A new ligand for an old lipocalin: induced circular dichroism spectra reveal binding of bilirubin to bovine β-lactoglobulin

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Abstract **This study reports that bilirubin–bovine β-lactoglobulin (BLG) complexes exhibit very characteristic induced circular dichroism (CD) spectra in the visible absorption region. Due to intramolecular chiral exciton coupling between the dipyrrinone chromophores, the long-wavelength negative and short-wavelength positive CD bands clearly prove that a single bilirubin molecule binds to BLG in a left-handed helical conformation (in pH 7.4 phosphate buffer $\Delta\varepsilon_{min}$ is -54 M^{-1} cm^{-1} at 467 nm and $\Delta\varepsilon_{max}$ is $+48.5$ M^{-1} cm^{-1} at 412 nm). The very low aqueous solubility and strong tendency of bilirubin molecules to aggregate around pH 7.4 meant that much more intense CD bands were measured at alkaline pH values owing to the increasing solubility of the ligand. Vanishing CD activity obtained upon titration of the complex with palmitic acid known to bind in the hydrophobic cavity of BLG indicates bilirubin to be bound at the open end mouth of the β-barrel. Reversible changes of the induced CD spectrum due to acidic pH shift of the sample solution lead to the same conclusion.**

Key words: β-Lactoglobulin; Bilirubin; Exciton coupling; Induced chirality; Lipocalin; Molecular recognition

1. Introduction

In recent years, considerable attention has been focused on the lipocalin protein family, of which members can be found in bacteria [1], plant cells [2], insects [3], mammals [4] and human body [5]. Lipocalins are small globular proteins typically comprising 160–180 residues designed mostly to bind and transfer hydrophobic compounds with physiological importance. To fulfill this function, they have an eight-stranded β-barrel providing a large interior cavity suitable to bind ligand molecules, including retinoids, carotenoids, biliverdin, pheromones, prostaglandins, steroid hormones and more [6,7]. Aside from the conserved β-barrel, there are four flexible loops at the open end of the hydrophobic pocket, of which structural variabilities determine molecular recognition and ligand specificity. With the aid of techniques from combinatorial molecular biology, the structurally rigid lipocalin scaffold [8] has recently been utilized to produce a novel class of engineered proteins, the so-called anticalins, which can be used – similarly to antibodies – for the detection of substances or for the targeting of cellular surface markers [9,10]. High affinity and specificity of these proteins towards given molecular structures make them promising tools for the wide field of biomolecular/biotechnology researches. Thus, it is especially interesting to study the ligand-binding properties of natural lipocalins and the underlying molecular mechanisms.

Because of its abundance and ease of purification, bovine β-lactoglobulin (BLG) found in cow milk has been studied extensively for many years [11,12]. It is a small, 162 amino acid globular protein of the lipocalin family composed of 10–15% of α-helix structures and of ca. 50% of anti-parallel β-sheet, forming a calyx in which small hydrophobic molecules (i.e. fatty acids, vitamin A and its derivatives) have been shown to be bound [13–15]. Bovine milk contains two main genetic variants of BLG, named A and B. Variant B differs in the amino acid sequence at positions 64 (Asp→Gly in var. B) and 118 (Val→Ala in var. B). Around neutral pH BLG is mainly dimeric, whereas near pH 3 it dissociates into monomers. Furthermore, between pH 6.5 and 8.0 reversible conformational change occurs in the loop region, the so-called N→R or Tanford transition [16]. Depending on actual pH values the EF loop (residues 85–90) blocks or opens the entrance of the calyx; it is closed at pH 6.2 and opened at pH 7.1 and 8.2 [17]. This conformational movement profoundly alters ligand binding property of molecules into the hydrophobic pocket; lowering the pH value below 7, ligand–BLG complexes dissociate in a reversible manner [18,19].

If an achiral ligand molecule having a good chromophore (i.e. extended aromatic π-system) becomes optically active upon binding to a protein, then this interaction can sensitively be traced by the circular dichroism (CD) spectroscopy method [19,20]. Using this technique, this paper reports that BLG is able to bind bilirubin (BR), the powerful antioxidant [21] lipophilic tetrapyrrole molecule which is the final product of heme catabolism. This surprising result offers a good opportunity to improve our knowledge of the molecular recognition mechanism of lipocalins and opens new possibilities for studying non-covalent interactions between BLG and other tetrapyrrole compounds having biochemical significance.

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Abbreviations: BLG, bovine β-lactoglobulin; BR, bilirubin; CD, circular dichroism; CE, Cotton effect; DMSO, dimethyl sulfoxide; L/P, ligand/protein molar ratio; mdeg, millidegree; UV/Vis, ultraviolet–visible

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2. Materials and methods

2.1. Materials

Cow milk β-lactoglobulin (L3908, a mixture of A and B variants; L7880, variant A; L8005, variant B) and palmitic acid (P5585) was obtained from Sigma and used as supplied. Crystalline BR (Sigma, B4126, lot No. 117H12831; 88% BR IXα, 7% BR IIIα, 5% BR XIIIα) was used without further treatment. Doubly distilled water and spectroscopy-grade dimethyl sulfoxide (DMSO; Scharlau Chemie, Barcelona, Spain) were used. All other chemicals were of analytical grade.

2.2. Preparation of β-lactoglobulin solution

To obtain $\sim 10^{-4}$ M sample solution, BLG was dissolved in 0.05 M pH 7.4 phosphate buffer solution. Protein concentration was determined spectrophotometrically from the UV spectrum of BLG by using a molar absorption coefficient $\varepsilon_{278\ nm} = 17\,600\ M^{-1}\ cm^{-1}$ [22]. Molecular weight of a BLG monomer was taken to be 18 300.

2.3. Preparation of BR stock solution

Freshly prepared solutions were made up by dissolving the pigment in DMSO to give 2×10^{-3} M concentration. Stock solutions were protected from light throughout the experiments. Concentration of BR was determined by measuring its ultraviolet–visible (UV/Vis) spectrum in DMSO with $\varepsilon_{458\ nm} = 64\,000\ M^{-1}\ cm^{-1}$ [23].

2.4. Stock solution of palmitic acid

2.25 mg of palmitic acid was dissolved in 2.2 ml DMSO to obtain 4.0×10^{-3} M solution.

2.5. Circular dichroism and UV/Vis absorption spectroscopy measurements

CD and UV/Vis spectra were recorded between 330 and 550 nm on a Jasco J-715 spectropolarimeter at 20 ± 0.2°C in a rectangular cuvette with 1 cm optical pathlength. Temperature control was provided by a Peltier thermostat equipped with magnetic stirring. All spectra were accumulated two or three times with a bandwidth of 1.0 nm and a resolution of 0.2 nm at a scan speed of 100 nm/min. Induced CD is defined as the CD of the BR–BLG mixture minus the CD of BLG alone measured at the same wavelengths and expressed as ellipticity in millidegrees (mdeg).

2.6. Titrations of BLG A, BLG B and the mixture of A+B with BR in pH 7.4 phosphate buffer solution at 20°C

Three independent titration experiments were performed using the genetic variants of BLG A, BLG B and the mixture of A+B. 2 ml protein solution was placed in a rectangular cuvette with 1 cm optical pathlength and small amounts of BR stock solution were added with an automatic pipette in 2.5 μl aliquots ($c_{BLG\ A} = 9.1\times10^{-5}$ M, $c_{BLG\ B} = 8.1\times10^{-5}$ M, $c_{BLG\ A+B} = 9.0\times10^{-5}$ M). Initial and final concentrations of BR were 2.5×10^{-6} M and 4.2×10^{-5} M, respectively. DMSO added with the ligand never exceeded 3% (v/v).

2.7. Measuring the induced CD and UV/Vis spectra of the BR–BLG complex at alkaline pH values

25 μl BR stock solution was added to 2 ml of 8.9×10^{-5} M BLG solution (a mixture of A and B variants) prepared in 0.05 M pH 7.4 phosphate buffer solution ($c_{BR} = 2.1\times10^{-5}$ M). After recording the CD and absorption spectra, μl volumes from 2 M NaOH solution were pipetted into the cuvette consecutively to achieve pH values of 7.7, 8.0, 8.2, 8.7 and 9.6 (t = 20°C). Spectra were taken at each pH value.

2.8. Titration of the BR–BLG complex with palmitic acid

25 μl BR stock solution was added to 2 ml of 9.0×10^{-5} M BLG solution (a mixture of A and B variants) in a rectangular cuvette with 1 cm optical pathlength. After formation of the complex, palmitic acid stock solution in 2.5 μl aliquots was added consecutively to achieve complete extinction of the induced CD signals.

2.9. Investigation of the acidic pH shift on the induced CD spectrum of the BR–BLG complex

25 μl BR stock solution was added to 2 ml of 8.9×10^{-5} M BLG solution (a mixture of A and B variants) in a rectangular cuvette with 1 cm optical pathlength ($c_{BR} = 2.1\times10^{-5}$ M). After recording the CD/UV/Vis spectra, the pH of the solution was measured (7.51). Then the pH value was decreased to 6.49 by mixing 50 μl 1 M HCl into the sample solution and a spectral measurement was taken again. Finally, the pH value was set back to 7.66 with addition of 30 μl 2 M NaOH.

For pH measurements, a digital pH meter (OP-115; Radelkis, Budapest, Hungary) with a combined glass electrode (OP-0864 P; Radelkis) was used.

3. Results and discussion

Despite its polar functionalities, BR is a strongly hydrophobic molecule due to extensive intramolecular hydrogen bonds formed between the propionic acid and lactam moieties. In solution, BR exists as an equimolar mixture of two intramolecularly hydrogen-bonded non-superimposable conformational isomers of P (plus) and M (minus) helicity referred to as 'ridge tile' structures in which the dipyrrinone chromophores are approximately perpendicular to each other

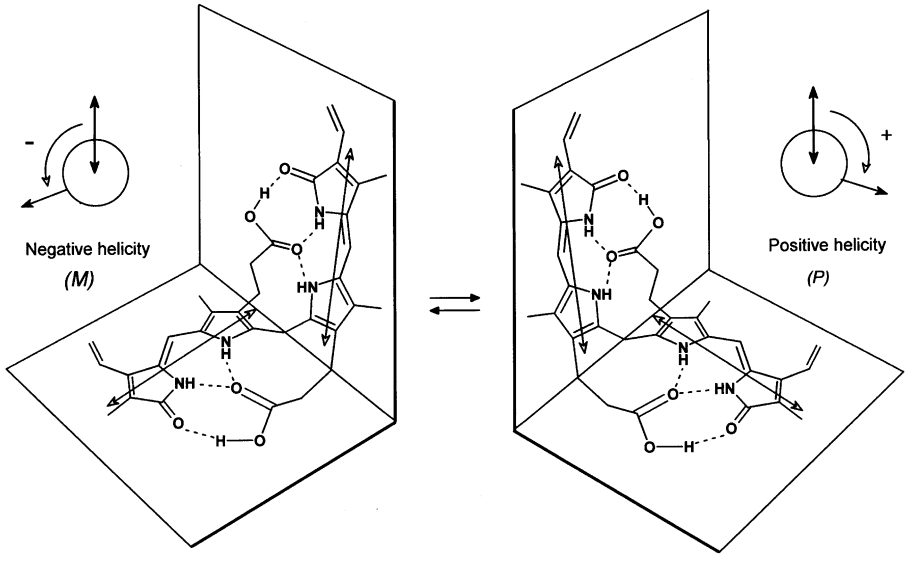

Fig. 1. Interconverting mirror image conformations of BR. Double-headed arrows show polarization of long-wavelength electronic dipole transition moments of dipyrrinone chromophores; dashed lines denote intramolecular hydrogen bonds. The relative orientation of dipole moments defines the helicity of the chiral conformers (M = minus or left-handed; P = plus or right-handed). The figure is taken from [25] by permission.

(Fig. 1) [24]. According to the mirror image relation of these interconverting conformers, BR shows no optical activity either in organic or aqueous solutions. However, if BR molecules interact with external chiral complexing agents (i.e. serum albumin, cyclodextrines, etc.) the equilibrium is shifted and, due to the excess of the P or M conformers, induced optical activity can be measured [24–26].

In the presence of BLG, which itself shows neither light absorption nor CD activity above 330 nm, BR exhibits definite, oppositely signed CD bands in the visible spectral region (Fig. 2). Long- and short-wavelength Cotton effects (CE) appear centered at 467 and 412 nm, respectively, with a zero cross-over point at 434 nm matched nearly with the maximum of the intense absorption band of BR. On the one hand, these induced CD spectra give proof of the binding of BR to BLG. On the other hand, the bisignate nature of CD curves suggests intramolecular chiral exciton coupling to occur between the electric dipole transition moments of dissymmetrically positioned dipyrrinone chromophores. According to the exciton chirality rule [24], the signed order of the induced CEs (a long-wavelength negative CE followed by a positive short-wavelength one) indicates BR molecules to bind to BLG in a left-handed chiral conformation (M-helicity, Fig. 1). It is important to note that such exciton CD bands might stem from intermolecular interactions between two neighboring, protein-bound ligand molecules (intermolecular exciton coupling).

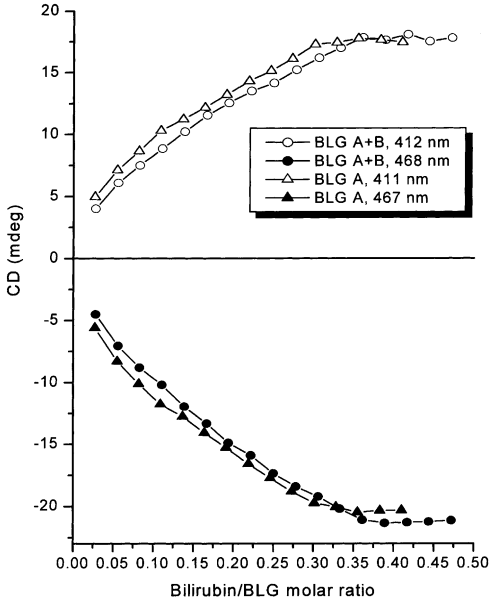

Fig. 3. CD titration curves of BLG A and the mixture of A and B variants with BR in pH 7.4 phosphate buffer solution at 20°C ($c_{BLG\,A} = 9.1\times10^{-5}$ M, $c_{BLG\,A+B} = 9.0\times10^{-5}$ M; cell length 1 cm). Ellipticity values measured at the maxima/minima of CEs are plotted versus the L/Ps. Concentrations of BR were varied between 2.5×10^{-6} and 3.7×10^{-5} M, and between 2.5×10^{-6} and 4.2×10^{-5} M, respectively.

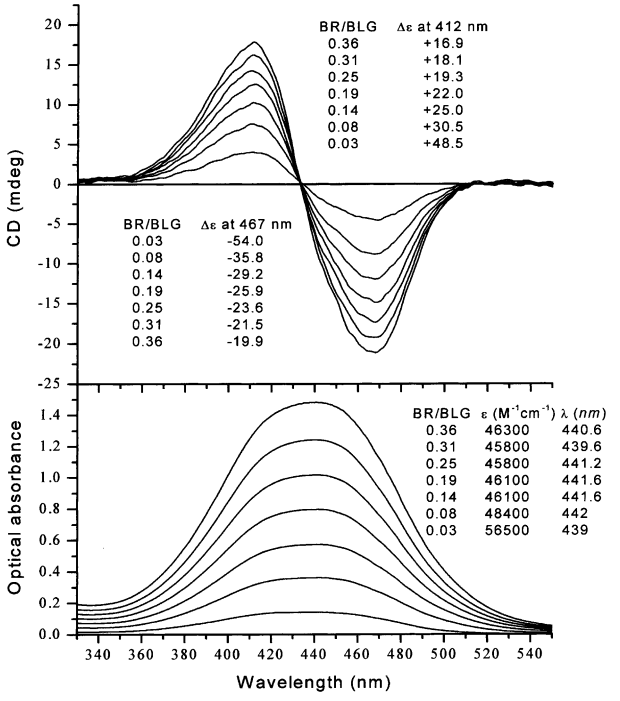

Fig. 2. Induced CD and UV/Vis spectra obtained by titration of BLG (a mixture of A and B variants) with BR in pH 7.4 phosphate buffer (for clarity, only selected curves are shown). Ligand was added as aliquots of DMSO stock solution (cell length 1 cm; $t = 20$°C). During the titration, concentration of BR was changed from 2.5×10^{-6} M to 4.2×10^{-5} M, concentration of BLG was changed from 9.0×10^{-5} M to 8.8×10^{-5} M. Insets: molar circular dichroic absorption coefficients ($\Delta\varepsilon$, in M^{-1} cm^{-1}) and molar absorption coefficients (ε, in M^{-1} cm^{-1}) of the induced CD and absorption bands calculated on the basis of total BR concentration in solutions.

This possibility, however, can be ruled out by the facts that induced CEs appear far below a ligand/protein molar ratio (L/P) of 1 and their shapes and spectral positions show no changes upon further addition of BR. Thus, it can be concluded that single, BLG-bound BR molecules are responsible for the observed induced CD spectrum.

It is worthwhile to note that the interaction between BR and BLG has already been investigated by the fluorescence spectroscopy method and solid-phase binding assay [27]. Since neither significant quenching of the intrinsic fluorescence of BLG nor binding in the solid-phase assay were observed, the authors concluded that BLG does not bind BR. It is not clear why these methods showed no interaction between BR and BLG.

In the course of the titration of BLG with BR the intensities of the induced CD bands increase with the added amount of the ligand, but above 0.33 BR/BLG molar ratio ellipticity values become constant (−21 mdeg at 467 nm and +18 mdeg at 412 nm), showing no changes despite further addition of BR (Fig. 3). Practically the same results were obtained using the A and B genetic variants of BLG alone, suggesting that there are no significant differences in the BR binding properties of the two isoforms (Fig. 3; for clarity, data points of the BLG B–BR complex are omitted). To understand that saturation of the CD titration curves already occurs at L/P = 0.30–0.35 (Fig. 3) an additional experiment was performed. At constant BR and BLG concentrations (L/P = 0.22) the pH value of the solution was gradually raised from 7.4 to 9.6 by addition of μl volumes of concentrated NaOH. Surprisingly, the intensities of the induced CD bands were greatly enhanced during this process (Fig. 4); there was a four-fold increase in the magnitudes of CE values, reaching $\Delta\varepsilon$ values of −86.5 (467 nm) and +77.1 (411 nm), while band

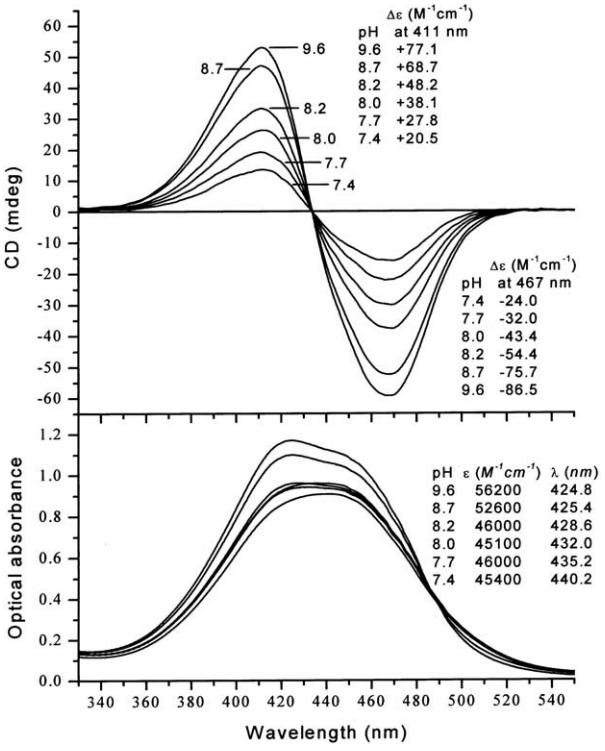

Fig. 4. Changes of the CD and UV/Vis spectra of the BR–BLG complex formed in pH 7.4 phosphate buffer upon addition of μl volumes of 2 M NaOH (BR/BLG = 0.23; $c_{BLG} = 8.9\times10^{-5}$ M, $c_{BR} = 2.1\times10^{-5}$ M; cell length 1 cm; $t = 20$ °C). Actual pH values, ε_{max} and $\Delta\varepsilon_{max/min}$ of the absorption and CD bands are indicated (BLG is the mixture of A and B variants).

shapes and wavelength positions remained the same (from a pH value of 10.2, the CD intensities begin to decrease due to the alkaline denaturation of BLG). Additionally, the magnitude of the visible absorption band of BR also increased at higher pH values. To explain these results it must be taken into account that BR has very limited aqueous solubility, especially below pH 8 [28–31]. For example, its solubility was reported to be about 10^{-7} M in 0.1 M pH 7.4 phosphate buffer [29]. At concentrations exceeding this value, BR shows a strong tendency to self-associate, forming a colloidal solution, although the sample seems to be clear to the naked eye. With higher pH values, however, the solubility increases and more and more monomeric BR molecules enter into solution [30,31]. Thus, at the concentrations used in the titration experiment (from 2.5×10^{-6} to 4.2×10^{-5} M at pH 7.4) BR molecules exist in three different states: molecules are bound to BLG, unliganded molecules are truly in solution and a certain fraction are in aggregated form. Titration data obtained at pH 7.4 suggest that with increasing concentrations of BR, equilibria between these states are shifted towards formation of aggregates, resulting in constant induced CD values (Fig. 3). Consequently, alkalization of the solution changes this process oppositely, yielding higher and higher ratios of monomeric molecules available for binding to BLG (Fig. 4). Simultaneously, light absorption of BR increases as well since the large aggregates, in which probability of photon absorption of individual molecules is reduced, disintegrate [32].

The pronounced optical activity of the BR–BLG complex can be utilized to ascertain the protein binding site of BR. A titration experiment was performed adding increasing amounts of palmitic acid to the BR–BLG complex prepared in phosphate buffer solution (pH 7.4, L/P = 0.28). Fatty acids, the well-known endogenous ligands of BLG, were found by X-ray crystallographic studies to occupy the central calyx [13,14]. Upon addition of palmitic acid stock solution, the magnitudes of the induced CD bands began to decrease and they completely vanished above the value of 2.5 of fatty acid/BR molar ratio (Fig. 5). According to this result, palmitic acid molecules compete with BR for a common binding site of BLG, namely for the central hydrophobic cavity.

The behavior of the induced CD spectrum upon an acidic

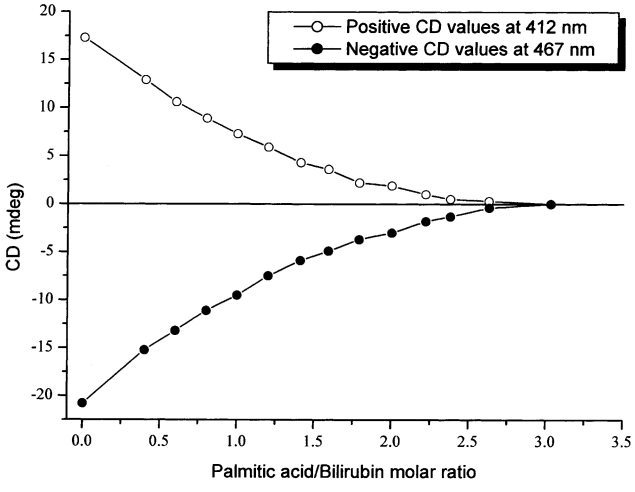

Fig. 5. Titration of the BR–BLG complex with palmitic acid in pH 7.4 phosphate buffer solution (L/P = 0.28; $t = 20$°C; cell length 1 cm). During the titration, concentration of palmitic acid was changed from 9.8×10^{-6} to 7.3×10^{-5} M. Due to the addition of palmitic acid solution c_{BLG} was varied between 8.9×10^{-5} and 8.7×10^{-5} M and c_{BR} was varied between 2.5×10^{-5} and 2.4×10^{-5} M (BLG is the mixture of A and B variants).

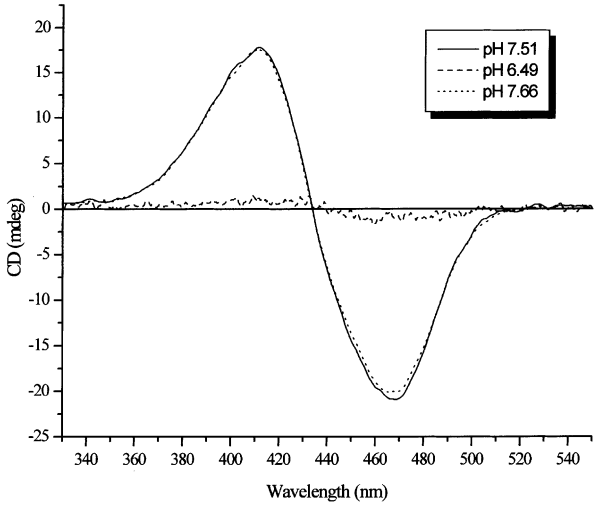

Fig. 6. pH dependence of the induced CD spectrum of the BR–BLG complex formed in pH 7.4 phosphate buffer solution (L/P = 0.24; c_{BLG} 8.9×10^{-5} M, c_{BR} 2.1×10^{-5} M; $t = 20$°C). Dotted line represents the spectrum of the reconstituted complex (pH 7.66) obtained by adding 30 μl 2 M NaOH to the slightly acidic (pH 6.49) solution (BLG is the mixture of A and B variants).

pH shift leads to the same conclusion. Changing the pH value of the buffer solution from 7.51 to 6.49, the amplitudes of the induced CEs drop instantly down to zero. However, this process is completely reversible since the induced CD bands appear again by setting the pH value back to 7.66 (Fig. 6). These striking changes can be explained by the Tanford transition of BLG [17]. In pH 7.51 solution, the EF loop located at the entrance of the interior cavity is in an open conformation, allowing dynamic binding of BR molecules in the hydrophobic pocket. Upon lowering of the pH, the EF loop flips back, blocking the access to the calyx and, due to unfavorable steric interactions arising between BR molecule and protein side chains, the complex dissociates. Since the EF loop movement is entirely reversible, BR–BLG complexes and the corresponding CD spectra can promptly be reconstituted by a small volume of concentrated NaOH (Fig. 6).

4. Conclusions and perspectives

Performed in pH 7.4 phosphate buffer solution at 20°C, CD spectroscopy measurements demonstrated the binding of BR to BLG. The protein acts as a chiral, water-soluble complexation agent that binds this highly lipophilic pigment. As a result of binding, oppositely signed induced CD CEs appear which fall in the visible absorption region of the ligand. The signed order of negative–positive CD bands derived from intramolecular exciton coupling between dipyrrinone chromophores of bound BR indicates selection of the BLG binding site for a negative chirality (left-handed) conformer.

Binding in the central cavity, palmitic acid displaces BR molecules. Additionally, conformational change of BLG during Tanford transition leads to the dissociation of the pigment-protein complex. Due to its molecular dimensions, BR is probably not able to penetrate deeply into the interior cavity; therefore, the flexible loop region of BLG might play an important role not only in the recognition of BR but also in its stabilization via non-covalent chemical interactions.

The main perspectives offered by these results are as follows:

- The results that prove the ability of BLG to bind structurally unrelated molecules, such as e.g. retinoic acid and BR, serve as a good starting point to study what kind of structural elements are responsible for plasticity and promiscuity of the ligand-binding mechanism of BLG.
- Comparing the structure of BLG with that of other lipocalins able to form complexes with tetrapyrrole molecules [27,33] gives better insight into the problem as to how distinct amino acid sequences form sites which bind the same ligand molecules.
- It can be expected that BLG binds not only BR but also other tetrapyrroles and related compounds. Systematic investigations of such complexes would help to extend our knowledge both of the molecular recognition mechanism of lipocalins and of the stereochemical features of these important pigment molecules.
- Because of its stability, abundance and ease of preparation, BLG complexed with BR seems to be a promising subject for X-ray crystallographic investigations [13,14].
- The dairy industry produces vast amounts of BLG (its concentration in cow milk is 5–6 g/l). Applying protein engineering methods, the molecular recognition mechanism of BLG could be modified to obtain small proteins uniquely suited for detection, transport or separation of certain tetrapyrroles in biological samples.
- In severe cases of neonatal hyperbilirubinemia, when very high levels of BR develop, exchange transfusion may be needed, which has many disadvantages (the baby's blood is replaced with other, compatible blood). To reduce the serum level of BR, patients' blood should pass through e.g. a column containing immobilized BLG molecules which would bind the free, unconjugated BR. For repeated use, the column would be regenerated by slightly acidic buffer solution.

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