Chromopeptides from Phytochrome and Phycocyanin. NMR Studies of the P_{fr} and P_r Chromophore of Phytochrome and *E,Z* Isomeric Chromophores of Phycocyanin

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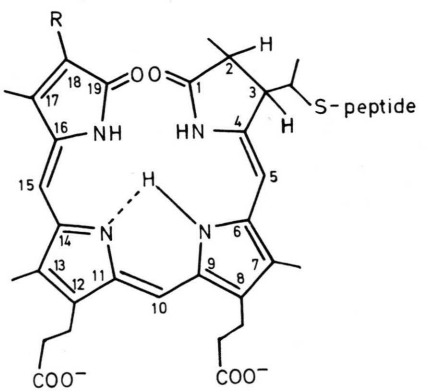
Chromopeptides were prepared by pepsin digestion of C-phycocyanin isolated from the cyanobacterium *Spirulina maxima* and of phytochrome isolated from seedlings of *Avena sativa* L. The chromopeptides were characterized by amino acid analysis. The *ZZZ* configurated chromophore of the phycocyanin peptide was transformed into its *ZZE* configurated isomer by the method of Falk *et al.* (Mh. Chemie **111,** 159–175, 1980) which had previously been applied to biliverdins. The 500 MHz ^{1}H NMR spectrum of the *ZZE* configurated chromopeptides confirmed that its chromophore has the 15 *E* configuration. Irradiation yielded the *ZZZ* configurated isomer for which the ^{1}H NMR spectrum was also recorded. Native phytochrome was irradiated at 660 nm to yield the maximum amount of the P_{fr} from (about 75% of total phytochrome). By digestion in the dark the previously described P_{fr} chromopeptide was obtained. The 500 MHz ^{1}H NMR spectrum was compared with that of the *ZZE* phycocyanin peptide. It confirmed the 15 *E* configuration of the P_{fr} chromopeptide. Irradiation yielded the 15 *Z* configurated P_r chromopeptide. Comparison of the high resolution ^{1}H NMR spectra of P_{fr} and P_r chromopeptides revealed that not only the chromophore resonances but also those of some amino acids are changed by the $P_{fr} \rightarrow P_r$ chromopeptide phototransformation. The results are discussed in terms of chromophore amino acid interaction.

Plant growth and development is effectively influenced by light. The most important photoreceptor for these processes in higher plants is the biliprotein phytochrome [1] which exists in a physiologically inactive form P_r ($\lambda_{max} = 665$ nm) and a physiologically active form P_{fr} ($\lambda_{max} = 730$ nm). Both forms are photoreversible, *i.e.* interconvertible by appropriate light treatments.

The chemical structure of the P_r chromophore, phytochromobilin (**1a**) has been established by investigations of small chromopeptides. On the one hand, the chromophore was cleaved with HBr [2] and identified with phytochromobilin obtained by total synthesis [3]. The nature of the covalent linkage had been established before by studies of elimination reactions after oxidative degradation [4]. On the other hand, high resolution NMR studies of the chromopeptides allowed to deduce the same structure **1a** independently [5]. The structure of the P_{fr} chromophore remained speculative in these studies. Similar studies with phycocyanin, the light harvesting chromoprotein of blue-green algae and red algae [6] showed, that its chromophore (Structure **1b**) differs from phytochromobilin only in the side chain at C-18, which is an ethyl group in phycocyanobilin and a vinyl group in phytochromobilin [7, 8].



1a: R = CH=CH$_2$

1b: R = C$_2$H$_5$

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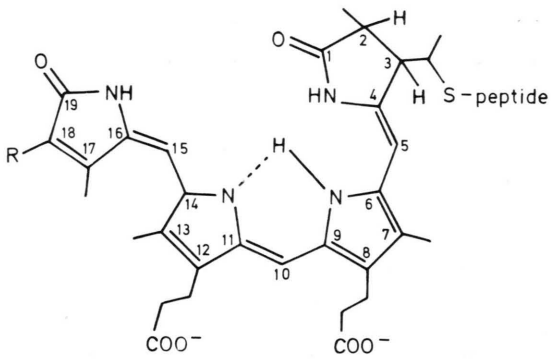


2a : R = $CH{=}CH_2$
2b : R = C_2H_5

Although it has been known since 1971 [9] that small chromopeptides from phytochrome (P_r form) are not photoreversible we succeeded to prepare light sensitive chromopeptides from the P_{fr} form of phytochrome which are different from the above mentioned P_r chromopeptides [10]. Irradiation converted these P_{fr} chromopeptides into P_r chromopeptides without change in the composition of the peptide moiety [10]. The conclusion that the P_{fr} chromophore in these chromopeptides is an *E* isomer (structure **2a** or isomers thereof) of the *ZZZ* configurated P_r chromophore was drawn by comparison of UV-vis spectra of chromopeptides from phytochrome and phycocyanin with an *E*-configurated chromophore (structure **2b** or isomers thereof) on the one hand and P_r and *Z*-configurated phycocyanin chromopeptides on the other hand [11]. *E*-configurated chromopeptides from phycocyanin were obtained from the *Z*-configurated components by a reaction sequence worked out by Falk *et al.* [12] with biliverdins. The *E*-configuration of the phytochrome chromophore was supported by comparison of the methine resonances of high resolution ^{1}H NMR spectra of phytochrome and phycocyanin chromopeptides [13]. We report here the full 500 MHz ^{1}H NMR spectrum of P_{fr} and P_r chromopeptides and of *E* and *Z* configurated chromopeptides from phycocyanin. These data confirm the 15 *E* configuration of the P_{fr} chromophore (structure **2a**) and furthermore indicate configuration dependent interaction of the chromophore with certain amino acids.

Materials and Methods

Small phytochrome (60 KD) was isolated from oat seedlings (*Avena sativa*, Pirol, from BAYWA München) and purified by brushite-chromatography as described [14] with little modifications: In all buffers mercaptoethanol was replaced by sodium sulfite. The brushite-column was equilibrated with 15 mM potassium phosphate buffer pH 7.5. The brushite-chromatography was followed by 50% ammonium sulfate fractionation and the precipitated phytochrome was dissolved in 10 mM tris-HCl/pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM NaN_3 and dialyzed against the same buffer. The samples were stored at − 20 °C in the dark until use. 130 mg of phytochrome were collected and applied to a DEAE-Sepharose-column (Pharmacia, 5 × 20 cm), equilibrated with the dialysis-buffer, then washed with the same buffer and eluted with the equilibration-buffer containing 250 mM KCl. The eluate was concentrated by 50% ammonium sulfate fractionation and the precipitate was dissolved with the equilibration buffer and dialyzed against the same buffer. The resulting solution (172 ml) contains 100 mg phytochrome, purity index $A_{665}:A_{280} = 1:20$. The solution does not contain any reducing agent which has to be avoided during pepsin digestion.

This phytochrome solution was then irradiated to saturation at 660 nm (6 min with a fluence rate of 63 Wm^{-2}; Leitz Prado projector and interference filter 660.3 nm, half width 12.8 nm, Searom, Argenteuil, France). The resulting P_{fr}-solution (ca. 75% P_{fr}, 25% P_r) was acidified with concentrated HCl to a final pH of 1.5, ascorbic acid was added to a final concentration of 50 mM and the mixture was then incubated with 100 mg pepsin (= enzyme-phytochrome ratio of 1:1) for 1 h at 37 °C under argon gas.

The resulting peptides were purified by chromatography on Biogel P-10 and on silica gel as described earlier [11]. The silica gel with the adsorbed P_{fr} chromopeptide was transfered into a small glass funnel. It was first washed with water and then with d_6-acetone to remove the non-deuterated water. The P_{fr} chromopeptide was desorbed from the silica gel with a mixture of d_6-acetone/H_2O/CF_3COOH (85:10:5, v:v:v) and stored in this solution under argon gas at − 18 °C until it was used for NMR measurements. Aliquots were taken for UV-Vis spectra and for amino acids analysis (see Tab. III).

The peptide concentration in the NMR tube was 9.4×10^{-5} M calculated from the UV-Vis spectrum with $\varepsilon_{660} = 32\,000$ [9].

C-Phycocyanin from *Spirulina maxima* was isolated and digested with pepsin as previously described [11]. Purification of chromopeptides by chromatograph on Biogel P-10 and silica gel and by isoelectric focusing was also performed as described [11]. The chromopeptide used in this study was the fraction which focused at pH 5.03. Servalyte was removed by washing the chromopeptide fraction adsorbed on a silica gel column with 0.5 N HCl. The chromopeptide was eluted with 50% aqueous formic acid and then lyophilized.

For photoisomerization, 2.6×10^{-3} mol purified chromopeptide were dissolved in the mixture of 0.1 M Tris-HCl (pH 8.5)/mercaptoethanol (3:2, v:v), illuminated and separated from mercaptoethanol by chromatography on Biogel P-10 and silica gel as previously described [11]. The *ZZE*-configurated chromopeptide which was adsorbed at silica gel was then treated in the same way as described for the P_{fr} chromopeptide, but desorption was achieved with a mixture of d_6-acetone/H_2O/CF_3OOH (80:10:10, v:v:v). The solution was stored under argon gas at − 18 °C until it was used for NMR measurements. Aliquots were taken for amino-acid analysis (see Table III). The peptide concentration in the NMR tube was 1.30×10^{-4} calculated from the UV-Vis spectrum with $\varepsilon_{665} = 35\,500$ [15]. The 500 MHz ^{1}H NMR spectra were recorded with a Bruker WM 500 FT NMR (quadrature detect.) with Aspect 2000 data system at 10 °C at Fa. Bruker, Analytische Meßtechnik, Karlsruhe. The H_2O peak was reduced with the gated decoupling method.

The chemical shift values are in ppm sodium trimethylsilyl propansulfonate (TPS) and acetone was used as the internal standard (2.050 ppm). Several tests verified that chemical shift from acetone is independent from temperature and concentration of the components of the solvent system used.

Results and Discussion

High resolution ^{1}H NMR spectra had already been obtained for a *ZZZ* phycocyanin peptide [7] and for a P_r peptide [5]. Both measurements were performed in pyridine-d_5 and in pure D_2O. These solvents are unsuitable for our purpose because P_{fr} peptides require an acid medium for stabilization [10, 11]. As outlined earlier [13] the solvent system acetone-d_6/water/trifluoracetic acid was designed for our measurements. It meets the requirements for stability of P_{fr} peptides (**2a**) and *ZZE* phycocyanin peptides (**2b**) and overcomes the problem of protoncatalyzed exchange of H-5 and, furthermore, elutes the chromopeptides from silica gel columns.

At first, ^{1}H NMR spectra of **2a** and **2b** were recorded. Since these compounds are light sensitive, they were handled, and their spectra recorded in the dark or under dim green safelight. Furthermore, the spectra were recorded at 10 °C to avoid too much of temperature dependent reversion during measurement [10]. Subsequently, the compounds were irradiated in the NMR tubes with white light to form **1a** and **1b**, respectively. Their ^{1}H NMR spectra were recorded immediately after this phototransformation.

For easier comparison the NMR spectra are arranged in the following manner: Fig. 1 displays the δ range downfield from the H_2O solvent peak at 5.33 ppm, namely 9.05 to 5.70 ppm, Fig. 2 contains the δ range upfield from the H_2O solvent peak namely 4.50 to 0.70 ppm. In Fig. 2 the δ range of the acetone solvent peak from 2.20 to 1.90 ppm was spared out. In each figure the spectra labelled A and A′ represent non-irradiated and irradiated phytochrome peptides, the spectra labelled B and B′ represent the corresponding samples of phycocyanin peptide. The ^{1}H NMR resonance frequencies assigned to the chromophores are found in Table I, the resonances assigned to the peptide moities are listed in Table II.

Bilin moiety of phycocyanin peptide

The ^{1}H NMR spectrum of the *ZZZ* configurated peptide (**1b**) is shown in Figs. 1−2B′, that of the *EZZ* configurated peptide (**2b**) is shown in Figs. 1−2B. Since our preparation of **2b** contains some **1b** due to dark reversion of **2b** [10] its ^{1}H NMR spectrum contains resonances of both **1b** and **2b**. The assignment of the methine resonances 5, 10, and 15 was achieved by comparison with suitable model compounds as described in detail in [13] and [16]. The assignment of the other resonances was based on chemical shifts and characteristic

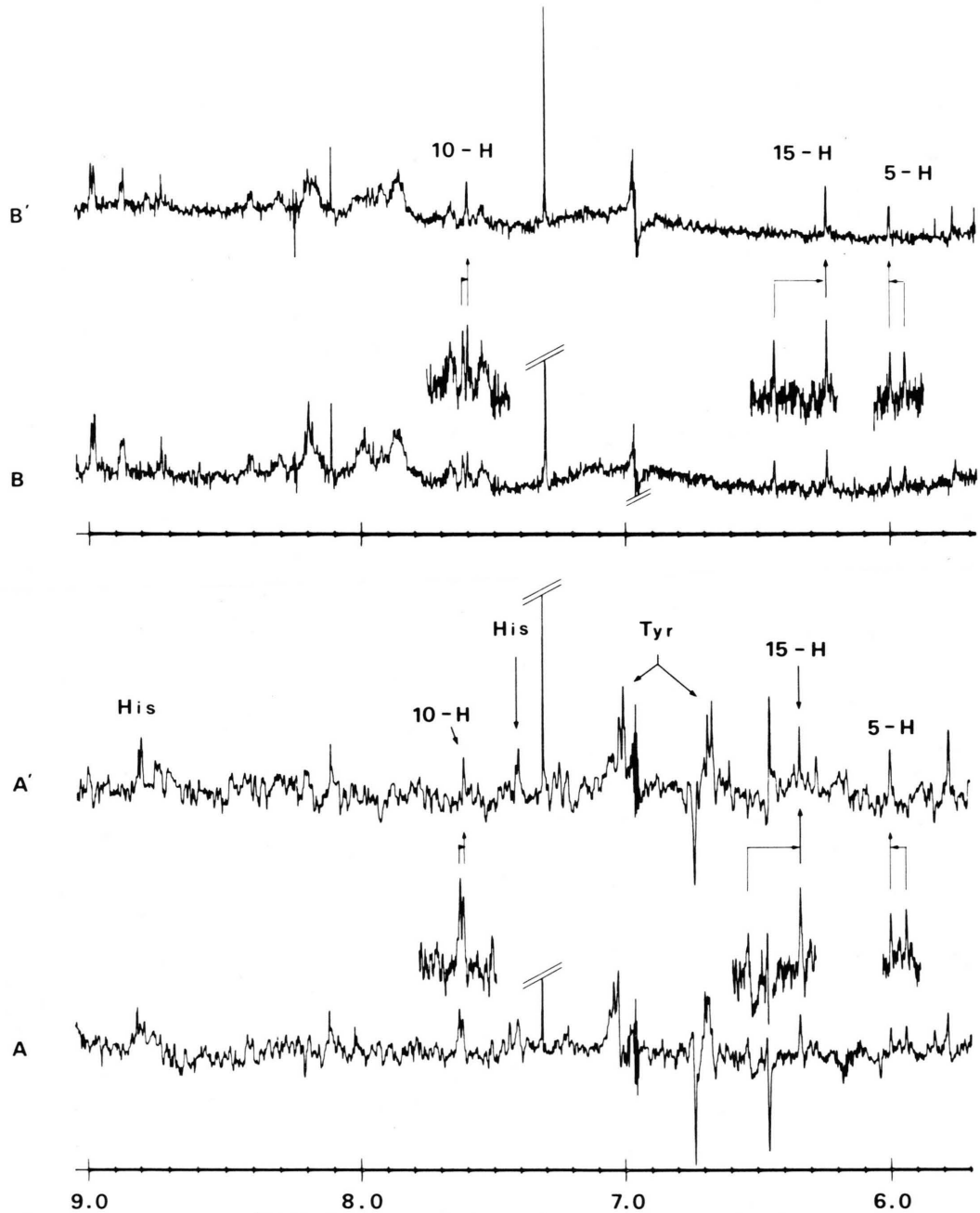


Fig. 1. Partial 500 MHz ^{1}H NMR spectra of phytochrome and phycocyanin chromopeptides in acetone-d_6/H_2O/CF_3COOH at 10 °C in the range of 9.05 to 5.70 ppm. A) P_{fr} chromopeptide (**2a**) containing some P_r chromopeptide (**1a**) due to dark reversion. A') P_r chromopeptide (**1a**) obtained from A) by irradiation with white light. B) *E*-phycocyanin peptide (**2b**) containing some *Z*-phycocyanin peptide (**1b**) due to dark reversion. B') *Z*-phycocyanin peptide (**1b**) obtained from B) by irradiation with white light.

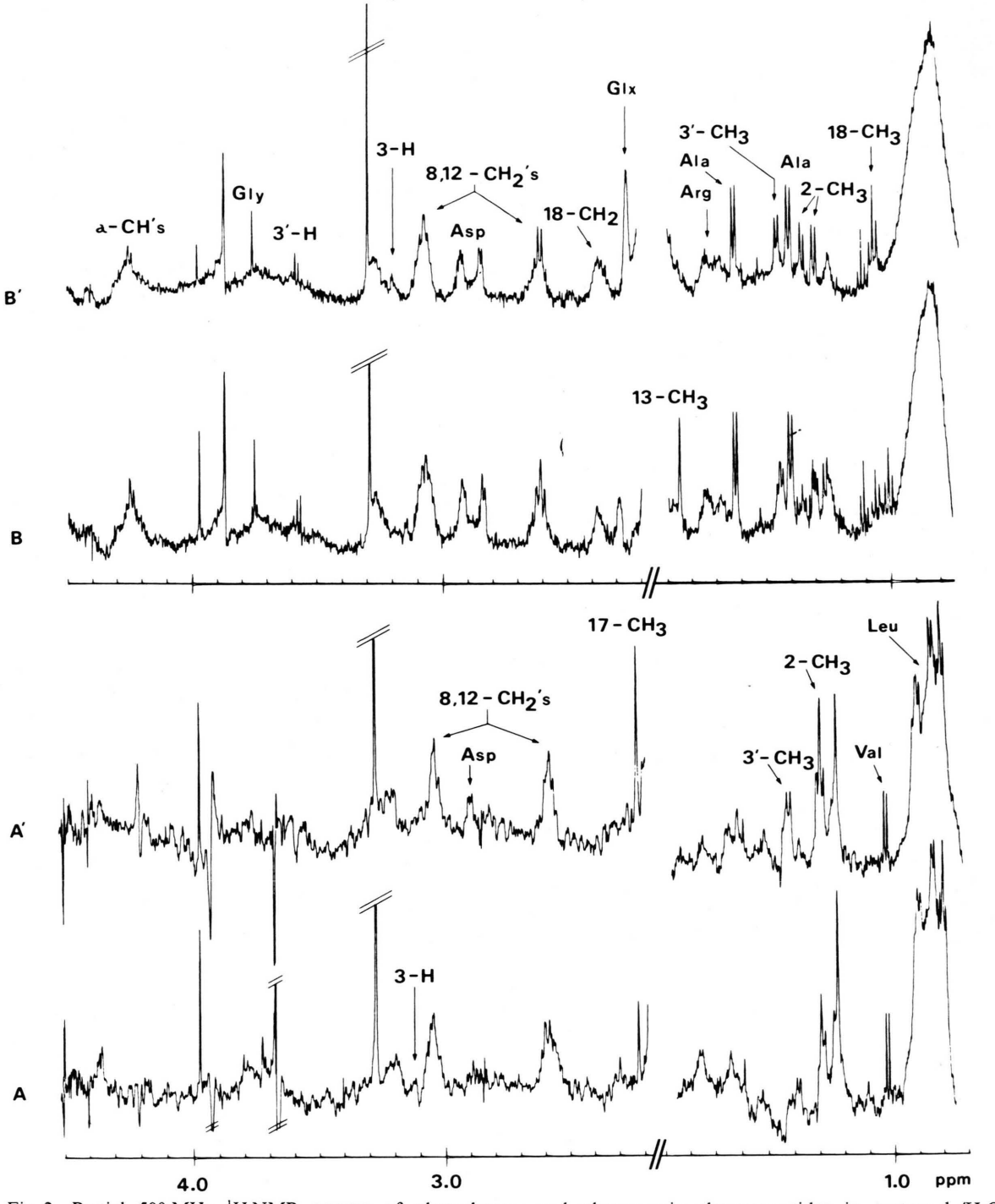
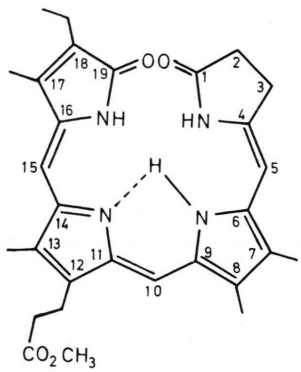


Fig. 2. Partial 500 MHz ^{1}H NMR spectra of phytochrome and phycocyanin chromopeptides in acetone-d_6/H_2O/CF_3COOH at 10 °C in the range of 4.50 to 0.70 ppm. The δ range of the acetone solvent peak from 2.20 to 1.90 ppm is spared out. Increase of the base line in the region upfield from about 1 ppm is due to a specific impurity in the probe head. A) P_{fr} chromopeptide (**2a**) containing some P_r chromopeptide (**1a**) due to dark reversion. A') P_r chromopeptide (**1a**) obtained from A) by irradiation with white light. B) *E*-phycocyanin peptide (**2b**) containing some *Z*-phycocyanin peptide (**1b**) due to dark reversion. B') *Z*-phycocyanin peptide (**1b**) obtained from B) by irradiation with white light.

multiplicities (Table I). Furthermore, the differences in the ^{1}H NMR spectra of the *E* and *Z*-configurated phycocyanin peptides were compared with these of reported 15 *E* and all *Z* configurated model chromophores (structures **3** and **4**; [17, 18]). The final assignment would require special NMR techniques which are not available to us. These techniques had, however, been previously applied to a *Z*-configurated phycocyanin peptide [7]. Comparison of these data with our measurements of **1b** shows a good correlation (see Table I). Differences between our data and the data reported for D_2O are within $\pm$ 0.1 ppm with the exception of 10-H, for which a difference of $-$ 0.15 ppm is observed.



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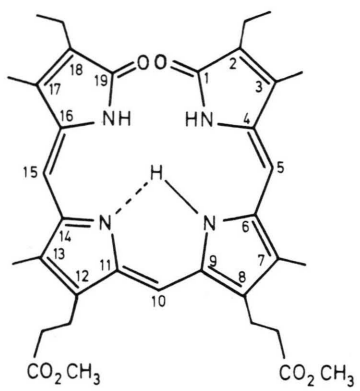

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Since only *ZZE* compounds are light sensitive, the resonances which are specific for **2b** disappear upon irradiation, whereas the resonances of **1b** increase (Compare Figs. 1–2B and B′). This facilitates the assignment of *ZZE* and *ZZZ* specific NMR resonances.

This is especially true for the methine resonances (see Figs. 1B and B′). Their assignment has been reported earlier [13] and is shown in Table 1. The triplet at 1.006 ppm was assigned to the methyl protons of the 18-ethyl group of **2b**. It disappears upon irradiation whereas the triplet at 1.059 ppm (18-CH_2–CH_3 of **1b**) increases. The difference of chemical shifts ($\Delta\delta$ **2b** minus **1b**: $-$ 0.053 ppm) corresponds to the differences reported for model compounds **3** ($\Delta\delta$: $-$ 0.05 ppm) and **4** ($\Delta\delta$: $-$ 0.03 ppm, 17, 18). The mixture of **1b** and **2b** exhibits four doublets in the region of 1.40 to 1.20 ppm, which overlap partly (and partly with a solvent resonance at 1.24 ppm) and coincide upon irradiation into two doublets of **1b** at 1.345 and at 1.295 ppm by a parallel shift ($\Delta\delta$: 0.04 ppm). Earlier NMR measurements [7] had revealed only one doublet for 2-CH_3. Contrary to those measurements, our *ZZE* configurated chromopeptide **2b** was prepared photochemically from **1b** according to the method of Falk *et al.* [12]. If such a reaction would be accompanied by an epimerisation at C-2, one would expect two resonances for the 2-CH_3 protons. The presence of four doublets in the spectrum of the mixture of **1b** and **2b** implicates, that the splitting of the 2-CH_3 resonance is present in the *E*-compound **2b**, too, and does not occure during reversion to the *Z*-compound **1b**. This question has to be investigated further.

The multiplet at 1.44 ppm yields a doublet at 1.448 ppm upon irradiation. The doublet was assigned to 3′-CH_3 protons (Table I). The multiplet most likely consists of the two overlapping doublets of **1b** and **2b** for 3′-CH_3 protons. The difference of the chemical shift is only about $-$ 0.01 ppm. Irradiation also leads to disappearance of the resonance at 3.14 ppm with corresponding increase of a resonance at 3.19 ppm. We have provisionally assigned these resonances to 3-H of **2b** and **1b** (Table I) although it cannot be excluded that they belong to cysteine β-CH_2 or arginine δ-CH_2 [7] (see Table II).

Upon irradiation, the singulet at 1.849 ppm disappears and is, therefore, a resonance specific for **2b**. It is tentatively assigned to the 17-CH_3 protons, because with respect to 7, 13, and 17-CH_3 resonances in model compounds **3** and **4**, the 17-CH_3 resonance shows the most pronounced shift ($\Delta\delta$: $-$ 0.16 and $-$ 0.07, respectively) upon transfor-

mation of the 15 *E* to the all *Z* compound. The corresponding resonance of **1b** is possibly covered by the solvent peak in our spectrum.

The resonances of the other aromatic methyl protons of **1b** and **2b** are also very close to the solvent peak in the region around 2 ppm. Their exact position and assignment is, therefore, uncertain.

Another resonance which disappears upon irradiation is that at 2.29 ppm. It was tentatively assigned to methylene protons of the 18 ethyl group of **2b** because the intensity of the multiplet at 2.352 ppm (18-CH_2–CH_3 of **1b**) is increased upon irradiation. However, the decrease in intensity at 2.29 ppm seems to be larger than the increase at 2.352 ppm. Furthermore, our assignment would mean a $\Delta\delta$ of -0.06 ppm, whereas values of $+0.11$ ppm were reported for **3** and $+0.01$ ppm for **4**. If we suppose a similar shift of the methylen-protons in our peptide their resonance would be hidden by the solvent peak in the *Z*-compound. Their final assignment, therefore, needs further experimentation. No shift can be detected in the resonance of the methylene protons of propionic acid side chains (2.61 and 3.07 ppm), and 3'H (3.58 ppm). However, the pattern of these resonances is clearly changed by irradiation. The resonance for the 2-H is probably covered by α-CH_2 resonances of the propionic acid side chains in both compounds (*cf.* [7]) and is therefore, probably only slightly shifted during the light induced reversion of **2b** and **1b**. The reaction sequence for $Z \rightarrow E$ isomerisation of phycocyanin peptide could theoretically lead to the 4 *E* or 15 *E* isomer [11]. The reaction sequence with 2.3 dihydrobilindiones, however, yields only 15 *E* compounds ([16] and H. Falk, personal communication). The ^{1}H NMR data of the *E* and *Z*-configurated phycocyanin peptide, especially the chemical shifts of the methine protons [13] clearly demonstrates that only the 15 *E* compound was obtained (structure **2b**).

Bilin moiety of phytochrome peptide

The ^{1}H NMR spectrum of the P_r peptide (**1a**) is shown in Fig. 1–2, A'. The low concentration of the phytochrome peptide (9.4×10^{-5} M) is the reason for its weak resonances in the ^{1}H NMR spectrum. Therefore, their assignment is more difficult than that of the phycocyanin peptide. As the chromophore resonances are very similar for *ZZ* phycocyanin peptide (**1b**) and **1a** as determined in pyri-

Table I. 500 MHz ^{1}H NMR assignments of the bilin moiety of chromopeptides from phycocyanin and phytochrome in aceton-d_6/H_2O/CF_3COOH.

Chemical Shift[a], Multiplicity and *J* [Hz]				Assignment	Chemical Shift Multiplicity and *J* [Hz] of Phycocyanin Peptide in D_2O [7]	
Phycocyanin Peptide		Phytochrome Peptide				
ZZZ (**1b**)	*ZZE* (**2b**)	P_r (**1a**)	P_{fr} (**2a**)			
1.295 d (7.5) 1.345 d (7.5)	1.255 d (7.5) 1.304 d (7.5)	1.29 m	1.24 m	2-CH_3	1.21	d (7.3)
1.448 d (6.8)	1.44 m	1.417 d (6.8)	1.386 d (6.8)	3'-CH_3	1.44	d (7.1)
⟨2.085 s⟩	1.849 s	⟨2.084 s⟩	n.d.		2.11	s
n.d.	n.d.	⟨2.089 s⟩	⟨2.098 s⟩	7,13,17-CH_3's	2.15	s
n.d.	n.d.	2.221 s	n.d.		2.17	s
1.059 t (7.5)	1.006 t (7.5)	–	–	18-CH_2–CH_3	1.02	t (7.4)
⟨2.35 m⟩	⟨2.29 m⟩	–	–	18-CH_2–CH_3	2.23	q
2.61 m	2.61 m	2.58 m	2.58 m	8,12-CH_2–CH_2–CO_2H	2.69	m
3.07 m	3.07 m	3.04 m	3.04 m	8,12-CH_2–CH_2–CO_2H	3.11	m
⟨2.61⟩	⟨2.61⟩	⟨2.58⟩	⟨2.58⟩	2-H	2.69	dq
⟨3.19 m⟩	⟨3.14 m⟩	⟨3.20⟩	⟨3.12 m⟩	3-H	3.11	m
3.58	3.58 m	n.d.	n.d.	3'-H	3.58	m
5.999 s	5.943 s	5.995 s	5.938 s	5-H	5.94	s
6.239 s	6.434 s	6.538 s	6.533 s	15-H	6.32	s
7.601 s	7.619 s	7.618 s	7.630 s	10-H	7.41	s

[a] The chemical shifts in aceton-d_6/H_2O/CF_3COOH are reported in parts per million from aceton-d_6 (2.050 ppm); n.d. = not detected; ppm values in ⟨ ⟩: assignment uncertain.

dine-d_5 [5, 7], we were able to assign the chromophore resonances of **1a** (Table I) by comparison with **1b** in our solvent mixture, because the reported similarities of both chromopeptides are apparently also found in our solvent system. In particular, methine protons at C-5, C-10 and C-15 were assigned by comparison with phycocyanin peptide and other model compounds [13, 16]. In the same way, other resonances of the P_r chromophore were assigned: The multiplets at 2.58 and 3.04 ppm to the methylene protons of propionic acid side chains, the doublet at 1.417 ppm to the 3′-CH_3 protons. The singulet at 2.221 ppm which increases upon irradiation is provisionally assigned to the 13-methyl protons because it is the most downfield of all aromatic methyls in pyridine-d_5 [5]. The resonances of the 17- and 7-CH_3 protons are only detected as singulets on the steep flank of the hugh acetone peak (not included in Fig. 2).

The 3′H is not detected because of the high noise level of the NMR spectrum. For the 2-CH_3 protons, we observe a multiplet at 1.29 ppm instead of the expected doublet [5]. This could be due either to splitting of the 2-CH_3 resonance (see under phycocyanin peptide) or to overlapping with another, unidentified resonance (for example the β-CH_3 protons of alanine). We consider resonance splitting due to epimerization of 2-CH_3 the less likely possibility: (i) The distance of doublets would be smaller in P_r peptide than in *ZZZ* phycocyanin peptide, (ii) the described photochemical reaction sequence was only applied to the phycocyanin peptide but not to the P_r peptide.

In accordance with the results of Lagarias and Rapoport [5], we do not find any resonance which could be attributed to an ethyl group. Since the ethyl group of phycocyanobilin is substituted by a vinyl group in phytochromobilin, we expect corresponding resonances in the range 5 – 7 ppm [5]. The assignment of resonances in this region of the spectrum (Fig. 1, A′) to the vinyl group is not unequivocal because of the high multiplicity of these resonances and the high noise level in the NMR spectrum.

Our preparation of P_{fr} chromopeptides (**2a**) contains some **1a** due to dark reversion. Therefore, the ^{1}H NMR spectrum (Fig. 1 – 2, A) contains resonances of **2a** and **1a**. Upon irradiation, the P_{fr} specific resonances disappear whereas P_r specific resonances increase. This was reported earlier [13] for the methine protons at C-5 (singulet of **2a** at 5.938 ppm), C-15 (singulet at 6.533 ppm), and C-10 (singulet at 7.630 ppm), and clearly observed for the 3′CH_3 protons (multiplet at 1.39 ppm).

The expected doublet of 2-CH_3 protons partially overlaps with a solvent peak at 1.23 ppm which was also detected in phycocyanin peptides. However, disappearance of part of the 2-CH_3 resonance at 1.243 ppm upon irradiation can be observed. This is accompanied by a corresponding increase of the P_r specific resonance at 1.29 ppm. Also the small multiplet at 3.12 ppm disappears upon irradiation. It is assigned to 3-H of **2a** but could also be assigned to cysteine or arginine protons (see discussion of phycocyanin peptide). The corresponding signal of **1a** is probably covered by a resonance at 3.20 ppm (not identified). The resonance of methylene protons of propionic acid side chains (2.58 and 3.04 ppm) and 2-H (2.58 ppm) are not shifted but change their pattern upon irradiation.

In summary, our NMR data demonstrate a striking similarity between *Z* phycocyanin peptide (**1b**) and P_r peptide (**1a**) on the one hand and between 15 *E* phycocyanin peptide (**2b**) and P_{fr} peptide on the other hand. It is, therefore, evident that the P_{fr} chromophore is the 15 *E* compound (structure **2a**). According to Lagarias and Rapoport [5], the aromatic methyl protons are downfield shifted by 0.01 – 0.04 ppm in **1a** versus **1b**. Such a shift could explain why solvent peaks (which are at the same position) can overlap with different resonances in phycocyanin and phytochrome peptides. This is apparently true for the singulet at 1.849 ppm in **2b** for which the counterpart in **2a** cannot be detected and for the singulet at 2.221 ppm in **1a** for which no corresponding resonance in **1b** is detected. The singulet at 2.25 ppm in the latter peptide is assigned to amino acid resonances (for instance the β-CH_2 of Glx, see below).

Amino acid moiety of phytochrome and phycocyanin peptides

For the assignment of the amino acid resonances in the ^{1}H NMR spectrum, knowledge of the amino acid composition of the investigated chromopeptide fractions is essential. Table III contains the data on the amino acid analyse of those chromopeptide fractions from phycocyanin and phytochrome which were used for NMR. Cysteic acid is derived by

Table II. 500 MHz ^{1}H NMR assignments of the peptide of chromopeptide from phycocyanin, phytochrome and of free amino acids in acetone-d_6/H_2O/CF_3COOH and 360 MHz ^{1}H NMR assignment of the peptide moieties of phycocyanin peptide in D_2O. Chemical shift, Multiplicity and *J* [Hz].

Phycocyanin peptide	Phytochrome peptide	Free amino acids[a]	Phycocyanin peptide [7]	Assignment	
Acetone-d_6/H_2O/CF_3COOH			D_2O		
–[b]	0.900 m	0.95 d (5.9)	0.77 d (5.3)	Leu^{1-3}	δ_1-CH_3
	0.840 d (5.4)	0.95 d (5.9)	0.83 d (5.3)	Leu^1	δ_2-CH_3
	0.815 d (5.4)			Leu^2	δ_2-CH_3
	0.793 d (5.4)			Leu^3	δ_2-CH_3
–	1.028 d (6.1)	1.09 (7.0)	–	Val	γ_1/γ_2-CH_3
1.401 d (7.5)	n.d.	1.61 d (7.4)	1.29 d (7.2)	Ala^1	β-CH_3
1.622 d (7.5)	n.d.		1.51 d (7.2)	Ala^2	β-CH_3
1.65–1.80	n.d.	1.6–2.0	1.54–1.64	Arg	β/γ-CH_2
2.25	n.d.	2.32 m	–	Glx	β-CH_2
2.84 m	2.90 m	3.13 d (5.4)	2.77 dd (7.8/16.8) β_1 2.87 dd (5.9/16.8) β_2	Asx^1	β-CH_2
2.92 m	–		–	Asx^2	β-CH_2
3.20–3.30	3.15–3.25	3.66 dd (4.0/7.6)	3.02 dd (7.6/13.9) β_1 3.11 dd (6.8/13.9) β_2	Cys	β-CH_2
		3.39 t (5.6)	3.11 t (6.8)	Arg	δ-CH_2
–		3.22 dd (2.8/6.5)	–	Tyr	β-CH_2
3.750 s	n.d.	3.95 s	–	Gly	α-CH_2
4.15–4.35	n.d.	4–5	4.1–4.6	amino acids	α-CH's
–	6.677 d (8.8)	6.75 d (8.7)	–	Tyr	ring H's (a)
	7.016 d (8.8)	7.15 d (8.5)	–	Tyr	ring H's (b)
–	7.42 m	7.59 d (1.4)	–	His	ring H (a)
	8.81 m	8.87 d (1.4)	–	His	ring H (b)

[a] The data were taken from 80 MHz ^{1}H NMR spectra which were taken on a Bruker WP 80 FTNMR with Aspect 2000 data system at 35 °C.
[b] – = amino acid not present in the peptide.
n.d. = not detected.

Table III. Amino acid analyses.

Amino acid	Chromopeptide from phycocyanin determined (nearest)	Chromopeptide from phytochrome determined (nearest)
Cya[a]	0.34 (1)	0.53 (1)
Asx	2.07 (2)	0.75 (1)
Thr	0	0.28
Ser	0	0.95 (1)
Glx	0.77 (1)	1.94 (2)
Gly	0.90 (1)	0.86 (1)
Ala	1.80 (2)	1.80 (2)
Val	0	0.57 (1)
Ile	0	0.37
Leu	0	2.77 (3)
Tyr	0	0.75 (1)
Phe	0	0.39
His	0	2.46 (2)
Lys	1.30 (1)	0.42
Arg	1.04 (1)	1.87 (2)

[a] Abbreviation: Cya = cysteic acid. Traces of cystine are found in addition (not given in Table).

autoxidation from cysteine which links the peptide chain with the tetrapyrrole chromophore. Because cleavage of the chromophore from cystein is incomplete under the applied conditions, only a small yield of cysteic acid appears in the analyses. Lagarias *et al.* [5] obtained a mixture of several chromopeptides with varying peptide length after digestion of phytochrome with pepsin and thermolysin. Subsequent purification was necessary to obtain single pure chromopeptides. We performed only a short digestion with pepsin and a rapid chromopeptide purification in the case of P_{fr} due to the instability of **2a** [10]. In the case of phycocyanin, we applied exhaustive purification of chromopeptides (see Material and Methods). This difference in preparation is reflected by amino acid analysis which shows contaminating amino acids in **2b** but not in **1b** (Table III).

The assignment of the peptide resonances (Table II) is based on comparison with the known data of the chromopeptides in other solvents [5, 7] and on control measurements of corresponding free amino acids in our solvent system. The ^{1}H NMR data of the amino acids correspond throughout with the results of amino acid analysis (Table III) with only a few exceptions: whereas the resonances for 2 alanines are present in the ^{1}H NMR spectrum of the phycocyanin peptide, alanine signals are not detected with certainty in the spectrum of phytochrome peptide. Other resonances which are uncertain in the spectrum of phytochrome peptide (but detected in the spectrum of phycocyanin peptide) are those of glutamic acid β-CH_2 aspartic acid β-CH_2, glycine α-CH_2, arginine β,γ-CH_2 and the α-CH's of all amino acids. The expected position of the latter resonances is very close to a solvent peak and is, therefore, eventually hidden by this strong peak. The resonances at 8.81 and 7.42 ppm have been provisionally assigned to the ring protons of histidines. We observe rather broad resonances (contrary of free histidine) because of slightly different chemical shifts of both histidines in our peptide. Both resonances show similar shifts upon irradiation (see Fig. 1 A and 1 A'). The resonance at 7.016 and 6.677 ppm are assigned to the ring protons of tyrosine. They also behave similarly during the P_{fr} peptide $\rightarrow$ P_r peptide transformation. Interestingly, no significant differences in the shifts of amino acid residues were found between the *E* and *Z* configurated chromopeptides from phycocyanin except the resonance at 2.25 ppm in the phycocyanin spectrum which appears upon irradiation. It seems rather to be an amino acid resonance (for instance the β-CH_2 of Glx) than that of aromatic methyl protons which give sharp singulets. Minor but significant differences were found in the chemical shifts of P_{fr} and P_r resonances assigned to histidine and tyrosine (see above). If the $E \rightarrow Z$ isomerisation of the chromophore changes the environment of the amino acid residues, the chromophore must be very close to the amino acid side groups (most pronounced with respect to histidine). We have recently demonstrated [19] that interaction between histidine and tetrapyrrol chromophores can occur. This interaction is probably of charge-transfer type [19]. Our presented data agree with such an interaction in the phytochrome peptide. The histidine residues are good candidates to play a role in the photoisomerisation $P_{fr} \rightleftharpoons P_r$. This possibility is presently being investigated further.

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