

Induced chirality upon binding of *cis*-parinaric acid to bovine β -lactoglobulin: spectroscopic characterization of the complex

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Abstract Binding of the polyunsaturated *cis*-parinaric acid to bovine β -lactoglobulin (BLG) was studied by circular dichroism (CD), electronic absorption spectroscopy and mass spectrometry methods. Upon protein binding, the UV absorption band of parinaric acid is red shifted by ca. 5 nm, showing hypochromism and reduced vibrational fine structure, suggesting that the ligand binds as a monomer in non-planar geometry. In the CD spectra measured at pH 7.36 and 8.5 a strong, negative Cotton band appears centered at 310 nm ($\Delta\epsilon = -25 \text{ M}^{-1} \text{ cm}^{-1}$) corresponding to the long-wavelength absorption band of *cis*-parinaric acid. The source of this induced optical activity is the helical distortion of the polyene chromophore caused by the chiral protein environment. From CD spectral data the value of the association constant was calculated to be $4.7 \times 10^5 \text{ M}^{-1}$ at pH 7.36. CD and mass spectrometry measurements showed that parinaric acid binds weakly to BLG in acidic solution, though small peaks at mass 18559 and 18645 can be obtained in the reconstructed electrospray mass spectrum; these correspond to the binding of parinaric acid in 1:1 stoichiometry to both monomer variants of BLG B and A. The hydrophobic interior cavity of BLG was assigned as the primary binding site of *cis*-parinaric acid. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Bovine β -lactoglobulin; *cis*-Parinaric acid; Induced chirality; Ligand binding; Circular dichroism spectroscopy; Mass spectrometry

1. Introduction

β -Lactoglobulin (BLG) is the major whey protein in the milk of ruminants and some other species. It was first isolated 60 years ago and despite the accumulation of a wealth of physico-chemical and biological information no function has been definitely ascribed to it [1].

BLG is an extremely acid stable protein which exists at the normal pH of bovine milk as a dimer, consisting of two monomeric subunits with a molecular weight of about 18300 Da. Several genetic variants of BLG have been detected, of which the bovine phenotype A and phenotype B are the most predominant [1,2]. As a member of the lipocalin

protein family [3], an important physico-chemical property of BLG is to bind hydrophobic substances of diverse nature, such as retinol [4,5], fatty acids [6,7], protoporphyrin [8], cholesterol, benzo[a]pyrene [9], etc. Two alternative sites have been proposed for ligand binding: the interior cavity of the β -barrel, and a surface pocket located in a groove between the α -helix and the barrel [1,10]. Recently, the X-ray structure of BLG complexed with 12-bromododecanoic [11] and palmitic acid [7] clearly showed fatty acid molecules to be accommodated within the central cavity. Detailed NMR investigation of the BLG–palmitic acid complex gave further evidence for cavity binding [6]. Beyond these findings, the ligand binding mechanism of BLG is not fully understood and the experimental data are still controversial [12–14].

In the present work, circular dichroism (CD), electronic absorption spectroscopy and electrospray ionization mass spectrometry (ESI-MS) were used to characterize the interaction of polyunsaturated *cis*-parinaric acid with BLG. *cis*-Parinaric acid (Fig. 1), a plant-derived 18-carbon fatty acid [15], shows selective toxicity against tumor cell lines in vitro [16,17]. It was initially developed as a membrane probe [18] and is widely used in fluorescence experiments [19]. Its strong UV absorption and conformational mobility also qualifies it for CD measurements to study stereospecific ligand-biopolymer interactions [20]. The strong, induced CD band found in our experiments makes *cis*-parinaric acid a promising molecular probe for further investigation of the binding properties of BLG and other members of the lipocalin protein family associated with growing biological significance [21]. Additionally, as *cis*-parinaric acid is practically insoluble in water, improving its bioavailability by non-covalent protein binding is highly preferred for enhancing its antitumor activity.

ESI-MS played important role in the complete structure elucidation of BLG [22]. Covalent adducts of BLG with acrylamide [23] and non-covalent complexes with 8-anilinoanthralene-1-sulfonic acid [23], 4-hydroxy-2-nonenal [24] and different pesticides [25] were also reported.

2. Materials and methods

2.1. Materials

Cow milk BLG (mixture of A and B variants, approximately 90% purity grade) was obtained from Sigma (catalogue No. L-3908, lot No. 119H7008). *cis*-Parinaric acid (9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid) was purchased from Calbiochem (catalogue No. 512730, lot No. B37507) and was used without further purification. Double distilled water and HPLC grade ethanol (Chemolab, Hungary) were used. All other chemicals used were of analytical grade.

cis-Parinaric acid was dissolved in 100% ethanol under nitrogen in the dark, to obtain a $2 \times 10^{-3} \text{ M}$ stock solution. Buffer solutions used

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Abbreviations: BLG, bovine β -lactoglobulin; CD, circular dichroism; CE, Cotton effect; ESI-MS, electrospray ionization mass spectrometry

to prepare BLG solutions were: 0.1 M phosphate buffer at pH 3.0 and 7.36, and 0.1 M Tris buffer at pH 8.5.

The following procedure was used for titration of BLG with *cis*-parinaric acid at pH 3.0, 7.36 and 8.5: 2 ml of 5×10^{-5} M or 1×10^{-5} M protein solution was placed in a cuvette and small amounts of the ligand stock solution was added with an automatic pipette to achieve 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 1, 1.5, 2 and 2.5 ligand/protein molar ratios. Ethanol added with the ligand never exceeded 2% (v/v). At pH 3.0, above 0.2 ratio *cis*-parinaric acid shows visual signs of aggregation, excluding further spectroscopic measurements. Similar phenomenon was observed above the ligand/protein ratio of 1.5 at both pH 7.36 and 8.5.

2.2. CD and UV absorption spectroscopy

CD and UV spectra were recorded between 240 and 380 nm on a Jasco J-715 spectropolarimeter at $25 \pm 0.2^\circ\text{C}$ in a rectangular cuvette with 1 cm pathlength. 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. Induced CD is defined as the CD of *cis*-parinaric acid–BLG mixture minus the CD of BLG alone at the same wavelengths and is expressed as ellipticity in millidegrees.

2.3. Electrospray mass spectrometry

Mass spectrometric measurements were performed on a Perkin-Elmer Sciex API2000 (Perkin-Elmer Sciex, Toronto, ON, Canada) triple quadrupole instrument equipped with a TurboIonSpray source. The instrument was scanned in a mass range of 900–1800 amu with a step size of 0.3 amu and dwell time of 1 ms. A Perkin-Elmer 200 micro HPLC pump was used as a solvent delivery system. Water was used as eluent at pH 3, which was adjusted by acetic acid. This relatively low pH had to be used because the maximum of the charge state distribution of the multiply charged proteins must be lower than 1800 Da (the upper mass limit of the instrument). The flow rate of eluent was 0.1 ml min^{-1} . BioMultiView 1.3.1 software was used for deconvoluting the raw electrospray data to obtain the real molecular weights.

2.4. Semiempirical quantum mechanical calculations

The geometry optimization of modified *cis*-parinaric acid was carried out with the Gaussian-98W program, using B3LYP functional and standard 6-31G** basis sets. Then the calculations of transition energies and rotatory strengths were performed by ArgusLab (ver. 3.0) using the INDO1/S method. ArgusLab is a free molecular modeling program for Windows 95/98 and NT 4.0 [26]. The electronic excited-state calculations consisted of a configuration interaction of the 20 lowest single-excited configurations.

2.5. Calculation of the binding constant of *cis*-parinaric acid–BLG complex

The stereospecific interaction between a ligand (L) and its primary site on the protein (P) may be quantified by the affinity constant referred to as the association equilibrium (K_a):

$$L + P = LP; K_a = \frac{[LP]}{[L][P]} \quad (1)$$

It is evident that

$$[L] = c_L - [LP] \quad (2)$$

and

$$[P] = c_P - [LP] \quad (3)$$

where c_L and c_P mean the total concentrations of the ligand and protein, respectively.

Assuming that the parinaric acid–BLG complex is responsible for the induced CD band, it can be written that:

$$\text{CD (mdeg)} = k[LP] \quad (4)$$

where k is a constant.

Using Eqs. 1–4, we obtain:

$$\text{CD (mdeg)} = \frac{k}{2} \left(c_P + c_L + K_a^{-1} - \sqrt{(c_P + c_L + K_a^{-1})^2 - 4c_P c_L} \right) \quad (5)$$

In order to calculate the optimal values of k and K_a , non-linear

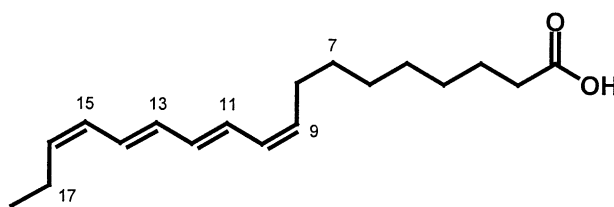


Fig. 1. Chemical structure of *cis*-parinaric acid (9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid).

regression analysis was applied (NLREG[®] statistical analysis program, ver. 3.4). The value of K_a was obtained to be $(4.7 \pm 1.7) \times 10^5 \text{ M}^{-1}$ at pH 7.36 ($r^2 = 0.997$).

3. Results and discussion

The structure of *cis*-parinaric acid is a linear, essentially planar polyene chain composed of four conjugated carbon–carbon double bonds with two uneven sequences attached in *cis* geometry at both ends (Fig. 1). In its electronic absorption spectrum only one intense absorption band can be seen between 210 and 370 nm showing a characteristic vibrational fine structure (Fig. 2). It has three resolved peaks at 292, 305 and 320 nm and a shoulder around 280 nm, separated by $\sim 1500 \text{ cm}^{-1}$ vibronic progressions according to the $\text{C}=\text{C}$ stretching frequency. The ground and excited states are denoted 1^1A_g and 1^1B_u corresponding to the C_{2h} symmetry of the polyene chromophore. The transition moment of this $\pi-\pi^*$ type excitation is electronic dipole allowed but magnetically forbidden and oriented along the long axis of the tetraene moiety. Lacking molecular chirality, the CD spectrum of *cis*-parinaric acid is a zero line (not shown).

3.1. CD spectrum of BLG

The near-UV CD spectrum of BLG alone shows a pattern of negative peaks between 250 and 300 nm (Fig. 3) due to the presence of aromatic residues: two tryptophans (Trp19 and 61), four tyrosines (Tyr20, 42, 99 and 102) and four phenylalanines (Phe82, 105, 136 and 151). Although all of them can contribute to the CD spectrum, tryptophan residues are often the major determinants of the near-UV CD curve; tryptophan signals are generally more intense than those of tyrosine and phenylalanine and they occur between 250 and 300 nm, whereas phenylalanines and tyrosines usually do not absorb above 270 nm and 290 nm, respectively [27]. According to this, the negative bands at 293.2 nm and 284.4 nm can be assigned to asymmetrically perturbed tryptophans while peaks below 280 nm are likely the result of the chiral environment of phenylalanine and tyrosine residues [28]. The optical rotatory strength calculated for the negative band between 255 and 320 nm is $-0.85 \times 10^{-39} \text{ cgs unit}$ (0.09 Debye–Bohr magneton).

3.2. Absorption and chiroptical properties of parinaric acid–BLG complex

Upon addition of *cis*-parinaric acid to BLG solution a new, negative CD band appears centered at 310 nm (Fig. 4). The difference spectra clearly show that aromatic amino acids of BLG have no contribution to this negative Cotton effect (CE) and it can be attributed exclusively to the chirally perturbed $1^1A_g \rightarrow 1^1B_u$ electronic transition of the tetraene chromophore. The shape and vibrational fine structure of the induced

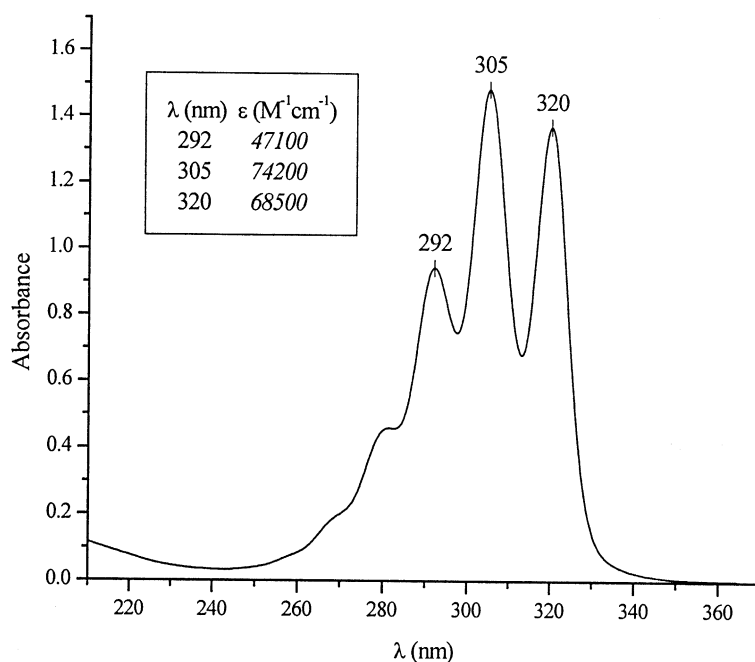


Fig. 2. UV absorption spectrum of *cis*-parinaric acid at room temperature in ethanol ($l=1$ cm; $c=2\times 10^{-5}$ M). Inset shows the peak positions and the molar extinction coefficients in $M^{-1} cm^{-1}$.

CE is highly similar to those of the corresponding absorption band and the wavelength positions of the CD subbands match with the red shifted absorption peaks. The measure of the red shift relative to the UV spectrum taken in ethanol is 5–6 nm and accompanied by sound hypochromism [29] (Table 1) while the band shape and the half width are hardly altered. As known, the absorption spectra of polyenes shift to longer wavelengths when the solvent polarizability increases [30].

Upon binding to BLG, *cis*-parinaric acid is accommodated in a protein environment of high polarizability, which promotes the induced dipole-induced dipole (dispersive) interactions, causing the bathochromic shift. By means of the Abs_{valley}/Abs_{max} quotient where Abs_{valley} is the absorption value at the minimum between the two resolved peaks around 310 and 326 nm and Abs_{max} denotes the absorption measured at λ_{max} (310 nm), the vibrational fine structure of free and

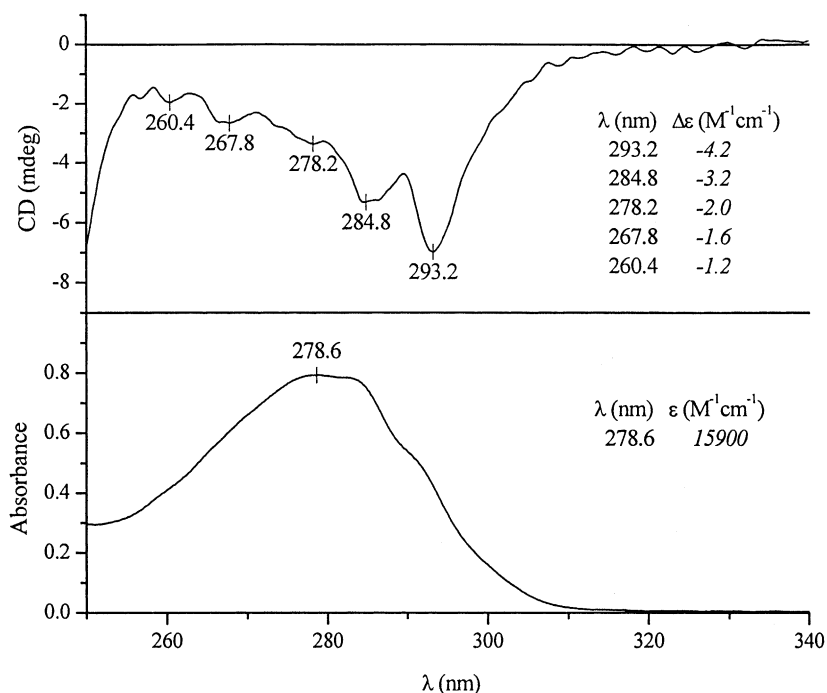


Fig. 3. CD and absorption spectra of BLG between 250 and 340 nm in 0.1 M phosphate buffer at pH 7.36 ($l=1$ cm; $c_{BLG}=5\times 10^{-5}$ M).

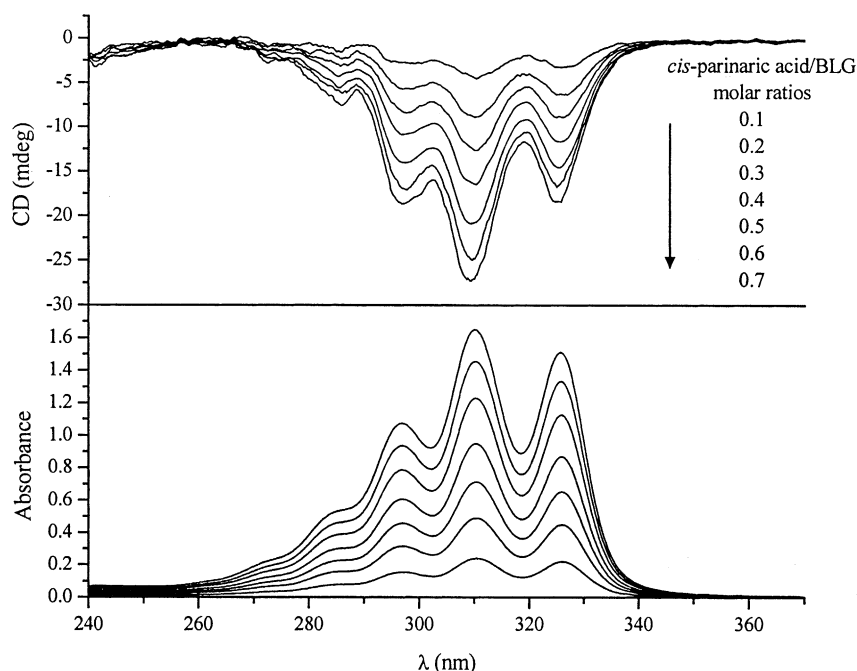


Fig. 4. Induced CD and UV spectra of *cis*-parinaric acid bound to BLG. The concentration of BLG was constant (5×10^{-5} M) and the ligand was added as aliquots of ethanolic stock solution ($l = 1$ cm; $t = 25$ °C). For clarity, curves measured above 0.7 ligand/protein ratio are omitted.

protein-bound ligand was compared. In ethanol, this value is 0.46, while between 0.1 and 0.7 ligand/protein ratios varies from 0.51 to 0.54 (pH 7.36), indicating that the vibrational structure is less pronounced in the presence of BLG. Low temperature absorption measurements performed on carotenes and carotenoids showed the spectral fine structure to be enhanced, indicating that the polyene chain becomes more planar upon cooling, which restricts the thermal deformation of the conjugated π -system [31,32]. Accordingly, our results indicate that the tetraene part of *cis*-parinaric acid bound to BLG is less planar and becomes slightly distorted

upon accommodation to the binding environment. Taken together, these spectral characteristics suggest that parinaric acid binds to a well-defined binding site of BLG in the form of a monomer.

With increasing ligand concentration the magnitude of the new CD band at 310 nm increases and shows very similar values at pH 7.36 and 8.5, respectively. The molar dichroic absorption coefficient ($\Delta\epsilon$) reaches a maximum value of ~ -25 M $^{-1}$ cm $^{-1}$ around 310 nm but begins to decrease as the ligand/protein ratio exceed the value of 0.7 (Table 1). It might be a sign for the free ligand starting aggregation. The

Table 1

CD and UV spectral data of *cis*-parinaric acid bound to BLG calculated at different ligand/protein molar ratios and expressed in $\Delta\epsilon$ and ϵ (M $^{-1}$ cm $^{-1}$) units

<i>cis</i> -Parinaric acid:BLG molar ratio	$\Delta\epsilon$ (M $^{-1}$ cm $^{-1}$)	ϵ (M $^{-1}$ cm $^{-1}$)
1:0 (in ethanol)	–	22 000 (279s), 47 000 (292)
[2.0×10^{-5} M]	–	74 000 (305), 68 500 (320)
0.1:1	–9.3 (285.4), –17.2 (298.8)	16 000 (284s), 31 000 (296.8)
[5.0×10^{-6} M]	–27.3 (310.4), –19.6 (325.6)	48 000 (310.4), 44 000 (325.8)
0.2:1	–6.9 (286.0), –17.3 (297.2)	15 000 (283.8s), 31 000 (296.6)
[1.0×10^{-5} M]	–26.8 (310.4), –19.0 (326)	49 000 (310.2), 45 000 (325.8)
0.3:1	–6.3 (285.6), –17.1 (297.6)	14 400 (283.8s), 30 500 (297)
[1.5×10^{-5} M]	–25.5 (310.2), –17.7 (325.6)	47 500 (310.4), 43 000 (326)
0.4:1	–6.5 (285.4), –16.4 (297)	14 400 (283.6s), 30 500 (296.8)
[2.0×10^{-5} M]	–24.9 (310.2), –17.5 (325.4)	47 500 (310.2), 43 500 (326)
0.5:1	–6.8 (285.4), –17.0 (297.4)	14 500 (283.4s), 31 500 (296.8)
[2.5×10^{-5} M]	–25.2 (309.4), –17.6 (325.4)	49 000 (310.2), 45 000 (325.8)
0.6:1	–6.3 (286), –17.2 (297.6)	15 000 (283.4s), 31 000 (296.8)
[3.0×10^{-5} M]	–25.2 (309.6), –16.8 (325)	48 500 (310.2), 44 500 (325.8)
0.7:1	–6.6 (286), –16.1 (297.2)	14 500 (283.2s), 31 000 (296.8)
[3.5×10^{-5} M]	–23.6 (309.2), –15.9 (324.8)	47 000 (310.2), 43 000 (325.8)
1:1	–5.4 (284.6), –13.0 (297.6)	16 000 (282s), 34 500 (296.4)
[1.0×10^{-5} M]	–18.7 (310.2), –12.6 (325)	52 000 (309), 46 000 (324.6)
1.5:1	–4.5 (285), –10.0 (297.8)	16 000 (281.6s), 33 000 (295.6)
[1.5×10^{-5} M]	–13.7 (310), –9.0 (325.4)	49 500 (308.4), 43 500 (323.4)

Wavelength positions are italicized, s denotes shoulder.

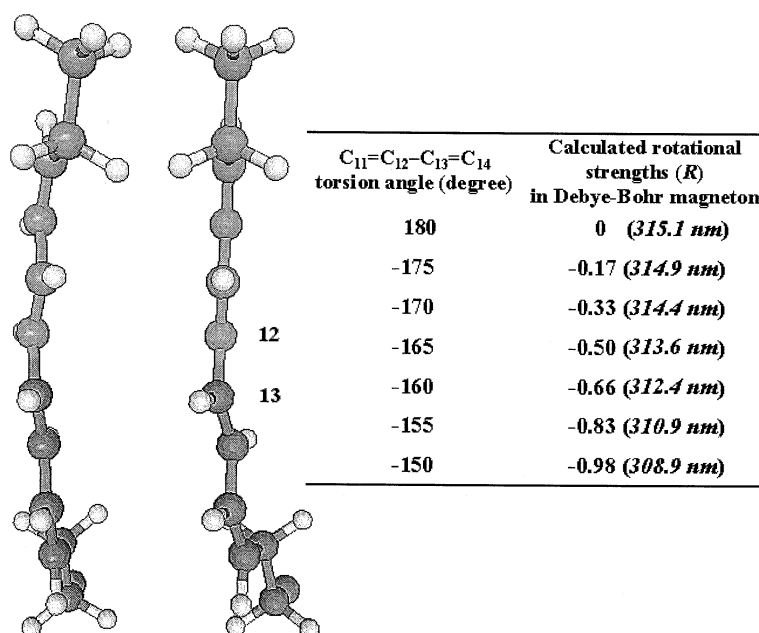


Fig. 5. Stereo view of the helically distorted model of the tetraene part of *cis*-parinaric acid (the carboxyl group containing saturated chain was omitted). The negative sense of helicity (the $C_{11}=C_{12}-C_{13}=C_{14}$ torsion angle is -160°) results in a negative rotational strength at the long-wavelength $\pi-\pi^*$ transition. Light and dark gray coloration represent the two parts of the chromophore distorted relative to each other. Inserted table shows computed values of the rotational strength at different chiral geometries obtained by INDO/S-CI method.

rotatory strength of the induced CD band at 0.3 ligand/protein ratio (pH 7.36) is 5.82×10^{-39} cgs units (0.63 Debye-Bohr magneton). This value is higher by one order of magnitude relative to that calculated for aromatic residues of BLG, indicating the presence of an inherently dissymmetric, chiral conformation of parinaric acid as the consequence of binding to BLG. It is worth mentioning that similar large values of the rotational strength were found at retinol-BLG and human retinol binding protein complexes [4,33]. The $1^1A_g \rightarrow 1^1B_u$ ex-

citation ($\pi-\pi^*$ transition) has a large electronic transition moment, but it is magnetically forbidden if the polyene chromophore is planar. The asymmetric protein environment, however, enforces a twist on the conjugated double bonds, resulting in a helically distorted, optically active chromophore which explains the observed intense CD band. In order to establish a qualitative connection between the sign of the induced CE and the sense of helicity, the so-called transoid diene rule was used which was developed for non-planar con-

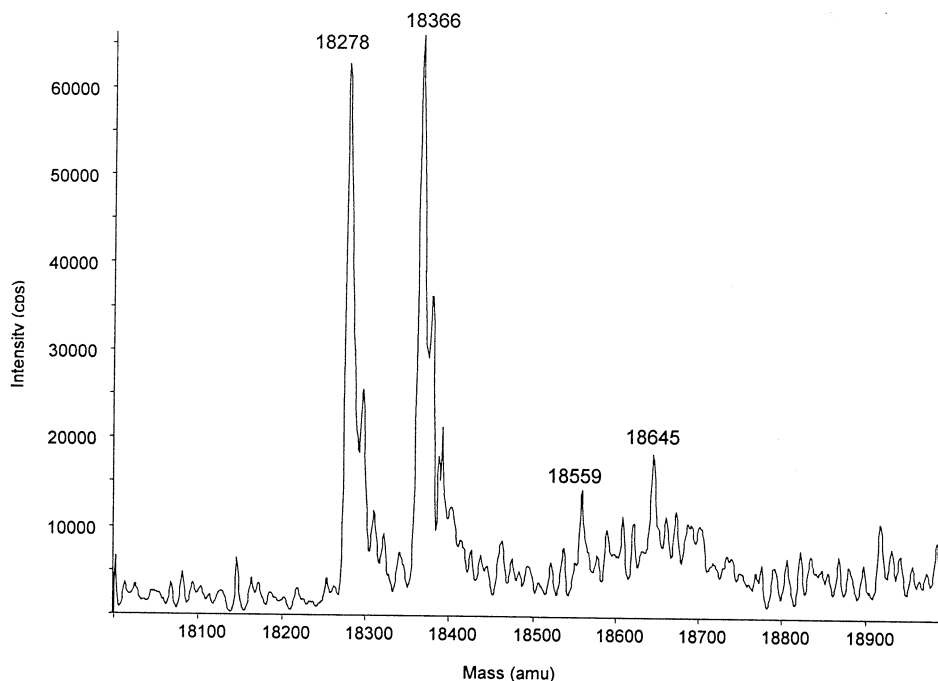


Fig. 6. Reconstructed electrospray mass spectrum of parinaric acid treated BLG.

jugated dienes [34]. According to this, the non-planar model conformer of *cis*-parinaric acid depicted in Fig. 5 having -160° torsion angle around the C(12)–C(13) bond results in a negative CE in the CD spectrum (the opposite torsion angle would give a positive CD band). Naturally, if a similar negative twist occurs around the C(10)–C(11) or C(14)–C(15) bonds, the rotatory strength would also be negative. Additionally, we have performed semiempirical quantum mechanical calculations to evaluate the extent of the helical distortion. The long, saturated tail of the parinaric acid was removed since it has no decisive contribution to the molecular chirality. As can be seen from Fig. 5, negative values were calculated for the rotatory strength when negative torsion angles were set for the C(12)–C(13) bond (the same, but positive values were obtained for positive torsion angles). Furthermore, the computational results suggest that a relatively small twist ($\sim -15^\circ$) may cause a rotatory strength found experimentally. It is worth mentioning that the saturated parts attached in *cis*-geometry at both ends of the polyene chain presumably play an important role in the helical twist by functioning as ‘handles’ for the protein environment to enforce distortion on the conjugated π -system.

Assuming that the magnitude of the induced CD band represents the total amount of ligand-protein complex, the binding constant was calculated (see experimental). Its value, $(4.7 \pm 1.7) \times 10^5 \text{ M}^{-1}$ at pH 7.36, is much lower than that found by Dufour et al. in their fluorescence measurements ($3.57 \times 10^7 \text{ M}^{-1}$ at pH 7.0) [9]. They also reported that parinaric acid binds strongly to BLG at pH 3.0 ($K_a = 4.76 \times 10^7 \text{ M}^{-1}$). CD spectra showed, however, that parinaric acid binding to BLG is weaker at pH 3.0 ($\Delta\epsilon_{311 \text{ nm}} = -14.8$ and $-3.7 \text{ M}^{-1} \text{ cm}^{-1}$ at 0.1 and 0.2 molar ratios, respectively). In acidic solution above 0.2 ligand/protein molar ratio, parinaric acid molecules visibly aggregate while at pH 7.36 and 8.5 this phenomenon was not observed until the ratio of 1.5. This finding is in good agreement with the results obtained by mass spectrometry (see below), revealing that only a minor fraction of BLG binds *cis*-parinaric acid at pH 3. However, we note that the decreased value of $\Delta\epsilon$ at 0.2 molar ratio at pH 3.0 may indicate starting aggregation. Likewise, at pH 7.36 and 8.5 the aggregation of free parinaric acid molecules may start when the ligand/protein molar ratio exceeds 0.7 (cf. Table 1).

In spite of the difference in flexibility between *cis*-parinaric acid and palmitic acid, the palmitic acid–BLG complex behaves in a similar way, as found by NMR spectroscopy [6]. Around neutral pH palmitic acid binds within the central cavity of BLG but below pH 6 it begins to dissociate due to the conformational changes of the protein (the so-called Tanford transition) [6,7,11]. Taking into account our results and the data mentioned above, it is reasonable to conclude that the primary binding site of parinaric acid is the hydrophobic cavity of BLG.

3.3. Electrospray mass spectrometry investigations

Two intense and two minor peaks were obtained in the reconstructed electrospray mass spectrum of pure BLG (not shown). Peaks at mass 18278 and 18366 correspond to the two variants of BLG B and BLG A, respectively. The minor peaks appearing at 18604 and 18692 correspond to the lactulose conjugates of the two BLG variants. It is well demonstrated that, according to the Maillard reaction, lactose can

react with the ϵ -amino group of lysine yielding the above-mentioned conjugates [35].

In the reconstructed electrospray mass spectrum of parinaric acid treated BLG (Fig. 6), two new peaks appear at 18559 and 18645. The mass differences between these peaks and the particular peaks of BLG variants (281 and 279) are close to the molecular weight of parinaric acid (276). There are no more peaks with this mass difference beyond these conjugate peaks, which means that one BLG can bind one parinaric acid. The intensity ratio of these conjugates and that of the BLG variants is quite similar. It suggests that there is no significant difference between the binding constants of the two variants.

4. Conclusion

New findings of this work are as follows:

1. the absorption band of *cis*-parinaric acid is red shifted in the presence of BLG;
2. the appearance of a strong induced negative CE in the CD spectrum indicated the formation of parinaric acid–BLG complex;
3. in an acidic environment, the induced CE became considerably weaker than at physiological pH;
4. the mass spectrum proved 1:1 stoichiometry of the complex formed by both A and B variants of BLG.

The findings suggest that *cis*-parinaric acid binds to the hydrophobic internal cavity of BLG.

Since the chiroptical technique sensitively detects ligand–biopolymer interactions, *cis*-parinaric acid as a new molecular probe opens new possibilities for studying ligand binding properties of BLG and other lipocalin molecules by CD spectroscopy.

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References

- [1] Sawyer, L. and Kontopidis, G. (2000) *Biochim. Biophys. Acta* 1482, 136–148.
- [2] Dong, A., Matsuura, J., Allison, S.D., Chrisman, E., Manning, M.C. and Carpenter, J.F. (1996) *Biochemistry* 35, 1450–1457.
- [3] Flower, D.R., North, A.C.T. and Sansom, C.E. (2000) *Biochim. Biophys. Acta* 1482, 9–24.
- [4] Fugate, R.D. and Song, P.S. (1980) *Biochim. Biophys. Acta* 625, 28–42.
- [5] Dufour, E. and Haertle, T. (1991) *Biochim. Biophys. Acta* 1079, 316–320.
- [6] Ragona, L., Fogolari, F., Zetta, L., Perez, D.M., Puyol, P., De Kruijff, K., Lohr, F., Ruterjans, H. and Molinari, H. (2000) *Protein Sci.* 9, 1347–1356.
- [7] Wu, S.Y., Pérez, M.D., Puyol, P. and Sawyer, L. (1999) *J. Biol. Chem.* 274, 170–174.
- [8] Dufour, E., Marden, M.C. and Haertle, T. (1990) *FEBS Lett.* 277, 223–226.
- [9] Dufour, E., Roger, P. and Haertle, T. (1992) *J. Protein Chem.* 11, 645–652.
- [10] Cho, Y., Batt, C.A. and Sawyer, L. (1994) *J. Biol. Chem.* 269, 11102–11107.
- [11] Qin, B.Y., Creamer, L.K., Baker, E.N. and Jameson, G.B. (1998) *FEBS Lett.* 438, 272–278.
- [12] Lange, D.C., Kothari, R., Patel, R.C. and Patel, S.C. (1998) *Biophys. Chem.* 74, 45–51.

- [13] Narayan, M. and Berliner, L.J. (1997) *Biochemistry* 36, 1906–1911.
- [14] Narayan, M. and Berliner, L.J. (1998) *Protein Sci.* 7, 150–157.
- [15] Tsai, A., Hudson, B.S. and Simoni, R.D. (1981) *Methods Enzymol.* 72, 483–485.
- [16] Cornelius, A.S., Yerram, N.R., Kratz, D.A. and Spector, A.A. (1991) *Cancer Res.* 51, 6025–6030.
- [17] Traynelis, V.C., Ryken, T.C. and Cornelius, A.S. (1995) *Neurosurgery* 37, 484–489.
- [18] Sklar, L.A., Hudson, B.S., Petersen, M. and Diamond, J. (1977) *Biochemistry* 16, 813–819.
- [19] Dergunov, A.D., Dobretsov, G.E., Visvikis, S. and Siest, G. (2001) *Chem. Phys. Lipids* 113, 67–82.
- [20] Sklar, L.A., Hudson, B.S. and Simoni, R.D. (1977) *Biochemistry* 16, 5100–5108.
- [21] Koistinen, H., Koistinen, R., Seppala, M., Burova, T.V., Choiset, Y. and Haertle, T. (1999) *FEBS Lett.* 450, 158–162.
- [22] Turula, V.E., Bishop, R.T., Ricker, R.D. and Dehaseth, J.A. (1997) *J. Chromatogr. A* 763, 91–103.
- [23] Bordini, E. and Hamdan, M. (1999) *Rapid Commun. Mass Spectrom.* 13, 1143–1151.
- [24] Bruenner, B.A., Jones, A.D. and German, J.B. (1994) *Rapid Commun. Mass Spectrom.* 8, 509–512.
- [25] Canosa, D., Daniel, R., Barcelo, D., Gelpi, E., Legoffic, F. and Abian, J. (1997) *Int. J. Mass Spectrom. Ion Process.* 160, 395–407.
- [26] Thompson, M.A. and Schenter, G.K. (1995) *J. Phys. Chem.* 99, 6374–6386.
- [27] Woody, R.W. and Dunker, A.K. (1996) in: *Circular Dichroism and the Conformational Analysis of Biomolecules* (Fasman, G.D., Ed.), pp. 109–158, Plenum Press, New York.
- [28] Wang, Q., Allen, J.C. and Swaisgood, H.E. (1997) *J. Dairy Sci.* 80, 1047–1053.
- [29] Chatterjee, S. and Srivastava, T.S. (2000) *J. Porphyr. Phthalocyan.* 4, 147–157.
- [30] Kuki, M., Nagae, H., Cogdell, R.J., Shimada, K. and Koyama, Y. (1994) *Photochem. Photobiol.* 59, 116–124.
- [31] Ke, B., Imsgard, F., Kjøsen, H. and Liaaen-Jensen, S. (1970) *Biochim. Biophys. Acta* 210, 139–152.
- [32] Frank, H.A., Bautista, J.A., Josue, J., Pendon, Z., Hiller, R.G., Sharples, F.P., Gosztola, D. and Wasielewski, M.R. (2000) *J. Phys. Chem. B* 104, 4569–4577.
- [33] Heller, J. and Horwitz, J. (1973) *J. Biol. Chem.* 248, 6308–6324.
- [34] Charney, E., Ziffer, H. and Weiss, U. (1965) *Tetrahedron* 21, 3121–3126.
- [35] Jones, A.D., Tier, C.M. and Wilkins, J.P.G. (1998) *J. Chromatogr. A* 822, 147–154.