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Studies on Plant Bile Pigments: Characterization of a Model for the Phytochrome P_r Chromophor

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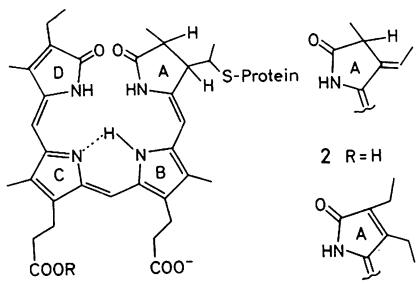
Dedicated to Prof. Dr. Dr. h. c. H. H. Inhoffen *on the Occasion of His 70tieth Birthday*

Bile Pigments, Phytochrome, Chromophor Model

The common A-dihydrobiliverdin chromophor proposed to be present in both phycocyanin and phytochrome P_r has been characterized by using octaethylbiliverdin and its A-dihydro derivative as model compounds. The UV-vis spectra for the free bases, cations, anions and zinc complexes are reported, and the related protonation-deprotonation equilibria have been studied. The data support the postulated structures for the biliproteid chromophor, and at the same time they indicate A-dihydrooctaethylbiliverdin to be a useful model.

Both the reaction center pigment of the photomorphogenetic phytochrome system, and the major photosynthetic antenna pigments in blue-green algae are biliproteids, in which the bile pigment chromophore is covalently linked by at least one bond to the protein [1]. In the series of reactions leading from phytochrome P_r to P_{fr} and *vice versa*, it is probable that the genuine photo reaction(s) involve a transformation of the bile pigment chromophor [2]. The detailed structure of this native chromophor its reactivity and its interactions with the protein is yet unknown.

Structural studies on biliproteid chromophors involve mainly degradation experiments [1], and spectral studies in which the influence of the protein is assumed to be impeded [1, 3]. Hydrolytic cleavage of biliproteids yields a variety of structurally altered bile pigments. The only one of established structure, *viz.* phycocyanobilin (**2**) derived from phycocyanin (**1**), bears a 3-ethylidene group which is introduced during methanolysis [3–8]. Degradation of phytochrome is even more ambigous due to its poor accessibility.



1 R=Protein

2 R=H

5 R=H

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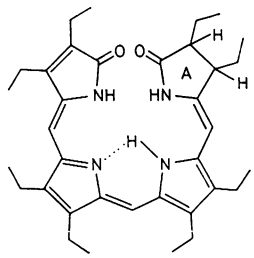

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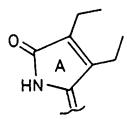

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Recently [9], closely related structures with a common A-dihydrobiliverdin chromophor have been advanced for the native chromophors of both phytochrome P_r [10] and phycocyanin on spectroscopic and reactivity arguments. In an attempt to characterize the salient properties of this A-dihydrobiliverdin chromophor, a comparative study of octaethylbiliverdin (**3**) and its A-dihydro analogue (**4**) is in progress. Here, the UV-vis spectra of the free bases, the cations, the anions and the Zn complexes, as well as the pK values of **3** and **4** are reported. The data support the A-dihydrobilin structure postulated for native biliproteid chromophors and at the same time they prove **4** to be a useful compound for model investigations of the phycocyanin and phytochrome pigments.

Experimental

Octaethylbiliverdin (**3**) and A-dihydrooctaethylbiliverdin (**4**) were prepared from octaethylporphyrin [11], *via trans*-octaethylchlorin [12] by the method of Cavaleiro and Smith [13]. Al reagents were reagent grade, guanidinium hydrochloride was recrystallized twice. *UV*-vis spectra were recorded under anaerobic conditions with a DMR-22 spectrophotometer

(Zeiss, W.Germany). Deoxygenated methanol containing sodium ascorbate (5×10^{-4} M) was used as solvent if not otherwise noted. The anions, cations and Zn-complexes of the bilins were prepared by adding small aliquots of concentrated solutions of KOH, HCl and Zn $(OAc)_2$ in MeOH under oxygen free conditions. All spectra are corrected for dilution. The pK determinations were carried out in the spectrophotometer cell under purified nitrogen by adding 1 N aqueous HCl or NaOH to solutions of the bilin (5×10^{-6} N) in a 1:1 mixture of MeOH and aqueous guanidinium-hydrochloride (6 M). The pH was monitored with a glass electrode and is uncorrected. The absorption changes of at least two bands were recorded independently. The reported pK values (averaged from three or more titrations) were determined from the first derivative of the *E vs* pH curves.

Results and Discussion

The UV-vis spectra (in dry methanol) of the two compounds **3** and **4**, and their cations, anions and Zn complexes, respectively, are listed in Table I. Band position and extinction coefficients of the free bases are markedly dependent on the solvent system used. These solvent induced shifts are comparable for **3** and **4**. There is a general red shift with increased solvent polarity according to the $\pi \rightarrow \pi^*$ type transitions involved, but pronouced shifts for the same solvent are frequently observed. In untreated reagent grade methylene chloride, extremes of $\lambda_{max} = 645$ and 670 nm, have been found for the red band of **3**. Comparably wide margins have been reported frequently for bile pigments and denaturated biliproteids[1, 9]. At least one important factor for these variations is the easy cation formation of **3** and **4**, which can be suppressed by treatment with $NaHCO_3$, but aggregation seems to be important, too. In methanol, both factors are reduced, and the absorption maxima found for **3** and **4** are reproducible within ± 2 nm.

The spectra of the free bases of **3** and **4** in methanol are very similar to each other, but that of the A-dihydrobilin **4** being shifted uniformly by about 1500 cm^{-1} to the blue. Upon cation formation, the red band of **3** is shifted to longer wavelengths, and increased about twice in intensity. At the same time, the band is sharpened, and is accompanied by a pronounced shoulder at shorter wavelengths. The latter is present in the free base, too, but is obscured there by the increased width of the bands. By contrast, the blue band is almost unchanged except for a small red shift. The spectral changes of **4** upon cation formation are very similar, the cation of **4** exhibiting a uniform blue shift of about 500 cm^{-1} as compared to that of **3**.

In the spectrum of the anion of **3**, the red band is shifted by 2280 cm^{-1} to 770 nm. The intensity is only slightly increased, but again the asymmetry of this band is increased and a shortwavelength shoulder is apparent. The blue band is considerably more complex. Although the major maximum remains almost at the same position, the extinction coefficient is considerably reduced and intensity is transferred to a new, resolved band at 328 nm and a shoulder at longer wavelengths. At very high concentrations of KOH (~ 6 mol/l), this anion of **3** is further deprotonated. The product is unstable and transforms to a variety of compounds with ill defined absorptions in the visible range. The spectral changes of **4** upon addition of small amounts of methanolic KOH are similar, the spectrum of the anion of **4**, as compared to that of **3**, being shifted to shorter wavelengths (Fig. 1). However, the pure monoanion of **4** is difficult to prepare. Not only leads the presence

Table I. UV-vis spectra [λ_{max} ($\varepsilon \times 10^{-3}$) in methanol] of the free bases, cations, anions and zinc complexes of A-dihydrooctaethylbiliverdin (**4**) and octaethylbiliverdin (**3**). *, shoulder.

	A-dihydrooctaethylbiliverdin (4)					Octaethylbiliverdin (3)				
free base	594 (17.6)		347 (39.4)		275 (20.4)	657 (15.6)		367 (51.2)	300 * (26.1)	275 * (21.6)
cation	665 (34.0)		351 (36.5)		279 (16.3)	693 (31.2)		357 (55.9)		
anion I	720 (19.2)	400 (27.1)	352 (32.6)			770 (16.6)		372 (39.6)	328 (29.0)	282 (23.5)
anion II	766 (19.8)	710 * (12.1)	406 (30.1)	357 (31.2)		810	735	400 *	375	
zinc complex	638 (21.3)		377 (28.3)	343 (30.0)	384 (19.7)	691 (22.3)		370 (41.2)	330 * (28.3)	275 (21.0)

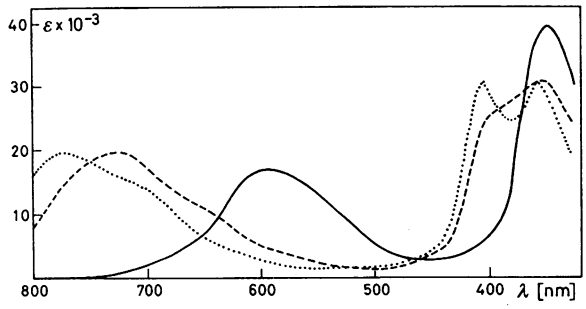

Fig. 1. UV-vis spectra of A-dihydrooctaethylbiliverdin (4) (———), its monoanion (— — —) and dianion (·····). In methanol and KOH/methanol, respectively.

of equilibrium amounts of the dianion (see below) to a shoulder around 770 nm and 400 nm, but it is also readily oxidized to a purpurin anion giving rise to additional peaks at 625, 577, 540 and 328 nm.

The equilibrium between the two anions of **4** is shifted with increasing KOH concentrations, and above 1.5×10^{-3} mol/l only the second anion is present. Its formation is accompanied by an additional shift of the red band to longer wavelengths ($\lambda_{max} = 766$ nm) and by a distinct split of the Soret band ($\lambda_{max} = 406, 357$ nm) (Fig. 1). Like the monoanion, it is readily oxidized by traces of oxygen to a purpurin anion, while it decomposes slowly under anaerobic conditions to products without pronounced absorptions in the visible range. Although there are indications that the dianion of **4** can be further deprotonated under the conditions leading to the deprotonation of **3**, the product is too unstable under these conditions for a detailed characterization.

In the spectrum of the Zn-complex of **3**, as compared to the free base, the red band is increased in intensity, and it is shifted by 495 cm^{-1} to longer wavelengths. In addition to the common blue shoulder, a red shoulder becomes apparent, too. The blue band of Zn-**3** is considerably broadened, without any apparent fine structure. While the spectral changes of **4** upon complexation with Zn are similar in the red region, the blue band shows increased fine structure. It is split and shows additional shoulders extending as far as 410 nm. Like the anion, the Zn complex of **4** is rather unstable in aerobic solution and is oxidized rapidly.

Bile pigments are generally characterized by two broad, poorly structured absorptions in the visible and near UV spectral range[1]. The position of the red band has been correlated empirically with the size of the conjugated system by Köst *et al.*[3]. Roughly linear relationships have been found between the number of conjugated double bonds and the bathochromic shift of the red band for the free base bilins, as well as for their cations and zinc complexes. These results suggest a similar conformation for the non-rigid tetrapyrrolic system in the compounds investigated, because pronounced variations in both intensity and position of the red band are expected for conformation changes from theoretical calculations[14–18]. The spectrum of the octaethyl-verdin **3** agrees well with that of mesobiliverdin IXα (**5**)[3] to exclude a significant contribution of the different substituents in **3** and **5**. These similarities extend to the cations, anions and zinc complexes (Tables I, II). As the same is true for **4** as compared to denatured phycocyanin (**1**), respectively, these spectral similarities are good evidence for the A-dihydrobiliverdin structure for the native PC and P_r chromophors. In both cases the incremental shifts as well as the intensity changes of the red band are similar for the free bases, cations, and zinc complexes (Table II).

	Meso biliverdin IXα (5) [a]	Denatured Phytochrome P_r [9]		Denatured C-Phycocyanin (1) (from *Pseudoanabena* spec. [21])		Δ [cm^{-1}]
free base	630–655	620–625	590 *	610 [b] 590 [c] 583 [a]	590 *	520–1132 1076–1682 1280–1885
cation	685	675–690	630 *	665–670 [b] 650 [a]	620 *	439– 327 786
anion	—	765–770		675–770 [b]		—
zinc-complex	688	650	590 [d]	640 [b] 630 [a]	590 *, [d] 583 *, [d]	1090

Tab. II. Absorption (λ_{max} of the red band of mesobiliverdin IXα (5) in methanol, and of denatured phycocyanin and phytochrome P_r. Δ = incremental shift of **1** *vs.* **5** due to hydrogenation of ring A.

a. from ref. 3; b. in 1 m guanidinium hydrochloride pH 8, from ref. 9; c. heat denatured in N/15 phosphate buffer, pH 7.2; d. probably free base absorption due to incomplete complexation. *, shoulder.

The only significant deviation is that between the anions of **4** and **1**. The anion of **4** absorbs at 720 nm, as compared to 770 nm for the anion of **1** [9]. However, **4** can be deprotonated further easily (in contrast to **3**) to form a second anion absorbing at 766 nm, which agrees well with the data reported for the anion of **1** at a pH > 11 [9]. From titration experiments, it is likely that **4** can be deprotonated easily in two stages leading subsequently to a monoanion ($\lambda_{max} = 720$ nm), and dianion ($\lambda_{max} = 766$ nm), and that denatured **1** was present as the dianion under these conditions. It should be noted that the pronounced red shift, shape and intensity of the anions makes them possible candidates for the P_{fr} chromophor. The long wavelength absorption has been accounted for by several authors to arise from an ionized chromophor [1, 5, 9, 18, 19], an idea which is especially attractive in view of the shorter conjugation system suggested recently for P_{fr} [9].

Both the easy abstraction of a second proton and the spectral similarities of the dianions of **4** (and **1**) and the monoanions of **3** (and **5**), respectively, can be rationalized in a straight-forward way. The biliverdin **3** contains formally one olefinic substituted 2H-pyrrole ring (C), one 1H-pyrrole ring (B), and two olefinic substituted 4-pyrrolin-2-one rings (A, D). The latter three bear NH-protons, of which the pyrrole ring B as expected to be deprotonated most easily, yielding the monoanion of **3**. The second deprotonation at the lactam-type nitrogens of rings A and D is considerably more difficult, the corresponding dianion is probably the species observed in concentrated methanolic KOH. In **4**, the first deprotonation is again expected to occur at the pyrrole type nitrogen of ring B, yielding a correspondingly shorter conjugation system than **3**, in agreement with the absorption at shorter wavelengths. However, the second deprotonation of **4** at ring A is facilitated for two reasons: The acidity of the hydrogenated pyrrolidin-2-one-ring A in **4** is expected to be higher than that of the pyrrolin-one-rings in **3**, if judged from the acidity of succinimide and maleimide, respectively, and the deprotonation of ring A leads to its conjugation with rings B, C and D. As a consequence, the conjugation system of the dianion of **4** is now similar tot hat of the monoanion of **3**, as reflected by its visible spectrum. Further deprotonation of both ions is difficult and proceeds only at high KOH concentrations. However, in spite of the spectral similarities of the anions of **3** and **4**, there are pronounced differences in their reactivity. This increased reactivity of the anions of **4** is obviously a direct result of the hydrogenated ring A, and the involved reactions are currently studied.

To characterize the protonation-deprotonation equilibria of **3** and **4** further, their pK values were determined by acid-base titrations in a mixed solvent system of aqueous concentrated guanidinium hydrochloride buffer and methanol. The main reason for the use of this solvent was that both denatured biliproteids and the octaethylbilins are sufficiently soluble and stable in it, and that data for a variety biliproteids have been recorded in guanidinium-HCl buffered solutions [1, 9, *]. An absolute pH scale for the system is yet to be defined.

For the cation formation of a series of bilins, increased pK values have been related empirically to a decreased extension of the conjugation system [1, 2, 9, 20]. Thus, in the series mesobiliverdin (4 conjugated rings), mesobiliviolin (3 rings), urobilin (2 conjugated rings), the pK increases from 3.0 to 7.4. The pK values of denatured biliproteids do not correlate with this series in having pK values which are about 2 units too high, an increase which has been attributed to the hydrogenation of ring A [1, 9]. The data obtained for the model compounds principally support this effect of the reduced double bond to increase the pK of the cation-free base transformation. For the biliverdin **3**, a pK of 5.11($\pm$0.1) has been obtained, while the A-dihydroverdin (**4**) has a pK of 5.55. This difference is less than the one observed for denatured biliproteids. To separate levelling effects of the solvent system, and possible influences from the protein, a more detailed characterization of the acid-base transformation in bilins is under way.

The observed similarities between the octaethylbilins **3** and **4**, and the natural bilins with the IXα-substitution pattern, respectively, support the proposed structure of the chromophors of phycocyanin and phytochrome P_r as being A-dihydrobiliverdins. The results indicate, that the influence of the β-pyrrolic substituents on the properties of the tetrapyrrole-chromophor is small enough to render **4** a suitable model for a more extensive characterization of the free biliproteid chromophors.

* Although aqueous detergent solutions are principally suitable, too, the used Triton X-100 (10^{-4} M) gave less reproducible results and facilitated side reactions during the titration experiments.

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