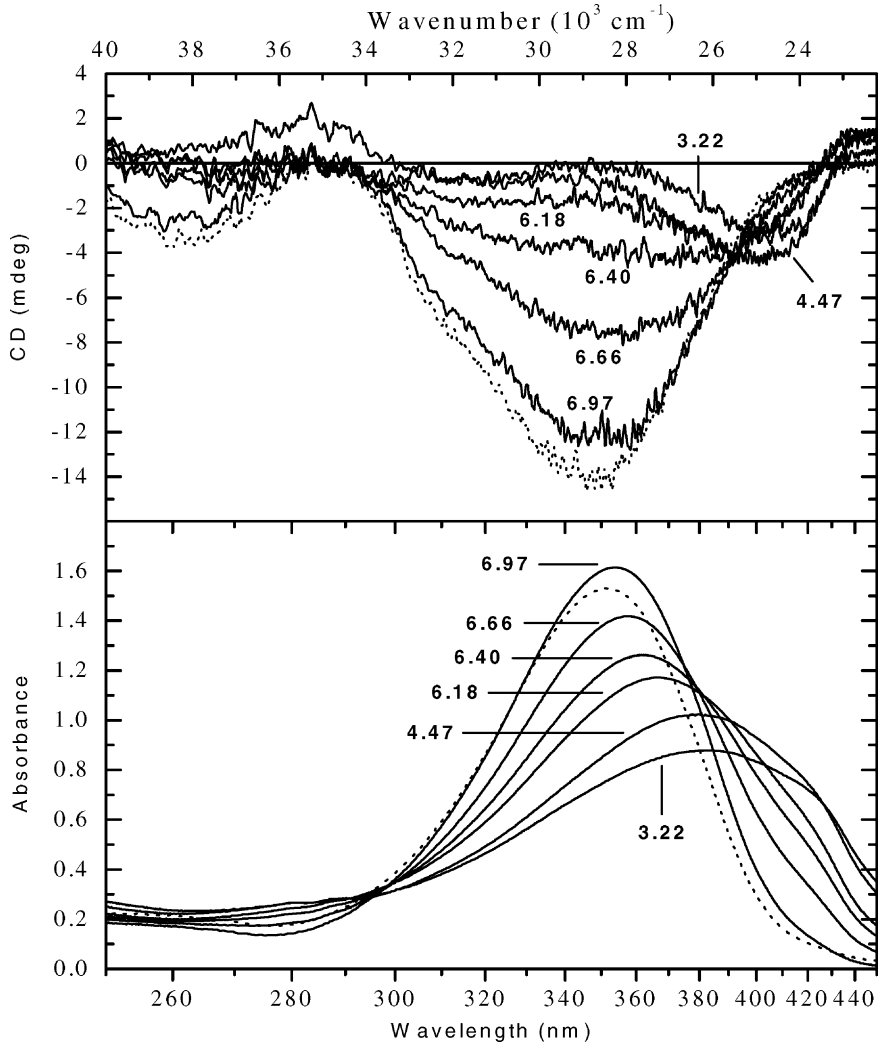

Fig. 7. pH dependence of the retinoic acid–BLG complex formed in distilled water. CD and absorption spectra were obtained through a titration procedure adding volumes (μL) of 0.1 M HCl to the 1 cm cuvette which contained 2 mL ligand + BLG solution (L/P ratio 0.87; $c_{BLG} = 4.6 \times 10^{-5}$ M; $c_{ligand} = 4.0 \times 10^{-5}$ M). The actual pH values are indicated. Dotted line represents the spectra of the reconstituted complex (pH 7.03) obtained by neutralization of the pH 3.22 solution by 1 M NaOH.

end of the titration procedure (pH 3.22) the pH was changed to 7.03 by the addition of 1 M NaOH. Upon doing this, the induced CD spectrum reappeared (Fig. 7) and it was identical to that measured at the beginning of the titration.

3.5. Molecular modeling studies on retinoic acid–BLG complex

As a starting point, the crystal structure of BLG complexed with palmitic acid [18] was used to model the retinoic acid binding in the central cavity. The resulting molecular model obtained by docking the ligand molecule into the hydrophobic cavity of the protein is shown in Fig. 8. The N-terminal end of the BLG is folded around the back of the barrel, locking that side of the pocket. In contrast, the front of the β-barrel, containing a flexible loop region, is open to provide a portal for the ligand. The β-ionone ring is buried in the hydrophobic pocket and the polyene chain is completely surrounded by the β-barrel (Fig. 8). Trp 19 faced to the base of the hydrophobic pocket is in the vicinity of the β-ionone ring but it is more distant from the isoprenoid tail; there is ≈12.5 Å between the C_7 atom of retinoic acid and the center of the indole ring. Practically the same distance was measured between C_{15} and Trp 61 located at the outer rim of the central cavity. There are a number of hydrophobic residues in the immediate vicinity of the ligand, mainly leucines and isoleucines. Among aromatic residues, Phe 82 and especially Phe 105 are in the closest contact to the cyclohexene ring. The π cloud of Phe 105 is oriented toward the axial hydrogens of C_3–C_4 and the C_{16} methyl group which is a favourable situation for CH/π interactions [39,40] and might contribute to the stabilization of the ligand in the hydrophobic cavity. The β-ionone ring is twisted around the C_6–C_7 bond, the C_5–C_6–C_7–C_8 torsion angle is −62°. The slightly

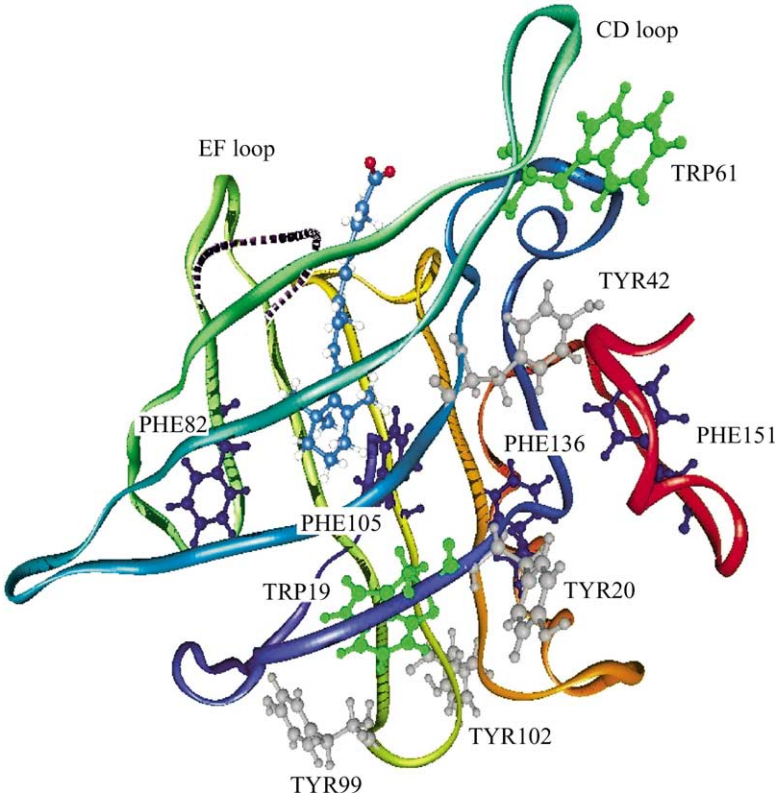

Fig. 8. Molecular model of the retinoic acid–BLG complex obtained by docking the ligand molecule into the central cavity of the β-barrel. Retinoic acid molecule is shown as ball-and-stick model (C, blue; O, red; H, white). Dotted ribbon shows the position of the EF loop at pH 6.2. Locations of aromatic amino acid residues of the protein are also indicated.

bent isoprene chromophore is streched along the barrel and it is helically distorted along its long axis. Semiempirical quantum mechanical calculation was performed on this chiral conformer which resulted in −1.47 Debye–Bohr magneton for the rotatory strength associated with the longest wavelength π–π^* excitation.

4. Discussion

All spectroscopic changes in CD and absorption spectra of retionic acid observed in the presence of BLG indicate that the Vitamin A derivative binds to a specific site of the protein. The red shift of the principal absorption band is due to protein binding; decrease of the excitation energy may be accounted for the hydrophobic, highly polarizable protein surrounding which favors the induced dipole-induced dipole interactions [41]. Furthermore, the band-width narrowing is also the consequence of complex formation since the binding environment sterically restricts the number of conformations produced by the twist around the C_6–C_7 bond. It should be noted that no vibrational fine structure is present in the absorption spectrum of the complex suggesting that the β-ionone ring preserve some, although decreased, conformational freedom in the binding site as well.

The induced CD spectrum of the complex proves that retinoic acid is bound to the asymmetric binding site of BLG which enforces a helical distortion along the long axis of the polyene chain resulting in an inherently chiral, optically active chromophore [42]. Due to the twist, the excited electron oscillates along a helical path causing a net magnetic transition moment associated with the π–π^* electronic excitation. Since the condition of the CD activity (parallel or anti-parallel electronic and magnetic transition moments) is satisfied, an induced Cotton effect appears at the longest wavelength absorption band. The sign of the CE is determined by the sense of the helical distortion. Semiempirical quantum mechanical calculation has been performed on the non-planar conformer of retinoic acid obtained after the protein docking procedure; this resulted in negative rotational strength (−1.47 Debye–Bohr magneton) in accordance with the negative value calculated from the CD spectrum.

It should be noted that the non-degenerate chiral exciton coupling [43] among the π–π^* transitions of the bound ligand and one or more adjacent aromatic amino acid residues is another mechanism which could explain the induced CD band as has been done by Fugate and Song in the case of retinol–BLG complex [26]. It is important, however, that the energies and intensities of the interacting electronic transitions should be close to each other; large

differences result in no or very weak coupling. In this respect the tryptophan residues (Trp 19 and 61) are the most likely candidates but the molecular model of the complex (Fig. 8) revealed that there are considerably large intermolecular distances between the polyene chain and these residues. Since the magnitude of exciton coupling is inversely proportional to the square of the interchromophoric distance it seems that excitonic interaction does not influence the CD spectral properties of the complex. This conclusion is supported by the fact that the induced CD spectra of *cis*-parinaric acid obtained in the presence of BLG lacks any sign of the excitonic interaction, either [42].

The reversible pH dependent changes of the CD and absorption spectra give valuable information on the location of the primary binding site of retinoic acid. These data suggest that at low pH, the binding site might become inaccessible for retionic acid due to changes in the tertiary structure of BLG. At the same time, high similarity of the near-UV CD spectra of BLG measured at pH 7.0 and pH 2 (data not shown) suggests that the aromatic amino acid residues are not involved in this structural change. As inferred from a variety of chemical and physical evidence, both bovine BLG variants A and B undergo pH-dependent conformational changes in the range of pH 6.5–7.5, the so called N ⇔ R or Tanford transition. The EF loop (Asp 85–Ala 86–Leu 87–Asn 88–Glu 89–Asn 90) forming a lid to the interior of the calyx is mainly involved in this conformational change (Fig. 8); at pH 7 and above this loop is folded back to reveal the interior of the calyx while at pH 6.2 and below it occludes the entrance of central cavity as demonstrated by X-ray diffraction methods [44,45]. This ''gating'' function is very important for ligands having binding site in the inner cavity. At low pH, when the EF loop is in closed conformation the added retinoic acid molecules are unable to bind into the cavity rather they form aggregates in the buffer solution. If the ligand is already bound in the hydrophobic cavity, the EF loop movement triggered by acidification also leads to the destabilization and dissociation of the complex, presumably due to unfavorable steric interactions arised between the loop residues and the polar end of retinoic acid (see Fig. 8). Thus, it can be concluded that retinoic acid binds within the hydrophobic central cavity of BLG. This conclusion is further supported by the fact that the same phenomenon was observed by NMR spectroscopy in the case of palmitic acid–BLG complex; around neutral pH palmitic acid binds within the central cavity of BLG but below pH 6 it begins to dissociate reversibly due to the Tanford transition of the protein [46]. Absence of indication for ligand–ligand interaction in the CD and absorption spectra refers to that of retinoic acid bound as a monomer in the β-barrel.

Our conclusions on the helically twisted conformation and binding location of retinoic acid are fully supported by a recent study on the X-ray structure of retionic acid–BLG complex [47].

5. Conclusion

The main findings of this work are, as follows:

(i) At neutral and alkaline pH all-*trans* retinoic acid forms a non-covalent complex with the lipocalin member bovine β-lactoglobulin.
(ii) Upon complex formation the absorption band of retinoic acid is bathochromically shifted and its bandwidth is narrowed due to the highly polarizable protein environment which restricts the β-ionone ring–polyene chain conformational mobility.
(iii) Corresponding to the red shifted absorption band, a negative Cotton effect appears in the CD spectrum induced by the asymmetric protein binding site which enforces a helical distortion on the polyene chain turning it into inherently chiral.
(iv) Below pH 7 retinoic acid starts to dissociate from its protein binding site as indicated by the vanishing CD band and the free ligand molecules aggregate in the aqueous environment. The ligand release is completely reversible upon re-neutralization of the solution proved by the reappearing of the induced CD band.
(v) Inability of BLG to bind retinoic acid at low pH and the reversible dissociation of the retinoic acid–BLG complex upon acidification were interpreted by the pH-dependent movement of the EF loop, opening and closing the entrance of hydrophobic central cavity of BLG.

Our results indicate that all-*trans* retinoic acid binds to the hydrophobic internal cavity of BLG. The ligand molecule bind to the BLG as a monomer in a chiral conformation. This work demonstrates that the circular dichroism spectroscopy is a valuable tool for the sensitive detection of retinoid–BLG interaction and offers further possibilities for studying ligand binding properties of BLG and related lipocalin molecules having great biological significance.

Acknowledgments

Financial support for the Hungarian National Research Foundation (grant no. OTKA T033109) and the 1/047 NKFP Medichem project are gratefully acknowledged.

References

[1] Drager UC, McCaffery P. Retinoic acid and development of the retina. Prog Retin Eye Res 1997;16:323–51.
[2] Miano JM, Berk BC. Retinoids—versatile biological response modifiers of vascular smooth muscle phenotype. Circ Res 2000;87:355–62.
[3] DeLuca LM, Kosa K, Andreola F. The role of Vitamin A in differentiation and skin carcinogenesis. J Nutr Biochem 1997;8:426–37.
[4] Ross SA, McCaffery PJ, Drager UC, De Luca LM. Retinoids in embryonal development. Physiol Rev 2000;80:1021–54.

[5] Zile MH. Function of Vitamin A in vertebrate embryonic development. J Nutr 2001;131:705–8.
[6] Gronemeyer H, Miturski R. Molecular mechanisms of retinoid action. Cell Mol Biol Lett 2001;6:3–52.
[7] Sun SY, Lotan R. Retinoids and their receptors in cancer development and chemoprevention. Crit Rev Oncol Hematol 2002;41:41–55.
[8] Sacchi S, Russo D, Avvisati G, Dastoli G, Lazzarino M, Pelicci PG, Bonora MR, Visani G, Grassi C, Iacona I, Luzzi L, Vanzanelli P. All-*trans* retinoic acid in hematological malignancies. An update. Haematologica 1997;82:106–18.
[9] Bruserud Ø, Gjertsen BT. New strategies for the treatment of acute myelogenous leukemia: differentiation induction—present use and future possibilities. Stem Cells 2000;18:157–65.
[10] Zouboulis CC. Retinoids—which dermatological indications will benefit in the near future? Skin Pharmacol Appl 2001;14:303–15.
[11] Orfanos CE, Zouboulis CC. Oral retinoids in the treatment of seborrhoea and acne. Dermatology 1998;196:140–7.
[12] Noy N. Retinoid-binding proteins: mediators of retinoid action. Biochem J 2000;348:481–95.
[13] Flower DR. The lipocalin protein family: structure and function. Biochem J 1996;318:1–14.
[14] Flower DR, North ACT, Sansom CE. The lipocalin protein family: structural and sequence overview. Biochim Biophys Acta 2000;1482:9–24.
[15] Sawyer L, Kontopidis G. The core lipocalin, bovine β-lactoglobulin. Biochim Biophys Acta 2000;1482:136–48.
[16] Brownlow S, Cabral JHM, Cooper R, Flower DR, Yewdall SJ, Polikarpov I, North ACT, Sawyer L. Bovine β-lactoglobulin at 1.8 Å resolution—still an enigmatic lipocalin. Structure 1997;5:481–95.
[17] Qin BY, Creamer LK, Baker EN, Jameson GB. 12-Bromododecanoic acid binds inside the calyx of bovine β-lactoglobulin. FEBS Lett 1998;438:272–8.
[18] Wu SY, Pérez MD, Puyol P, Sawyer L. β-Lactoglobulin binds palmitate within its central cavity. J Biol Chem 1999;274:170–4.
[19] Monaco HL, Zanotti G, Spadon P, Bolognesi M, Sawyer L, Eliopoulos EE. 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.
[20] Chen SX, Hardin CC, Swaisgood HE. Purification and characterization of β-structural domains of β-lactoglobulin liberated by limited proteolysis. J Protein Chem 1993;12:613–25.
[21] Cho Y, Batt CA, Sawyer L. Probing the retinol-binding site of bovine β-lactoglobulin. J Biol Chem 1994;269:11102–7.
[22] Lange DC, Kothari R, Patel RC, Patel SC. Retinol and retinoic acid bind to a surface cleft in bovine beta-lactoglobulin: a method of binding site determination using fluorescence resonance energy transfer. Biophys Chem 1998;74:45–51.
[23] Dufour E, Haertle T. Binding of retinoids and β-carotene to β-lactoglobulin. Influence of protein modifications. Biochim Biophys Acta 1991;1079:316–20.
[24] Dufour E, Roger P, Haertle T. Binding of benzo(α)pyrene, ellipticine, and *cis*-parinaric acid to β-lactoglobulin: influence of protein modifications. J Protein Chem 1992;11:645–52.
[25] Beuckmann CT, Aoyagi M, Okazaki I, Hiroike T, Toh H, Hayaishi O, Urade Y. Binding of biliverdin, bilirubin, and thyroid hormones to lipocalin-type prostaglandin D synthase. Biochemistry 1999;38:8006–13.
[26] Fugate RD, Song PS. Spectroscopic characterization of β-lactoglobulin–retinol complex. Biochim Biophys Acta 1980;625:28–42.
[27] Downing JW. Program package BDZDO/MCDSPD. Department of Chemistry and Biochemistry, University of Colorado, Boulder, USA; modified by Fleischhauer J, Schleker W, Kramer B, University of Aachen, Germany; ported to LinuX by Gulden KP.
[28] Das PK. Spectroscopy of polyenes. 6. Absorption and emission spectral properties of linear polyenals of the series CH_3–(CH=CH)–CHO. J Phys Chem 1982;86:921–7.
[29] Zsila F, Bikádi Z, Simonyi M. Induced chirality upon crocetin binding to human serum albumin: origin and nature. Tetrahedron Asymmetry 2001;12:3125–37.
[30] Christensen RL, Kohler BE. Low resolution optical spectroscopy of retinyl polyenes: low lying electronic levels and spectral broadness. Photochem Photobiol 1973;18:293–301.
[31] Hemley R, Kohler BE. Electronic structure of polyenes related to the visual chromophore. A simple model for the observed band shapes. Biophys J 1977;20:377–82.
[32] Myers AB, Trulson MO, Pardoen JA, Heeremans C, Lugtenburg J, Mathies RA. Absolute resonance Raman intensities demonstrate that the spectral broadening induced by the β-ionone ring in retinal is homogenous. J Chem Phys 1986;84:633–40.
[33] Woody RW, Dunker AK. Aromatic and cystine side-chain circular dichroism in proteins. In: Fasman GD, editor. Circular dichroism and the conformational analysis of biomolecules. New York: Plenum Press; 1996. p. 109–58.
[34] Farrell HM, Behe MJ, Eneyeart JA. Binding of *p*-nitrophenyl phosphate and other aromatic compounds by β-lactoglobulin. J Dairy Sci 1987;70:252–8.
[35] Wang Q, Allen JC, Swaisgood HE. Binding of retinoids to beta-lactoglobulin isolated by bioselective adsorption. J Dairy Sci 1997;80:1047–53.
[36] Heller J, Horwitz J. Conformational changes following interaction between retinol isomers and human retinol-binding protein and between the retinol-binding protein and prealbumin. J Biol Chem 1973;248:6308–16.
[37] Horwitz J, Heller J. Interactions of all-*trans*, 9-, 11-, and 13-*cis*-retinal, all-*trans* retinyl acetate, and retinoic acid with human retinol-binding protein and prealbumin. J Biol Chem 1973;248:6317–24.
[38] Han CH, Wiedmann TS. Spectral properties and ion dissociation behavior of retinoids. I. Aqueous solutions. Int J Pharm 1998;172:241–53.
[39] Nishio M, Hirota M, Umezawa Y. The CH/π interaction. Evidence, nature, and consequences. New York: Wiley-VCH, 1998.
[40] Takahashi H, Tsuboyama S, Umezawa Y, Honda K, Nishio M. CH/π interactions as demonstrated in the crystal structure of host/guest compounds. A database study. Tetrahedron 2000;56:6185–91.
[41] Frank HA, Bautista JA, Josue J, Pendon Z, Hiller RG, Sharples FP, Gosztola D, Wasielewski MR. Effect of the solvent environment on the spectroscopic properties and dynamics of the lowest excited states of carotenoids. J Phys Chem B 2000;104:4569–77.
[42] Zsila F, Imre T, Szabó PT, Bikádi Z, Simonyi M. Induced chirality upon binding of *cis*-parinaric acid to bovine β-lactoglobulin: spectroscopic characterization of the complex. FEBS Lett 2002;520:81–7.
[43] Berova N, Nakanishi K. Principles and applications of exciton chirality method. In: Berova N, Nakanishi K, Woody R, editors. Circular dichroism—principles and applications. New York: Wiley-VCH; 2000. p. 337–82.
[44] Qin BY, Bewley MC, Creamer LK, Baker HM, Baker EN, Jameson GB. Structural basis of the Tanford transition of bovine β-lactoglobulin. Biochemistry 1998;37:14014–23.
[45] Qin BY, Bewley MC, Creamer LK, Baker EN, Jameson GB. Functional implications of structural differences between variants A and B of bovine beta-lactoglobulin. Protein Sci 1999;8:75–83.
[46] Ragona L, Fogolari F, Zetta L, Perez DM, Puyol P, De Kruif K, Lohr F, Ruterjans H, Molinari H. Bovine β-lactoglobulin: interaction studies with palmitic acid. Protein Sci 2000;9:1347–56.
[47] Kontopidis G, Holt C, Sawyer L. The ligand-binding site of bovine β-lactoglobulin: evidence for a function? J Mol Biol 2002;318:1043–55.