Protonated 2,3-Dihydrobilindiones—Models for the Chromophores of Phycocyanin and the Red-Absorbing Form of Phytochrome

Michael Stanek and Karl Grubmayr*

Abstract: New structural and thermodynamic data on the protonation of 2,3-dihydrobilindiones are presented with respect to the ionic aspects of the chromophore–protein interactions in biliproteins. When intermolecular protonation with stoichiometric quantities of strong (sulfonic) acids was investigated by NMR spectroscopy, it was found that the positive charge is localized at the nitrogen atom of the azafulvenic ring B moiety. Complete protonation by weaker (carboxylic) acids can be achieved only intramolecularly at low temperature. The (2*R*,3*R*,3′*R*,Cys*R*)-cysteine adduct of phycocyanobilin dimethyl ester was synthesized to mimic the acid–base chemistry between protein and chromophore. Thermodynamic data for the equilibrium between its neutral form **1** and zwitterion $\mathbf{1}^{\pm}$ were calculated from the temperature dependence of the visible spectra ($\Delta H^{\circ} = -20.8\ \mathrm{kJ\,mol^{-1}}$, $\Delta S^{\circ} = -71\ \mathrm{J\,mol^{-1}\,K^{-1}}$). These data are in accord with others from intramolecular proton transfers, explaining the perfect order of highly conserved consensus sequences necessary for the effective protonation of the chromophores in various light-harvesting biliproteins such as phycocyanin. On the other hand, the highly negative value of ΔS° indicates the possibility of the reprotonation of the protein in case of steric interference. The geometrical changes of the protonated Pr chromophore of phytochrome as a result of the $Z \rightarrow E$ photoisomerization may trigger proton transfer back to the protein and thus initiate the sequence of dark reactions that lead to the physiologically active Pfr form of phytochrome.

Keywords: chromophores · phycocyanin · phytochrome · proteins · zwitterionic states

Introduction

The photoactivity of the phycocyanins[1] and phytochromes[2] depends on the unique structure of the 2,3-dihydrobilindione chromophore,[3] which enables perfect tuning of their absorption spectra by distinct chromophore–protein interactions. Among these the covalent linkage of the chromophores to the apoproteins by thioethers and the formation of ion pairs between the chromophores in the protonated state and the corresponding carboxylates of the proteins are thought to be essential for photophysical and photochemical functionality: for instance, excitation energy transfer in the light-harvesting complexes of cyanobacteria or red algae,[4] and the regulation of plant gene expression by light and phytochrome.[5]

From crystal structures of phycocyanin,[6] as well as other phycobiliproteins,[7] such as allophycocyanin,[8] phycoerythrocyanin,[9] and phycoerythrin,[10] a common principle for the protonation of their chromophores becomes evident: protonation always takes place at the dipyrrin moiety of rings B and C and is caused exclusively by aspartic acid residues. In the form of the various phytochromes that absorbs red light, commonly named the Pr form, the chromophores are also protonated by acidic residues of the protein. Protonation is indicated best by significant bathochromic shifts of the long-wavelength absorption maxima of phytochromobilin and phycocyanobilin during self-assembly with recombinant apophytochromes.[11]

In spite of all that is known about the holoproteins, structural details of their protonated chromophores remain uncertain. With respect to the distribution of the positive charge three distinct structures can be taken into consideration (**I**–**III**, Figure 1b). In structure **I** the symmetry of the protonated dipyrrin moiety[12] principally remains resulting in charge delocalization as found in protonated bilindiones of symmetric substitution patterns,[13] whereas in structures **II** and **III** the different oxidation state of the two lactam substituents, the 2,3-dihydropyrrolinone of ring A and the pyrrolinone of ring D, may cause a localization of the positive charge on either the nitrogen of ring B or that of ring C. In addition to this the question arises as to whether protonation by aspartic acid residues can be achieved quantitatively. At first glance the formation of stable ion pairs seems to be unlikely comparing the acidities of protonated 2,3-dihydrobilindiones ($pK_a = 4.6$)[14] and aliphatic carboxylic acids, for instance, acetic acid ($pK_a = 4.8$).

[*] a. Univ.-Prof. Dr. K. Grubmayr, Dipl.-Ing. M. Stanek
Institut für Chemie, Johannes Kepler Universität Linz
Altenbergerstrasse 69, A-4040 Linz (Austria)
Fax: (+43) 732-2468-747
E-mail: karl.grubmayr@jk.uni-linz.ac.at

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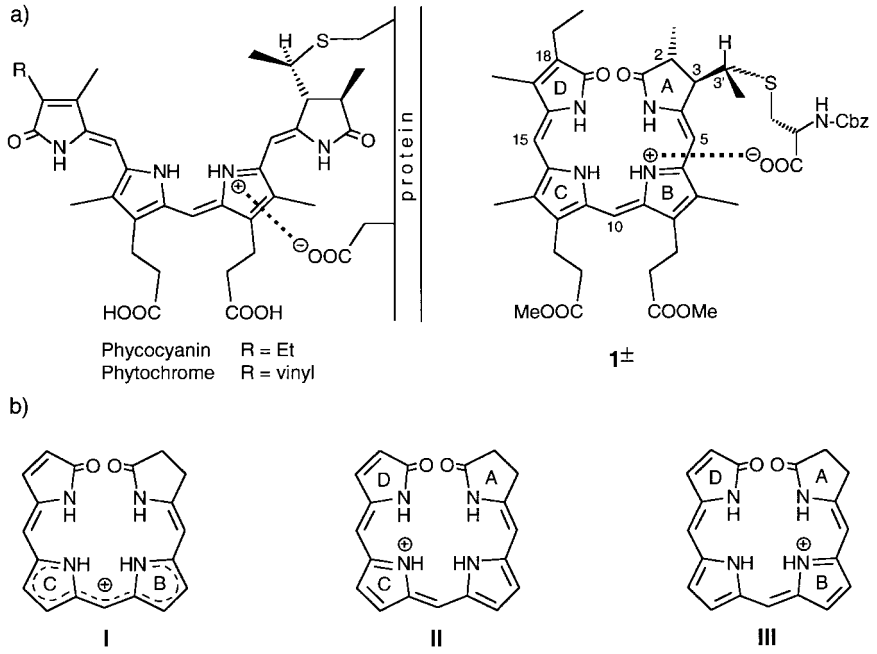

Figure 1. a) Comparison of the extended (5*anti*,10*syn*,14*anti*) conformation of the protein-bound chromophores of phycocyanin and the Pr form and the helical (5*syn*,10*syn*,14*syn*)-conformation of model compound **1** in its zwitterionic form **1**±. b) Hypothetical chromophore structures of protonated 2,3-dihydrobilindiones.

Here, we report on the structure and the spectral properties of the synthetic model compound **1**, which is in equilibrium with its zwitterionic species **1**±, representing the adduct of cysteine and phycocyanobilin in its (2*R*,3*R*,3′*R*,Cys*R*)-configuration. With respect to the extended chromophores of phycocyanin and the Pr form (Figure 1a) the main difference is the helical conformation of **1** and **1**±, respectively, adopted by bilindiones in solution as the most stable one. Nevertheless, **1** is able to mimic the acid–base chemistry between protein and chromophore. The protonation of the dipyrrin moiety by the cysteinic carboxyl group proceeds intramolecularly. Stereochemical influences can be quantified in comparison with the (2*S*,3*S*,3′*S*,Cys*R*)-diastereomer **2** and **2**±, respectively. Investigations are based on the results of the intermolecular protonation of the 2,3-dihydrobilindione models **3** and **4** (Figure 2). They will be presented first.

Abstract in German: *Neue strukturelle und thermodynamische Daten zur Protonierung von 2,3-Dihydrobilindionen werden im Hinblick auf die ionischen Anteile der Chromophor–Protein Wechselwirkung in Biliproteinen vorgestellt. Die intermolekulare Protonierung wurde mit stöchiometrischen Mengen starker Säuren (Sulfonsäuren) NMR-spektroskopisch untersucht. Die positive Ladung wird am Stickstoffatom der Azafulveneinheit von Ring B lokalisiert. Die vollständige Protonierung mit schwachen Säuren (Carbonsäuren) erfolgt nur intramolekular bei tiefen Temperaturen. Das (2R,3R,3′R,CysR)-Cystein-Addukt des Phycocyanobilindimethylesters wurde synthetisiert um die Säure–Base-Chemie zwischen Protein und Chromophor nachzuahmen. Die thermodynamischen Daten des Gleichgewichts zwischen der Neutralform **1** und dem zugehörigen Zwitterion **1**± wurden aus der Temperaturabhängigkeit der UV/Vis-Spektren ermittelt ($\Delta H^\circ = -20.8$ kJ mol^{-1}, $\Delta S^\circ = -71$ J mol^{-1} K^{-1}). Die Übereinstimmung mit den Daten anderer intramolekularer Protonentransfer-Reaktionen erklärt die Notwendigkeit für einen hohen Ordnungszustand aus streng konservierten Konsensussequenzen für die Protonierung der Chromophore in Biliproteinen mit Lichtsammelfunktion, beispielsweise dem Phycocyanin. Andererseits deutet der stark negative ΔS°-Wert auf die Möglichkeit einer Reprotonierung des Proteins nach sterischer Änderung hin. Demnach vermag die Geometrieänderung des protonierten Chromophors in der Pr-Form des Phytochroms nach erfolgter Z→E-Photoisomerisierung den Protonentransfer zum Protein hin auszulösen und damit die Sequenz jener Dunkelreaktionen einzuleiten, die zur physiologisch aktiven Pfr-Form des Phytochroms führen.*

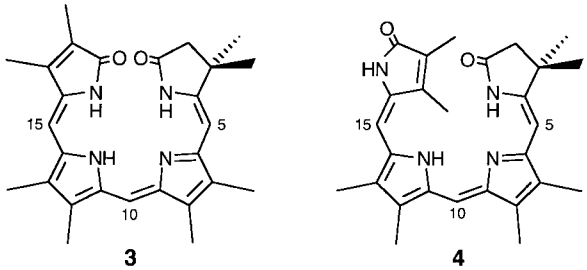

Figure 2. Structural formula of the diastereomeric model compounds **3** and **4** in the (4*Z*,9*Z*,15*Z*,5*syn*,10*syn*,14*syn*)- and (4*Z*,9*Z*,15*E*,5*syn*,10*syn*,14-*syn*) geometry.

Results and Discussion

Intermolecular protonation: The results on the structure of protonated 2,3-dihydrobilindiones are based predominantly on NMR spectroscopic investigations. At first, model compounds **3** and **4** were selected on account of their clear NMR spectra: their ^{1}H NMR spectra only consist of singlets, and because of the peripheral methyl substituents the assignment of all quaternary carbons by gradient-enhanced heteronuclear multiple bond correlation (HMBC) is unambiguous even for the protonated chromophores. Compounds **3** and **4** are *Z*/*E* diastereomers with respect to the exocyclic double bond of ring D representing those species, which are believed to be important for the photochemistry of phytochrome.

Because of its high acid strength *p*-toluenesulfonic acid (*p*-TsOH) was used for protonation forming the stable hydrotosylates **3** · H$^+$OTs$^-$ and **4** · H$^+$OTs$^-$. Compared with the salts of weaker acids, for instance hydrodichloroacetates, hydrotosylates are advantageous to NMR experiments by slowing down intermolecular proton exchange rates and thus prevent-

0947-6539/98/0409-1654 $ 17.50+.50/0 *Chem. Eur. J.* **1998**, *4*, No. 9

ing the formation of averaged signals. This is shown in Figure 3 for mixtures of **3** and its hydrodichloroacetate as well as its hydrotosylate within the range of the methine proton signals: addition of dichloroacetic acid to solutions of **3** resulted in the formation of shifted averaged signals, whereas the use of *p*-TsOH enables detection of the signals of $\mathbf{3}\cdot H^+$ as well as those of **3**.

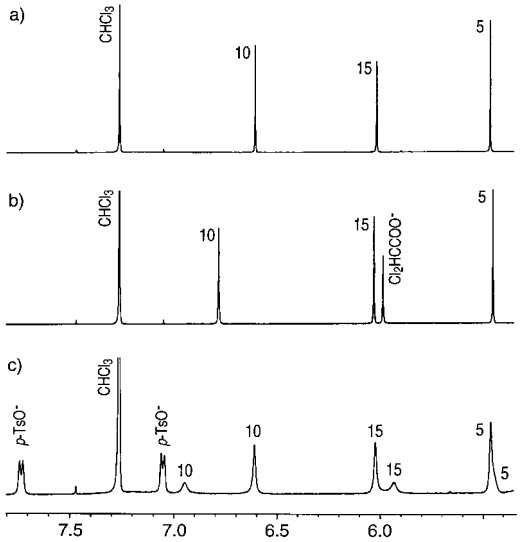

Figure 3. ^{1}H NMR spectra of the methine proton region of **3** in $CDCl_3$ at 25 °C. a) **3** (6.4×10^{-3} M), not protonated. b) **3** (6.4×10^{-3} M) on addition of 0.6 equiv dichloroacetic acid showing averaged signals of **3** and its hydrodichloroacetate. c) **3** (2.6×10^{-3} M) on addition of 0.4 equiv *p*-TsOH showing separated signals of **3** and its hydrotosylate.

NH signals cannot be seen in the ^{1}H NMR spectra of the hydrodichloroacetates, but under similar conditions the hydrotosylates show four NH singlets slightly broadened within the range $\delta = 9-12$. On protonation only the signal of the methine proton in position 10 is shifted significantly downfield ($\Delta\delta$ (ppm) = 0.416 [$\mathbf{3}\cdot H^+ - \mathbf{3}$]; 0.686 [$\mathbf{4}\cdot H^+ - \mathbf{4}$]) indicating protonation at the dipyrrin substructure. The $\Delta\delta$ values of all other ^{1}H NMR signals are less than 0.25 ppm and cannot be used for a detailed structure determination. However, a comparison of the $\Delta\delta$ values of all ^{13}C NMR signals belonging to the quaternary carbons of the chromophore is helpful (Figure 4, Table 1). In particular, the upfield shifts of the C-6 signal ($\Delta\delta$ (ppm) = −13.57 [$\mathbf{3}\cdot H^+ - \mathbf{3}$]; −14.07 [$\mathbf{4}\cdot H^+ - \mathbf{4}$]) and the C-9 signal ($\Delta\delta$ (ppm) = −17.36 [$\mathbf{3}\cdot H^+ - \mathbf{3}$]; −17.84 [$\mathbf{4}\cdot H^+ - \mathbf{4}$]) are not only remarkable in value but also conclusive. They exceed all other $\Delta\delta$ values and belong to both carbons in the α-position to the nitrogen of ring B where the positive charge must be localized. Concerning signal shifts, this finding is in accordance with ^{13}C NMR data of pyridine and pyridinium ions.[15] In addition, different chemical shift values of the carbons positioned quasisymmetrically within the dipyrrin skeleton, in particular the δ values of C-6 ($\delta = 152.4$ [$\mathbf{3}\cdot H^+$]; 152.8 [$\mathbf{4}\cdot H^+$]) and C-14 ($\delta =$ 142.1 [$\mathbf{3}\cdot H^+$]; 142.0 [$\mathbf{4}\cdot H^+$]), argue against charge delocalization and exclude structure **I** (Figure 1 b). Thus, protonated 2,3-dihydrobilindiones correspond to structure **III**.

Stereochemical properties of the chromophores are not changed on protonation. According to the ROESY spectra the (4*Z*,9*Z*,15*Z*,5*syn*,10*syn*,14*syn*)-geometry of **3** remains in $\mathbf{3}\cdot H^+OTs^-$. The same applies to the (4*Z*,9*Z*,15*E*,5*syn*,10-*syn*,14*syn*) geometry of **4** and $\mathbf{4}\cdot H^+OTs^-$. The extension of the

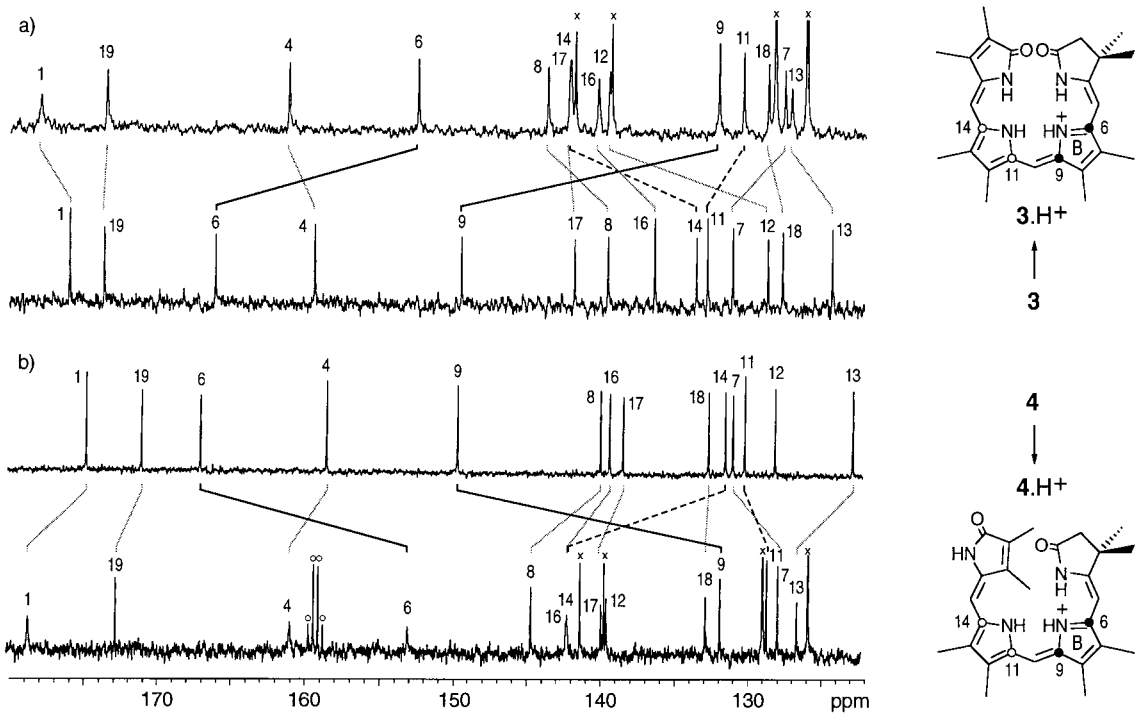

Figure 4. Correlation of ^{13}C NMR signals in the region of the quaternary carbons of a) $\mathbf{3}\cdot H^+OTs^-$ and **3** and b) **4** and $\mathbf{4}\cdot H^+OTs^-$. Signals of TsO^- are indicated by crosses, those of the *o*- and *m*-carbons below $\delta = 130$ are truncated for clarity. Complete dissolution of $\mathbf{4}\cdot H^+OTs^-$ at 2 °C in $CDCl_3$ necessitates the addition of a small amount of TFA. The corresponding quartet of the carbonyl carbon near $\delta = 160$ is indicated by circles.

Table 1. ^{13}C chemical shifts (δ) in $CDCl_3$ assigned by HMQC and HMBC spectroscopy.

	3 (298 K)	**3**·H$^+$OTs$^-$ (278 K)	**4** (298 K)	**4**·H$^+$OTs$^-$ (275 K)
C1	175.9	177.9	174.7	178.5
C2	44.2	44.0	44.5	43.8
C3	39.5	40.9	39.3	41.1
C3′	29.4	28.5	28.9	28.4
C4	159.3	161.1	158.4	160.8
C5	89.9	87.4	89.9	88.0
C6	166.0	152.4	166.9	152.8
C7	131.0	127.5	130.9	127.7
C7′	9.9	9.8	9.7	9.6
C8	139.5	143.6	139.8	144.5
C8′	9.9	10.6	9.7	10.5
C9	149.4	132.0	149.5	131.7
C10	111.3	114.8	112.3	116.0
C11	132.7	130.3	130.1	128.5
C12	128.6	139.4	128.0	139.4
C12′	9.5	10.4	9.5	10.3
C13	124.2	127.1	122.7	126.4
C13′	9.4	9.8	9.7	10.1
C14	133.5	142.1	131.4	142.0
C15	96.6	97.5	101.3	104.6
C16	136.3	140.2	139.2	142.1
C17	141.7	142.1	138.3	139.7
C17′	10.0	9.8	12.6	11.8
C18	127.6	128.6	132.5	132.7
C18′	8.7	8.2	8.8	8.4
C19	173.6	173.5	170.9	172.6

chromophores to the (*anti*,*syn*,*anti*) conformation as found in phycobiliproteins seems to be unlikely for 2,3-dihydrobilindiones intermolecularly protonated in solution.

Intramolecular protonation: Dissolved in chloroform the chromophores of the compounds **1** and **2** are protonated quantitatively at low temperature by the carboxyl group of the covalently attached cysteine moiety. In contrast to carboxyl-protected derivatives, for instance, the trimethylsilylethyl esters **1**·Tmse and **2**·Tmse, the chromophores cannot be protonated under similar conditions by acetic acid, not even in large excess. Assuming that the pK_a values of acetic acid and the carboxyl groups in **1** and **2** are more or less equal, the proton transfer in **1** and **2** must proceed intramolecularly resulting in the formation of the zwitterions **1**$^{\pm}$ and **2**$^{\pm}$. Interconversion of the neutral and the zwitterionic forms is temperature-dependent and can be followed by reversible changes of their ^1H NMR and UV/Vis spectra.

On cooling, the formation of the zwitterion can be observed in the UV/Vis spectra by the batho- and hyperchromic shifts of the long-wavelength absorption band (Figure 5). The equilibrium is shifted almost quantitatively towards **1**$^{\pm}$ at $-57\,^{\circ}C$ and towards **2**$^{\pm}$ at $-33\,^{\circ}C$. At these temperatures the protonation of the chromophores is practically complete and the absorption maxima remain quite constant despite further cooling.

The temperature dependence of the equilibrium between neutral and zwitterionic species is also characterized by isosbestic points. For the calculation of the thermodynamic data the extinction values of the pure neutral species **1** and **2**, which cannot be measured directly from the absorption spectra, were set equal to those of carboxyl-deprotonated **1** and **2** (Table 2). The fit of their absorption spectra to the isosbestic points was sufficient and differences in absorbance compared with **1** and **2** were therefore neglected. The negative values of ΔH° and ΔS°, which are typical of intramolecular proton transfer reactions,[16] regulate the equilibrium.

Table 2. Data for the proton transfer equilibria.

	λ_{max} [nm]	$\lambda_{max\pm}$ [nm]	ΔG° [kJ mol^{-1}]	ΔH° [kJ mol^{-1}]	ΔS° [J mol^{-1}K^{-1}]
1 $\rightleftharpoons$ **1**$^{\pm}$	584	641	+0.2	−20.8	−71
2 $\rightleftharpoons$ **2**$^{\pm}$	581	650	−1.3	−26.8	−86

Depending on the temperature, the ^1H NMR spectra of **1** and **2** show similarities with the spectra of the dichloroacetates as well as the tosylates of **3**·H$^+$ and **4**·H$^+$. On cooling, the averaged signal of the methine proton in position 10 is shifted downfield and no NH signals are detectable. However, below $-50\,^{\circ}C$ all four NH signals can be observed in the case of compound **2**$^{\pm}$**,** resembling the signal pattern of the hydrotosylates of **3** and **4** at room temperature. Thus, the intramolecular protonation by the cysteinic carboxyl group at low temperature seems to be equivalent to intermolecular protonation by *p*-TsOH at room temperature. Consequently, we conclude that protonation of 2,3-dihydrobilindione chromo-

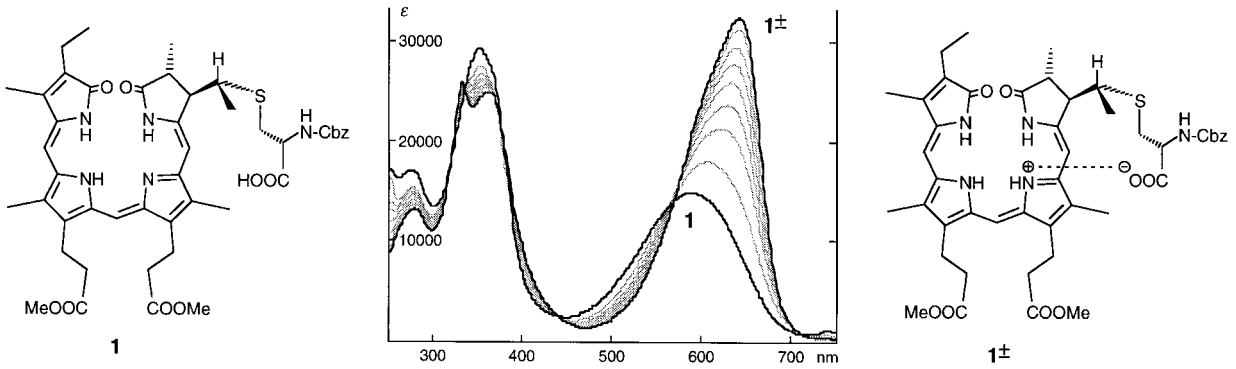

Figure 5. UV/Vis spectra showing the temperature dependence of the proton transfer equilibrium of **1** and **1**$^{\pm}$ in chloroform. Spectra were recorded at $-57\,^{\circ}C$ for **1**$^{\pm}$ (black), and at -45, -30, -15, 0, 15, 30, and $58\,^{\circ}C$ for equilibrium mixtures of **1** and **1**$^{\pm}$ (gray). All values of ε are corrected to room temperature. The spectrum indicated by **1** originates from the diisopropylethylammonium carboxylate of **1** at room temperature, because in chloroform the equilibrium cannot be shifted completely towards **1** below the boiling point of the solvent.

0947-6539/98/0409-1656 $ 17.50+.50/0 *Chem. Eur. J.* **1998**, *4*, No. 9

phores by carboxylic acids requires intramolecularity and low temperature, as demonstrated for the quantitative formation of $\mathbf{1}^{\pm}$ and $\mathbf{2}^{\pm}$. In phycobiliproteins protonation by aspartic acid residues is favored by the highly ordered arrangement of short consensus sequences[17] enabling the correct geometry necessary for effective ion-pair formation. On the other hand, conversion of the zwitterions $\mathbf{1}^{\pm}$ and $\mathbf{2}^{\pm}$ into the neutral forms **1** and **2** by raising the temperature indicates that in biliproteins proton transfer from the protonated chromophore back to the aspartate could be induced by geometrical strain on the ion pair. Regarding phytochrome, geometrical change by $Z \rightarrow E$ photoisomerization[18] of the Pr chromophore may induce deprotonation in this way. Some results from resonance Raman spectroscpy on phytochrome[19] suggest that such chemistry may be relevant in the sequence of dark reactions towards the physiologically active far-red-absorbing form of phytochrome.

Synthesis and stereochemistry: The synthesis of $\mathbf{1}^{\pm}$ and $\mathbf{2}^{\pm}$ started with the addition of *N*-(benzyloxycarbonyl)-cysteine trimethylsilylethyl ester (Cbz-Cys-OTmse) to the 3-ethylidene double bond of phycocyanobilin dimethyl ester (PCBDME) according to ref. [20], resulting in the formation of the four diastereomeric Tmse esters: **1**·Tmse (2*R*,3*R*,3′*R*,Cys*R*), **2**·Tmse (2*S*,3*S*,3′*S*,Cys*R*), and two Tmse esters with the (2*R*,3*R*,3′*S*,Cys*R*) and (2*S*,3*S*,3′*R*,Cys*R*) configuration. From this mixture **1**·Tmse was separated by thin-layer chromatography on silica gel. After deprotection with tetra-*n*-butylammonium fluoride (TBAF) in tetrahydrofuran (THF), followed by chromatography and treatment first with aqueous HCl and then with water, compound **1** was obtained in equilibrium with $\mathbf{1}^{\pm}$. Analogously, the remaining three diastereomeric Tmse esters were transformed into their carboxylic acids yielding optically pure **2** in equilibrium with $\mathbf{2}^{\pm}$ after separation by column chromatography on silica gel followed by successive treatment with aqueous HCl and water.

The assignment of the relative configuration with respect to the centers 2, 3, and 3′ in **1**, **1**·Tmse, and **2** is based on distinct H,H-ROESY cross-peaks: $2 \leftrightarrow 3''$; $2' \leftrightarrow 3 \leftrightarrow 3''$; $3 \leftrightarrow 5 \leftrightarrow 3'$. The absolute configuration of center 2 was determined by a comparison of the CD spectra of optically pure PCBDME.[21] The (2*R*) enantiomer was obtained by thermal elimination of Cbz-Cys-OTmse from **1**·Tmse in toluene at 83 °C.

Model compounds **3** and **4** were synthesized according to the protocol of Gossauer[22] by condensation of (4*Z*)- or (4*E*)-9-formyl-2,3,7,8-tetramethyldipyrrin-1(10*H*)-one with (4*Z*)-2,3-dihydro-9-*tert*-butyloxycarbonyl-3,3,7,8-tetramethyldipyrrin-1(10*H*)-one[23] in trifluoroacetic acid (TFA). For spectroscopic investigations the tosylates of $\mathbf{3}\cdot H^+$ and $\mathbf{4}\cdot H^+$ were prepared by adding stoichiometric quantities of *p*-TsOH to the solutions of **3** and **4**.

Conclusion

In summary, we have shown that the chromophores of 2,3-dihydrobilindiones are protonated without change of their overall geometry at the nitrogen of ring B where the positive charge is localized. This result, based on NMR spectroscopic investigations of synthetic model compounds, contributes to a better understanding of the ionic chromophore–protein interactions in biliproteins. With respect to phycocyanin, we conclude that protonation of the chromophores by aspartic acid residues is entropically controlled. In general, the formation of ion pairs in biliproteins depends on perfect order among chromophore and amino acid residues, but interference by steric strain may result in the reverse reaction. This may be relevant to the photochemically initiated conversion of the red-absorbing form of phytochrome, when geometrical changes upon the $Z \rightarrow E$ photoisomerization of the protonated Pr chromophore are followed by proton transfer back to the protein.

Experimental Section

General techniques: All chemicals were reagent grade. Solvents were generally distilled prior to use; THF was distilled from sodium benzophenone ketyl. *p*-TsOH·H_2O was melted under high vacuum prior to use. Column chromatography was performed on silica gel (E. Merck, silica gel 60, 0.063–0.200 mm). Preparative thin-layer chromatography was performed on precoated glass-backed plates (E. Merck, silica gel F_{256}, 0.5 mm). NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer. The assignment of ^{13}C signals is based on gradient-enhanced HMQC and HMBC experiments. IR spectra were recorded on a Perkin Elmer FT-IR spectrometer Paragon 1000 PC. UV/Vis and CD spectra were recorded on a Hitachi U-3210 spectrometer and a Jobin-Yvon Mark V circular dichrograph. Isosbestic points are indicated by λ_{ip}. Electrospray and electron-impact mass spectra were measured on Hewlett Packard MS-Engines 5989 API and 5989 A.

(4*Z*,9*Z*,15*Z*,2*R*,3*R*,3′*R*,Cys*R*)-3-(1-(*N*-benzyloxycarbonyl-cystein-*S*-yl)-ethyl)-18-ethyl-2,3-dihydro-8,12-bis-(2-methoxycarbonylethyl)-2,7,13,17-tetramethyl-23*H*-bilin-1,19(21*H*,24*H*)-dione (**1**): TBAF (10 mg, 31.7 μmol) was added to a solution of **1**·Tmse (11.5 mg, 11.8 μmol) in THF (2 mL). The reaction mixture was stirred under an argon atmosphere for 30 min, diluted with CH_2Cl_2 (40 mL), washed with H_2O (3 × 120 mL), with aqueous $NaHCO_3$ (0.2 M, 60 mL), and dried over Na_2SO_4. After evaporation of the solvent the residue was purified by thin-layer chromatography (silica gel, CH_2Cl_2/MeOH = 10/1). Elution with MeOH gave **1** (8.2 mg, 80 %) as a blue solid. Dissolution in CH_2Cl_2 and treatment with aqueous HCl (0.1 N, 50 mL) and H_2O (3 × 100 mL) afforded a solution of **1** in equilibrium with $\mathbf{1}^{\pm}$ at a ratio near to 1:1 at room temperature. R_f (silica gel, CH_2Cl_2/MeOH = 10/1): 0.3; ^1H NMR (500 MHz, $CDCl_3$; δ corresponds to the top of the broad single averaged signals of **1** and $\mathbf{1}^{\pm}$ at 278 K): $\delta = 7.28$ (5 H; H-C-Ph), 7.08 (1 H; H-C10), 5.97 (1 H; H-C15), 5.93 (1 H; H-N-Cys), 5.66 (1 H; H-C5), 4.98 (2 H; H_2-C-Bzl), 4.30 (1 H; H-$^{\alpha}$C-Cys), 3.65 (6 H; 2 × CH_3-O), 3.41 (1 H; H-C3′), 3.16 (1 H; H(1)-$^{\beta}$C-Cys), 3.09–2.80 (6 H; H_2-C8′, H_2-C12′, H(2)-$^{\beta}$C-Cys, H-C3), 2.60–2.48 (5 H; H_2-C8″, H_2-C12″, H-C2), 2.32 (2 H; H_2-C18′), 2.18, 2.08 (6 H; H_3-C17′, H_3-C13′), 2.03 (3 H; H_3-C7′), 1.39 (3 H; H_3-C3″), 1.25 (3 H; H_3-C2′), 1.13 (3 H; H_3-C18″); (H,H)-ROESY NMR (500 MHz, $CDCl_3$, 278 K): $2' \leftrightarrow 3$, $2 \leftrightarrow 3''$, $3 \leftrightarrow (5,3'')$, $3' \leftrightarrow 5$, $5 \leftrightarrow 7'$, $7' \leftrightarrow (8',8'')$, $10 \leftrightarrow (8',12')$, $13' \leftrightarrow (12',12'')$, $15 \leftrightarrow (13',17')$, 2,6-Ph ↔ CH_2-Bzl; IR ($CHCl_3$): $\tilde{\nu} = 3372$, 3270, 3151, 2973, 2954, 2934, 2875, 1732, 1687, 1633, 1609 cm^{-1}; UV/Vis ($CHCl_3$, 298 K): λ_{max} (ε) = 624 (22000), 352 (26600), 274 nm (15100); λ_{ip} [**1**/$\mathbf{1}^{\pm}$] (ε) = 575 (15960), 442 (2050), 367 (24200), 334 (25100), 319 nm (19300); CD ($CHCl_3$, 298 K): λ_{max} ($\Delta\varepsilon$) = 593 (−35), 350 (52), 275 nm (−16); $C_{46}H_{55}N_5O_{10}S$; MS (ESIp): *m*/*z* (%) = 870 (100) $[M+H]^+$, 615 (6) $[PCBDME+H]^+$.

(4*Z*,9*Z*,15*Z*,2*S*,3*S*,3′*S*,Cys*R*)-3-(1-(*N*-benzyloxycarbonyl-cystein-*S*-yl)-ethyl)-18-ethyl-2,3-dihydro-8,12-bis-(2-methoxycarbonylethyl)-2,7,13,17-tetramethyl-23*H*-bilin-1,19(21*H*,24*H*)-dione (**2**): TBAF (60 mg, 230 μmol) was added to a solution of **2**·Tmse and its diastereomers with the (2*R*,3*R*,3′*S*,Cys*R*) and (2*S*,3*S*,3′*R*,Cys*R*) configuration (90 mg, 93 μmol) in THF (4 mL). The reaction mixture was stirred under an argon atmosphere for 40 min, diluted with CH_2Cl_2 (80 mL), washed with H_2O (3 × 120 mL),

with aqueous $NaHCO_3$ (0.2 M, 60 mL), and dried over Na_2SO_4. After evaporation of the solvent the residue was subjected to column chromatography (silica gel, CH_2Cl_2/MeOH = 15/1) separating **2** from its diastereomers. Fractions containing **2** were treated with aqueous HCl (0.1 N, 50 mL) and H_2O (3 × 100 mL). The ratio of **2** and $\mathbf{2}^{\pm}$ was near 1:2 at room temperature. R_f (silica gel, CH_2Cl_2/MeOH = 10/1): 0.4; ^{1}H NMR (500 MHz, $CDCl_3$; δ corresponds to the top of the broad averaged signals of **2** and $\mathbf{2}^{\pm}$ at 273 K): δ = 7.30 (5H; H-C-Ph), 7.23 (1H; H-C10), 5.89 (2H; H-C15, H-N-Cys)), 5.69 (1H; H-C5), 5.07 (2H; H_2-C-Bzl), 4.41 (1H; H-$^{\alpha}$C-Cys), 3.66, 3.65 (6H; 2 × CH_3-O), 3.55 (2H; H-C3′, H(1)-$^{\beta}$C-Cys), 3.10 – 2.80 (7H; H_2-C8′, H_2-C12′, H(2)-$^{\beta}$C-Cys, H-C3, H-C2), 2.55 (4H; H_2-C8″, H_2-C12″), 2.32 (2H; H_2-C18′), 2.13, 2.04, 2.02 (9H; H_3-C17′, H_3-C13′, H_3-C7′), 1.43 (3H; H_3-C3″), 1.30 (3H; H_3-C2′), 1.07 (3H; H_3-C18″); (H,H)-ROESY NMR (500 MHz, $CDCl_3$, 299 K): 2′ ↔ 3, 2 ↔ 3″, 3 ↔ (5, 3″), 3′ ↔ (5, $^{\alpha}$C-Cys), 5 ↔ (7′, $^{\alpha}$C-Cys), 7′ ↔ (8′, 8″), 10 ↔ (8′, 12′), 13′ ↔ 12′, 15 ↔ (13′, 17′), 2,6-Ph ↔ CH_2-Bzl; IR ($CHCl_3$): $\tilde{\nu}$ = 3294, 2973, 2954, 2934, 2875, 1732, 1690, 1630, 1602 cm^{-1}; UV/Vis ($CHCl_3$, 298 K): λ_{max} (ε) = 638 (24100), 350 (24800), 330 (25400), 277 nm (12300); λ_{ip} [**2**/$\mathbf{2}^{\pm}$] (ε) = 581 (15800), 432 (2500), 376 (18700), 333 (24500), 318 nm (19500); CD ($CHCl_3$, 298 K): λ_{max} ($\Delta\varepsilon$) = 623 (38), 349 (−56), 273 nm (19); $C_{46}H_{55}N_5O_{10}S$; MS (ESIp): *m*/*z* (%) = 914 (22) $[M - H + 2Na]^+$, 892 (100) $[M + Na]^+$, 870 (15) $[M + H]^+$.

Synthesis of 3 and 4: A solution of (*Z*)-9-formyl-2,3,7,8-tetramethyldipyrrin-1(10*H*)-one[22] (60 mg, 235 μmol) in MeOH (200 mL) was irradiated with a high-pressure mercury immersion lamp (Hanau, TQ 150 – 1725) under an argon atmosphere for 2 h in a Pyrex well. After evaporation of the solvent under reduced pressure the residue was added at once to a stirred solution of (*Z*)-2,3-dihydro-9-*tert*-butyloxycarbonyl-3,3,7,8-tetramethyldipyrrin-1(10*H*)-one[23] (72 mg, 235 μmol) in TFA (5 mL). After stirring for 90 min at room temperature MeOH (15 mL) was added. The reaction mixture was diluted with CH_2Cl_2 (200 mL), washed with H_2O (3 × 150 mL), with aqueous $NaHCO_3$ (0.5 M, 100 mL), and dried over Na_2SO_4. After evaporation of the solvent the residue was subjected to column chromatography (silica gel, CH_2Cl_2/MeOH = 30/1) separating **3** (80 mg, 61%) from **4** (25 mg, 19%).

(4*Z*,9*Z*,15*Z*)-2,3-Dihydro-3,3,7,8,12,13,17,18-octamethyl-23*H*-bilin-1,19(21-*H*,24*H*)-dione (**3**): M.p. 244 °C; R_f (silica gel, CH_2Cl_2/MeOH = 30/1): 0.5; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.60 (s, 1H; H-C10), 6.01 (s, 1H; H-C15), 5.45 (s, 1H; H-C5), 2.35 (s, 2H; H_2-C2), 2.18 (s, 3H; H_3-C12′), 2.13 (s, 3H; H_3-C8′), 2.11 (s, 3H; H_3-C17′), 2.09 (s, 3H; H_3-C13′), 1.99 (s, 3H; H_3-C7′), 1.84 (s, 3H; H_3-C18′), 1.42 (s, 6H; 2 × H_3-C3′); (H,H)-ROESY NMR (500 MHz, $CDCl_3$): 2 ↔ 3′, 5 ↔ (7′, 3′), 10 ↔ (8′, 12′), 15 ↔ (13′, 17′), 17′ ↔ 18′; IR ($CHCl_3$): $\tilde{\nu}$ = 3430, 3006, 2921, 2862, 1738, 1716, 1688, 1635, 1596 cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (ε) = 584 (14100), 347 (32500), 275 nm (17400); $C_{27}H_{32}N_4O_2$; MS (70 eV; EI): *m*/*z* (%) = 444 (100) $[M]^+$.

(4*Z*,9*Z*,15*E*)-2,3-Dihydro-3,3,7,8,12,13,17,18-octamethyl-23*H*-bilin-1,19(21-*H*,24*H*)-dione (**4**): M.p. 242 °C; R_f (silica gel, CH_2Cl_2/MeOH = 30/1): 0.3; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.56 (s, 1H; H-N24), 6.58 (s, 1H; H-C10), 6.23 (s, 1H; H-C15), 5.38 (s, 1H; H-C5), 2.33 (s, 2H; H_2-C2), 2.15 (s, 3H; H_3-C12′), 2.12 (s, 3H; H_3-C8′), 2.01 (s, 3H; H_3-C17′), 1.98 (s, 3H; H_3-C13′), 1.97 (s, 3H; H_3-C7′), 1.86 (s, 3H; H_3-C18′), 1.35 (s, 6H; 2 × H_3-C3′); (H,H)-ROESY NMR (500 MHz, $CDCl_3$): 2 ↔ 3′, 5 ↔ (7′, 3′), 10 ↔ (8′, 12′), 15 ↔ (13′, 24), 17′ ↔ 18′; UV/Vis ($CHCl_3$): λ_{max} (ε) = 546 (15100), 350 (22500), 276 nm (14500); $C_{27}H_{32}N_4O_2$; MS (70 eV; EI): *m*/*z* (%) = 444 (100) $[M]^+$.

Hydrotosylates of 3 and 4: Hydrotosylates were prepared in solution by adding stoichiometric quantities of *p*-TsOH dissolved in $CDCl_3$ or $CHCl_3$ to the corresponding solutions of **3** and **4**.

3·H$^+$OTs$^-$: ^{1}H NMR (500 MHz, $CDCl_3$, 278 K): δ = 11.73, 11.46, 10.66, 9.92 (4 × s, 4H; 4 × H-N), 7.02 (s, 1H; H-C10), 6.03 (s, 1H; H-C15), 5.40 (s, 1H; H-C5), 2.31 (s, 3H; H_3-C8′), 2.28 (s, 3H; H_3-C12′), 2.27 (s, 2H; H_2-C2), 2.08 (s, 3H; H_3-C13′), 2.05 (s, 3H; H_3-C7′), 1.91 (s, 3H; H_3-C17′), 1.60 (s, 3H; H_3-C18′), 1.27 (s, 6H; 2 × H_3-C3′), tosylate: 7.75 (d, ^3J = 8 Hz, 2H; H-C2,C6), 6.97 (d, ^3J = 8 Hz, 2H; H-C3,C5), 2.26 (s, 3H; H_3-C4′); (H,H)-ROESY NMR (500 MHz, $CDCl_3$, 298 K): 2 ↔ 3′, 5 ↔ (7′, 3′), 10 ↔ (8′, 12′), 15 ↔ (13′, 17′), 17′ ↔ 18′; IR ($CHCl_3$): $\tilde{\nu}$ = 3440, 3006, 2921, 1710, 1688, 1633, 1600 cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (ε) = 624 (36900), 352 (28100), 277 nm (11500).

4·H$^+$OTs$^-$: ^{1}H NMR (500 MHz, $CDCl_3$, 275 K): δ = 11.65, 11.51, 10.41, 9.45 (4 × s, 4H; 4 × H-N), 7.02 (s, 1H; H-C10), 6.42 (s, 1H; H-C15), 5.47 (s, 1H; H-C5), 2.43 (s, 2H; H_2-C2), 2.30 (s, 3H; H_3-C8′), 2.25 (s, 3H; H_3-C12′), 2.06 (s, 3H; H_3-C7′), 1.94 (s, 3H; H_3-C13′), 1.81 (s, 3H; H_3-C17′), 1.78 (s, 3H; H_3-C18′), 1.33 (s, 6H; 2H_3-C3′), tosylate: 7.54 (d, ^3J = 8 Hz, 2H; H-C2,C6), 7.05 (d, ^3J = 8 Hz, 2H; H-C3,C5), 2.29 (s, 3H; H_3-C4); (H,H)-ROESY NMR (500 MHz, $CDCl_3$, 280 K): 2 ↔ 3′, 5 ↔ (7′, 3′), 10 ↔ (8′, 12′), 15 ↔ (13′), 17′ ↔ 18′; UV/Vis ($CHCl_3$): λ_{max} (ε) = 610 (34000), 353 (23400), 304 nm (12900).

Acknowledgments: This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Austria (FWF-Project P-9166-CHE). We thank Dr. G. Niessner and Dipl.-Ing. W. Ahrer for mass spectral measurements. K.G. thanks Prof. W. Wehrmeyer for drawing his attention to ref. [17].

Received: January 14, 1998 [F963]

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