Thioether Formation of Phycocyanobilin: A Model Reaction of Phycocyanin Biosynthesis

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Dedicated to Prof. Dr. H. Plieninger *on the Occasion of His 65th Birthday*

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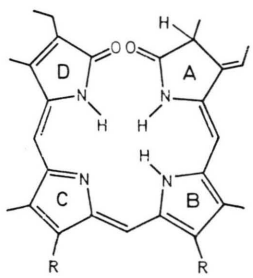
Bile Pigments, Ethanethiol, Chromic Acid Degradation

The reaction of phycocyanobilin dimethylester (**1 a**) with ethanethiol is studied. The spectral shifts during a slow reaction correspond to the disappearance of the double bonds at the ethylidene group (595 → 580 nm) and at the methine bridge between rings B and C (365 → 395 nm). Chromic acid degradation of the reaction products reveiles that the thiol has been added at the ethylidene double bond yielding two stereoisomers as expected. The reaction is considered as a model reaction for the presumed last step of phycocyanin biosynthesis, namely the formation of the thioether between cysteine residues of the apoprotein and phycocyanobilin.

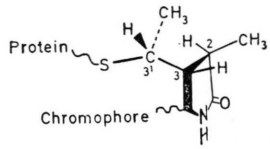
Phycocyanins are accessory pigments of photosynthesis in red (Rhodophyta), blue-green (Cyanophyta-Cyanobacteria) and cryptomonad (Cryptophyta) algae [1, 2]. These pigments are chromoproteins in which the chromophore is covalently attached to the protein. Cleavage of the chromophore-protein bond by a variety of methods yields the free chromophore phycocyanobilin * (**1**) characterized by an ethylidene side chain at ring A [3–10]. Structure **1** has been confirmed by total synthesis [11–13]. The chromophore *in situ* **2** contains a thioether linkage at C-3^1 (*i. e.* the side chain of ring A). This has been deduced from the following data: spectral characterization showed the absence of the ethylidene group *in situ* [14]. Sequence analysis reveiled cysteine as the linkage amino acid in all cases [2, 15, 16]. Comparison of the elimination reaction **2** → **1** with phycocyanin and model compounds did not only prove the thioether linkage [17] but also allowed deduction of the absolute configuration at the chiral centers C-2, C-3 and C-3^1 [18].

When the rhodophyte *Cyanidium caldarium* is incubated with 5-aminolevulinate, the cells synthesize and excrete the free phycocyanobilin **1** [19, 20]. The chromophore with ethylidene group is therefore believed to be an intermediate in the phycocyanin biosynthesis; the formation of the thioether linkage should then be the last step of the chromoprotein biosynthesis [20]. This implies that the ethylidene compound easily adds thiols to form the thioether.



1: R = CH_2CH_2COOH

1a: R = $CH_2CH_2COOCH_3$



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Such a reactivity has been checked with ethylidene methyl succinimide (**5**) [17, 18, 21], but not with **1**. Only the addition of methanol at the ethylidene group of **1** has been described [9, 12, 13]. We describe here experiments to check the reactivity of phycocyanobilin with ethanethiol.

Materials and Methods

Phycocyanobilin dimethyl ester (**1 a**), obtained by total synthesis [12, 13] was a gift from Professor Gossauer. For spectral measurements, 1–3 mg **1 a** were dissolved in 10 ml ethanethiol. Spectra were recorded with a spectrophotometer type DB-GT (Beckman, München) after various incuba-

Reprint requests to Prof. Dr. W. Rüdiger.

* Whereas the chromophore *in situ* (**2**) has been named phycocyanobilin by all authors, the term phycobiliverdin has been introduced for the cleaved chromophore **1** [8]. This name, however, has not been recommended by the IUPAC-IUB Commission of Biochemical Nomenclature and is therefore replaced here by the term phycocyanobilin for **1**.

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tion time at room temperature under a nitrogen atmosphere or at 35 °C under reflux of ethanethiol. The incubation mixture was applied to TLC on precoated plates (silica gel HPTLC grade, Merck, Darmstadt) and developed 3 – 6 times with the same solvent (carbon tetrachloride/ethyl acetate 9 : 1). Because true R_F values cannot be given with repeated development, relative migration based on $R_{\mathbf{1a}} = 1.0$ is given here: violet pigment (main product of reaction at room temperature): $R_{rel.} = 0.2$; blue-green pigment: $R_{rel.} = 0.5 - 0{,}6$; blue pigment (main product of reaction at refluxing): $R_{rel.} = 1.4$; yellow pigment: $R_{rel.} = 2.5$; several minor components over the range $R_{rel.} = 0.2 - 2.0$.

For the preparation of sulfone imides **4 a** and **4 b**, 50 mg **1 a** were refluxed for 24 hours with 30 ml ethanethiol. The solution was evaporated under nitrogen, the residue was stirred after addition of 130 ml chromic acid (1% w/v in 2 N H_2SO_4) for 2.5 hours. The reaction mixture was then diluted with 200 ml water and exhaustively extracted with ethyl acetate (10 × 50 ml). The organic phase was washed with water, dried with sodium sulfate and concentrated under reduced pressure to 4 ml. Aliquots of this solution were applied to TLC for determination of imides (precoated plates, silica gel HPTLC grade, Merck, Darmstadt; solvent carbon tetrachloride/ethyl acetate/cyclohexane = 4 : 4 : 1; staining with chloride/benzidine [22]). The preparative separation of imides was achieved by column chromatography on silica gel 60 (Merck, Darmstadt) with the same solvent. The fractions which contained the sulfone imides **4 b** or the compound X, resp. were combined, evaporated and applied to mass spectrometry (mass spectrometer IMS-D 100, Jeol, at 75 V [23]).

Results

Phycocyanobilin dimethylester (**1 a**) can easily be dissolved in ethanethiol. The pigment reacts with the solvent as shown by spectral shifts (Fig. 1 and Table I). The red band is shifted in a fast reaction from 600 – 630 nm (two bands) to 595 nm and then slowly to 580 nm. The slow reaction corresponds to the disappearance of the ethylidene double bond [14]. Moreover, the blue band is shifted from 365 nm to 395 nm. This corresponds to the formation of a bilirubinoid pigment, *i. e.* addition of the thiol at the middle methine bridge. Such a reaction

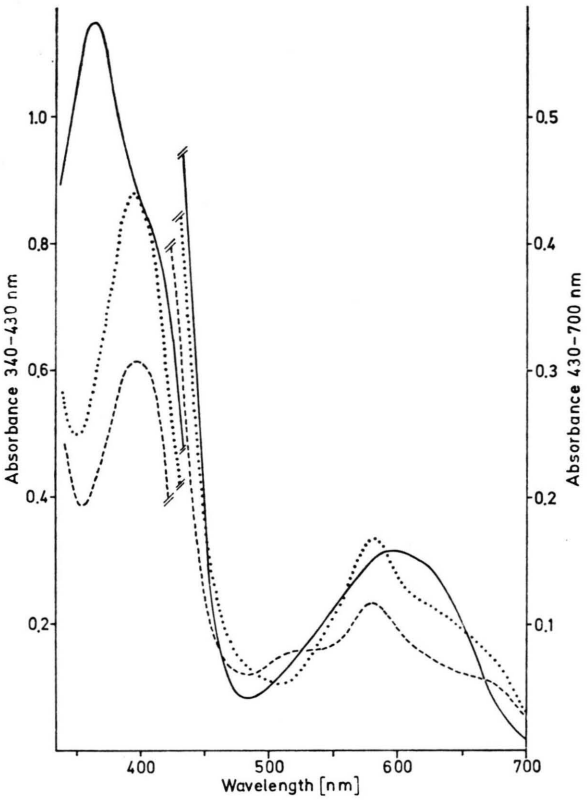


Fig. 1. Electronic spectra of phycocyanobilin (**1 a**) in ethanethiol. ——— **1 a** 10 min after dissolving; – – – – **1 a** 18 hours after dissolving; ····· **1 a** 22 hours refluxed (35 °C). Left absorbance scale for left part of the spectrum, right absorbance scale for right part of the spectrum.

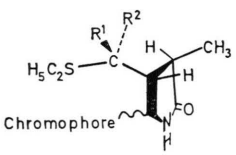
Table I. Absorption maxima (red band) of phycocyanobilin dimethylester (**1 a**) and related compounds.

Compound	Solvent	λ_{max} [nm]	ref.
1 a	methanol	603	[14]
1 a	ethanethiol (immediately after dissolving)	600 – 630 *	this paper
1 a	ethanethiol (10 min at room temperature)	595	this paper
1 a	ethanethiol (18 hours at room temperature)	580	this paper
1 a	ethanethiol (22 hours under reflux)	580	this paper
phycocyanin-peptide (**2**)	methanol	585	[14]

* Two bands at about 600 – 610 (band A) and 620 – 630 (band B) nm; band B disappears very fast after dissolving.

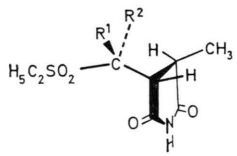
type has been demonstrated and investigated in detail with thiols and other reducing agents [25]. Some by-products are also formed as indicated by shoulders in the absorption spectra of the reaction mixtures. This is confirmed by thin-layer chromatography: **1 a** (blue spot) disappears after several hours reaction time. A number of slower and faster migrating pigments appear instead of which a violet pigment predominates if the reaction is carried out at room temperature.

Because several of these pigments may contain ethanethiol added at the ethylidene double bond (*i. e.* structure **3 a/3 b**) no preparative separation of pigments was tried but the pigment mixture was directly applied to chromic acid degradation. This should yield the known sulfone imides **4 a** and **4 b** if the presumed addition of ethanethiol has taken place.



3a : R^1 = H, R^2 = CH_3

3b R^1 = CH_3, R^2 = H



4a R^1 = H, R^2 = CH_3

4b R^1 = CH_3, R^2 = H

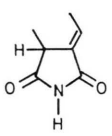
Analysis of the chromic acid degradation of **1 a** by thin-layer chromatography [22] yields the expected products ethylidene methyl succinimide (**5**), ethyl methyl maleimide (**6**), and hematinic acid imide methyl ester (**7**). After reaction of **1 a** with ethanethiol either at room temperature or under reflux for 18 – 24 hours, chromic acid degradation still yields **6** and **7** but **5** cannot be detected any more. This proves that the ethylidene group of **1 a** has been quantitatively removed by the reaction with ethanethiol. The expected addition to **3 a/3 b** is proven by detection of the imides **4 a** and **4 b** in the degradation mixture. **4 b** is identified not only by TLC but also by mass spectrometry (Table II). The yield of **4 b** has been higher in all experiments than that of **4 a**. Products of an eventual angular addition of ethanethiol [21] – the thioether formed at first should be oxidized to the sulfone **8** under the applied conditions – cannot be detected in the reaction mixture. However, an unknown product X is formed which migrates in TLC near to **5**. According to its mass spectrum (Table II) it is presumably no succinimide or maleimide. It could be derived from a pigment which has added ethanethiol at one of the methine bridges.

Table II. Mass spectra of sulfone imides **4 a** and **4 b** and unknown compound X.

m/e	Relative intensities			*m/e*	Relative intensity
	4 a *	**4 b** *	**4 b** **		X
233 (M^+)	1	1	ca. 1	181	5
140	78	20	30	169	5
139	23	7	26	139	8
97	13	10	24	138	11
96	31	8	20	131	8
69	100	100	100	119	14
68	40	12	18	101	9
67	45	13	27	96	14
66	15	5	11	69	28
53	38	11	17	68	10
41	78	31	60	67	19
39	35	14	23	41	14
29	41	12	16	32	56
28	61	12	28	28	100
27	44	13	15	18	56

* Synthetic samples [24], source temperature 180 °C.

** Isolated from chromic acid degradation products of **3 a/3 b**, source temperature 210 °C. The main difference between **4 a** and **4 b** is the ratio of peaks at *m/e* 96 and *m/e* 97.



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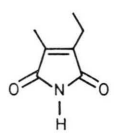

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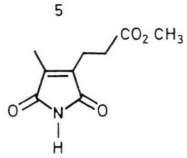

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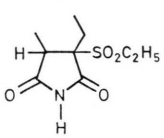

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Discussion

The ethylidene side chain at ring A is unique for plant bile pigments. It is formed during cleavage of the chromophores from the protein by an elimination reaction of the thioether (structure **2**). The formation of the thioether linkage starting with a bile pigment which contains an ethylidene group has never been described although such a reaction has been assumed to be the last step of phycocyanin biosynthesis [20]. It is demonstrated in the present

paper by spectral investigation (Fig. 1, Table I) and thin layer chromatography that phycocyanobilin dimethyl ester reacts with ethanethiol to a variety of pigments. Because some reactions (*e. g.* the well-known reduction and addition at the middle methine bridge [25]) may occur independent of a possible addition at the ethylidene group only the latter question was investigated by analysis of the oxidation products.

The detection of the expected oxidation products **4a** and **4b** clearly proves that the addition of the thiol has occurred at the ethylidene double bond. Furthermore, it shows that the addition occurs at the expected position, namely C-3[1] and not at the angular position C-3. Such a specific addition has been explained in the case of the imide **5** by nucleophilic attack of the thiolate anion to the polarized ethylidene double bond of **5** [21]. It is not clear whether this explanation holds also for the reaction of the pigment **1a** because the ethylidene double bond is not necessarily polarized in **1a**. The thiol apparently attacks **1a** from both sides leading to be stereoisomers **4a** and **4b**. This again is analogeous to the reaction with the imide **5** [17, 18]. However, both reactions are different concerning the relative yield of products: **4a** and **4b** are formed in equal amounts starting from **5**, whereas more **4b** than **4a** is formed from **1a**. This could be due to steric hindrance in **1a** which is not found in **5** or to kinetic selection: if the addition were reversible, the isomer **3b** should be accumulated with time because the elimination (to **1a**) presumably takes place easier with **3a** than with **3b**. Such a difference in the elimination has been found at least with the stereoisomers **4a** and **4b** [18]. The isomers **3a** (with the configuration in the native biliprotein) should be selected during biosynthesis by enzyme specificity.

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