Monatshefte für Chemie 120, 163 – 168 (1989)

Monatshefte für Chemie
Chemical Monthly

UV-VIS and CD-Spectroscopic Investigations of Intermolecular Interactions of Bile Pigments with Small Proteins

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Summary. The intermolecular complex formation of bilirubin and biliverdin with two proteins (basic pancreatic trypsin inhibitor and lysozyme) were studied by optical and chiroptical methods. Evidence for specific intermolecular interactions of biliverdin with both proteins was found. Bilirubin forms a soluble complex only with lysozyme.

Keywords. Bilirubin; Biliverdin; Protein complex; Circular dichroism.

UV-VIS und CD-spektroskopische Untersuchungen intermolekularer Wechselwirkungen von Gallenpigmenten mit kleinen Proteinen

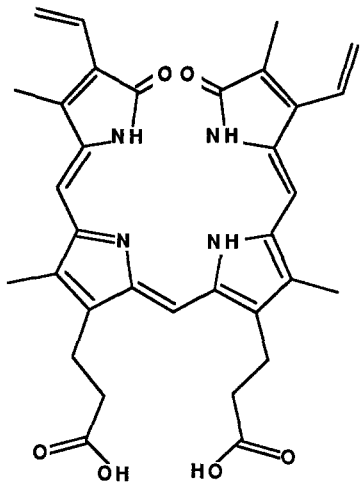
Zusammenfassung. Die intermolekulare Komplexbildung von Bilirubin und Biliverdin mit zwei Proteinen (basischer pankreatischer Trypsininhibitor und Lysozym) wurden mittels optischer und chiroptischer Spektroskopie untersucht. Für Biliverdin konnten intermolekulare Wechselwirkungen mit beiden Proteinen nachgewiesen werden. Bilirubin bildet nur mit Lysozym einen löslichen Komplex.

Introduction

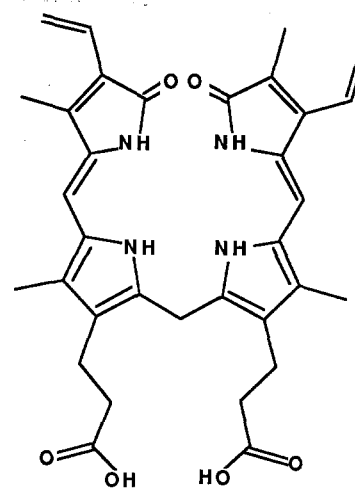
Bile pigments in their native states are frequently in close interaction with proteins [1–3]. In the animal kingdom, for example, the transport proteins of serum albumin type [1] or the bile pigment binding protein in *Pieris brassicae* [2] form non-covalent complexes. In plants, bile pigments are covalently attached to the protein in the phycobiliproteins and in phytochrome [3]. The conformations and consequently the spectroscopic and chiroptical properties of linear tetrapyrroles have been shown by experimental and theoretical methods [4–6] to depend strongly on intermolecular interactions.

So far very little is known, however, on the fundamental mechanisms of interaction and their structural consequences for the bile pigment chromophore. In the present communication we report spectroscopic data on complexes of the two naturally occuring pigments bilirubin (BR) and biliverdin (BV) with two well characterized small proteins.

The proteins used, i.e. the porcine basic pancreatic trypsin inhibitor (BPTI) and hen egg white lysozyme, were chosen for several reasons: both proteins contain lysine amino acid residues on their surfaces, which are potential sites for the for-



BV



BR

mation of hydrogen bonds or salt bridges to the free carboxylic acid residues present in the bile pigments. They are among the most thoroughly investigated small proteins, and they are commercially available in high purity. These two proteins are also readily amenable to NMR analysis because of their sizes and solubilities (their NMR spectra have been nearly completely assigned in the past [7, 8]); therefore, future structural investigations employing NMR methods may be facilitated.

Materials and Methods

Bilirubin and lysozyme were obtained from "Sigma". Biliverdin was prepared by oxidation of BR with 2,3-dichloro-5,6-dicyanobenzoquinone [9]. Solid BPTI was obtained by lyophilisation of solutions of Trasylsol® (Bayer). Xanthobilirubinic acid was prepared according to Ref. [10].

The BV complexes were prepared from $4.5 \cdot 10^{-5}$ *M* solutions of BV in 0.01 *M* sodium phosphate buffer (*pH* 7.45) to which the appropriate amounts of solid protein was added. Precipitates (in case of the lysozyme complexes) were removed by centrifugation. The *pH* was adjusted using 0.1 *M* solutions of sodium hydroxide and NaH_2PO_4. The BR complexes were prepared similarily from solutions of BR in 0.1 *M* NaOH after bubbling with argon gas for 10 min to prevent oxidation.

UV-vis spectra were recorded on a Perkin-Elmer 330 spectrophotometer, CD-spectra on an Auto-Dichrograph Mark V (Jobin-Yvon).

Stoichiometric compositions were determined by the method of Job [11]. Formation constants were determined by an iterative fit procedure to the formation curves obtained from UV-vis difference spectra [12].

Results and Discussion

Biliverdin forms a moderately soluble 1 : 1 complex with BPTI, which is stable in a *pH* range of 2.5 to 8.5. Both the UV-vis and CD-spectra shown in Fig. 1 clearly indicate specific interaction. As the most intensive Cotton effects are observed around a neutral *pH*, the formation of salt bridges is evident.

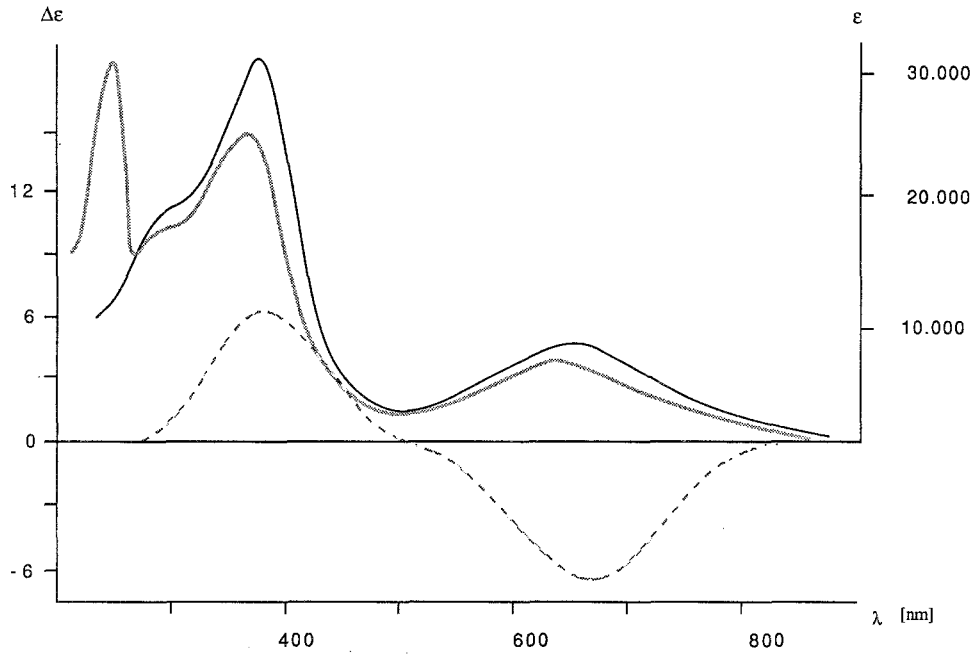


Fig. 1. UV-vis spectrum of BV (−) and UV-vis (gray) and CD (− − −) spectra of its complex with BPTI in 0.01 *M* aqueous phosphate buffer at *pH* 7.45, 25 °C

It was impossible to prepare a complex of bilirubin and BPTI, since self-aggregation of BR [13] dominates in this case. Lysozyme forms complexes both with BR and BV; however, the situation is complicated by the fact that besides the soluble 1 : 1 complex also insoluble complexes with a higher bile pigment content are formed. Although no significant change in the UV-vis spectra is induced through interaction with lysozyme, the CD-spectra of the soluble complexes show clear evidence for chiral discrimination between the interconvertible enantiomeric forms of the tetrapyrrolic compounds (Figs. 2 and 3). Below *pH* 3.8, however, the insoluble bile pigment complex dissolves. The solutions obtained thereby cannot be distinguished spectroscopically from those formed if solutions of the soluble complex are acidified. No CD is observed below *pH* 3.8. Nevertheless, the high solubility of the bile pigments in low *pH* lysozyme solutions is a strong indication of an interaction, which due to lack of spectroscopic effects could not be tracked down so far.

All bile pigment protein reactions investigated appear to be reversible and the insoluble lysozyme complexes with high bile pigment content may be dissolved in excess of protein solution. Although all complexes studied exhibit solubilities below 10^{-3} *M* and form insoluble aggregates at higher concentrations, they can be readily lyophylized and redissolved. It is also noteworthy that, although the solubilities of all complexes investigated decrease drastically below *pH* 7.0 (and increase again below *pH* 3.8 in the case of lysozyme), the tetrapyrrole chromophore concentrations obtainable in the presence of these proteins exceed the chromophore concentrations in saturated aqueous solutions of bile pigments at the same *pH* by more than an order of magnitude. No investigations were conducted above *pH* 8.5 since BV undergoes nucleophilic attack and also the proteins become less stable.

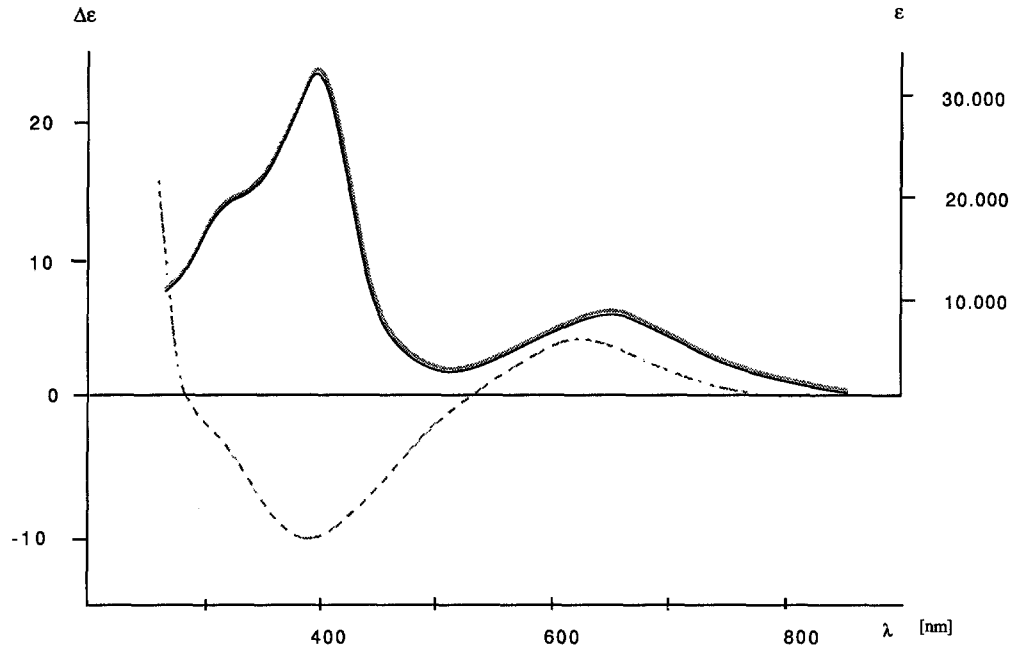


Fig. 2. UV-vis spectrum of BV (−) and UV-vis (gray) and CD (− − −) spectra of its complex with HEW lysozyme in 0.01 *M* aqueous phosphate buffer at *pH* 7.45, 25 °C

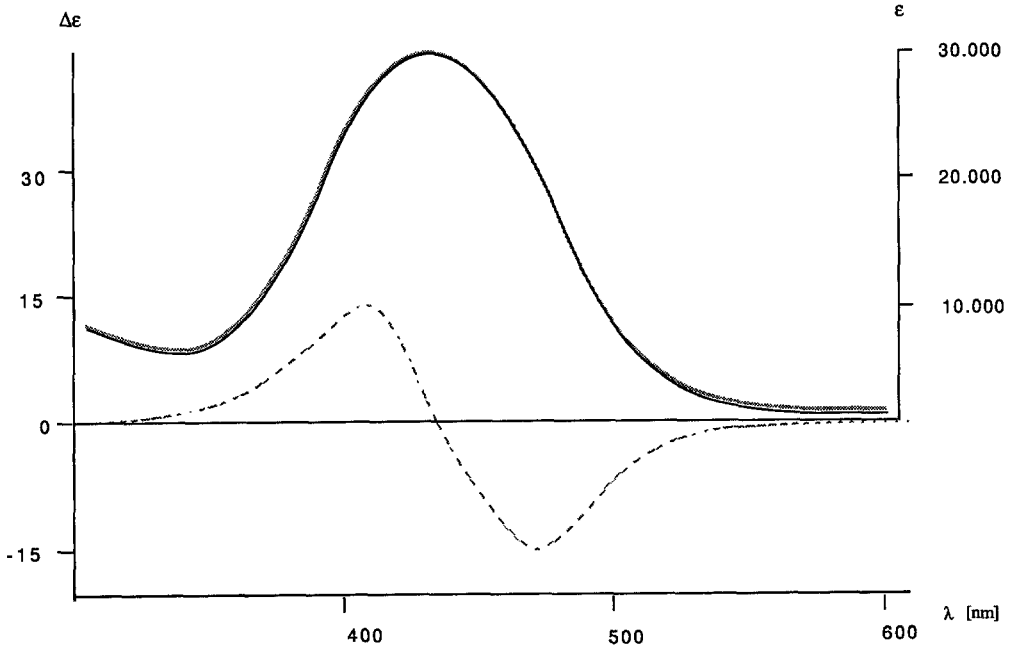


Fig. 3. UV-vis spectrum of BR (−) and UV-vis (gray) and CD (− − −) spectra of its complex with HEW lysozyme in 0.01 *M* aqueous phosphate buffer at *pH* 7.45, 25 °C

The formation constants are larger than 10^4. For the BV-BPTI complex an equilibrium constant of $2.5 \cdot 10^4 \, l \, mol^{-1}$ was determined at *pH* 7.45 from the UV-vis difference spectra obtained in a titration experiment (Fig. 4). Only approximate formation constants (10^4–10^6) could be deduced for the lysozyme complexes (10^4–10^6) as the precipitating bile pigment rich complexes interfere with the spectroscopic measurements. All complexes are kinetically labile – they decompose into their constituents upon sephadex chromatography.

Several structural implications can be derived from the spectroscopic results. As the protein CD-spectra below 300 nm remain unchanged upon complex formation, no major conformational change in the protein occurs. The long wavelength absorption band of BV is shifted from 670 to 655 nm. This indicates a change in the helix conformation towards a larger overall torsion of the three exocyclic formal single bonds. The overall geometry, however, remains circular-helical as can be deduced from the practically unchanged ratio of the extinction coefficients at the long and short wavelength main absorption bands [6]. No significant shifts of the absorption maxima occur in the complexes with lysozyme. Therefore a change in the geometry of the chromophores seems highly improbable. Applying the C_2-rule [2, 16, 17] to the CD spectra the dominating helicity of the biliverdin chromophore can be deduced to be (*M*) in its complex with BPTI and (*P*) in the lysozyme complex.

The low values of $\Delta\varepsilon$ (5 to 10) as compared to those derived and found for pure helix enantiomers in the case of verdins (80 to 120) [2, 5, 17, 18], however, indicate that the chiral discrimination is weak – only approximately 10% enantiomeric excess of the helix antipodes is found. Similar $\Delta\varepsilon$ values have been found for interactions with chiral solvents [5].

The CD spectrum of the BR lysozyme complex is in accordance with the one found by Blauer [19]. However, the C_2-rule should not be applied in a straightforward manner to the conformation of the bilirubin chromophore in the lysozyme complex, as the spectrum represents a typical bisignate exciton couplet. Therefore Lightner and coworkers have analysed the exciton coupling in chiral BR complexes with cyclodextrins [14] and alkaloids [15]. By comparison of the CD spectrum

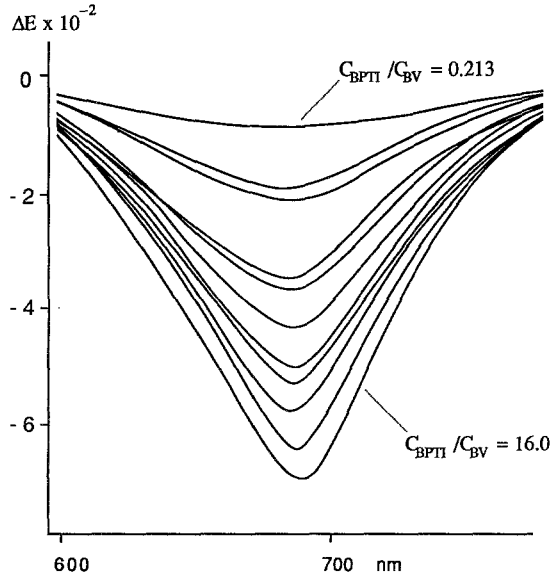


Fig. 4. UV-vis difference spectra for the titration of BV with BPTI, BV-concentration: $4.5 \cdot 10^{-5} M$

shown in Fig. 3 with the spectra and computational results in Ref. [14], the preferred chirality of the bilirubin chromophore in the lysozyme complex may be assigned as (*M*). The possibility that the bisignate CD band is not due to exciton splitting can be ruled out by the fact that no Cotton effect is observed for the complex between lysozyme and the dipyrrolic compound xanthobilirubinic acid. The magnitude of the CD of the BR-lysozyme complex exceeds the values observed for the complexes with cyclodextrins [14] but remains below the Cotton effect found for serum albumin bound bilirubin [20] and the quinine complex [15]. From the theoretical values ($\Delta\varepsilon_{max}^{450\,nm} = -260$, $\Delta\varepsilon_{max}^{395\,nm} = +190$) for a pure BR enantiomer [15] the enantiomeric excess of BR in the lysozyme complex can be calculated to be 12%.

So far, attempts to investigate the structure of these complexes by NMR methods were unsuccessful as this approach is severely hampered by their low solubilities and the instability of concentrated solutions.

Acknowledgements

Trasylol® was a gift from Bayer Austria, Ges.m.b.H. This work has been supported by the "Fonds zur Förderung der wissenschaftlichen Forschung" (Project no. 6213).

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Received June 29, 1988. Accepted August 27, 1988