

# 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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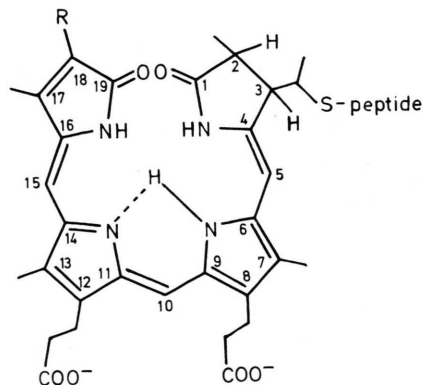
Bilipeptides, C-Phycocyanin, High Resolution NMR Spectra, Photoisomerization, Phytochrome

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  $ZZZ$  configured chromophore of the phycocyanin peptide was transformed into its  $ZZE$  configured isomer by the method of Falk *et al.* (Mh. Chemie **111**, 159–175, 1980) which had previously been applied to biliverdins. The 500 MHz  $^1\text{H}$  NMR spectrum of the  $ZZE$  configured chromopeptides confirmed that its chromophore has the 15  $E$  configuration. Irradiation yielded the  $ZZZ$  configured isomer for which the  $^1\text{H}$  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  $^1\text{H}$  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  $Z$  configured  $P_r$  chromopeptide. Comparison of the high resolution  $^1\text{H}$  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  $P_r$  ( $\lambda_{\text{max}} = 665$  nm) and a physiologically active form  $P_{fr}$  ( $\lambda_{\text{max}} = 730$  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].

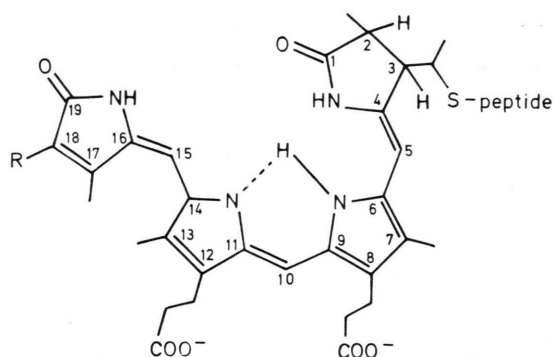


**1a** : R = CH = CH<sub>2</sub>

**1b** : R = C<sub>2</sub>H<sub>5</sub>

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2a : R = CH=CH<sub>2</sub>

2b : R = C<sub>2</sub>H<sub>5</sub>

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  $ZZZ$  configured  $P_r$  chromophore was drawn by comparison of UV-vis spectra of chromopeptides from phytochrome and phycocyanin with an  $E$ -configured chromophore (structure **2b** or isomers thereof) on the one hand and  $P_r$  and  $Z$ -configured phycocyanin chromopeptides on the other hand [11].  $E$ -configured chromopeptides from phycocyanin were obtained from the  $Z$ -configured 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  $^1\text{H}$  NMR spectra of phytochrome and phycocyanin chromopeptides [13]. We report here the full 500 MHz  $^1\text{H}$  NMR spectrum of  $P_{fr}$  and  $P_r$  chromopeptides and of  $E$  and  $Z$  configured chromopeptides from phycocyanin. These data confirm the  $15E$  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<sub>3</sub> and dialyzed against the same buffer. The samples were stored at  $-20^\circ\text{C}$  in the dark until use. 130 mg of phytochrome were collected and applied to a DEAE-Sepharose-column (Pharmacia,  $5 \times 20$  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  $A_{665} \cdot A_{280} = 1:20$ . 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  $63 \text{ W m}^{-2}$ ; 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  $37^\circ\text{C}$  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 transferred 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/ $\text{H}_2\text{O}/\text{CF}_3\text{COOH}$  (85:10:5, v:v:v) and stored in this solution under argon gas at  $-18^\circ\text{C}$  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  $9.4 \times 10^{-5}$  M calculated from the UV-Vis spectrum with  $\epsilon_{660} = 32\,000$  [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,  $2.6 \times 10^{-3}$  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-configured chromopeptide which was adsorbed at silica gel was then treated in the same way as described for the P<sub>fr</sub> chromopeptide, but desorption was achieved with a mixture of d<sub>6</sub>-acetone/H<sub>2</sub>O/CF<sub>3</sub>OOH (80:10:10, v:v:v). The solution was stored under argon gas at  $-18^\circ\text{C}$  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  $1.30 \times 10^{-4}$  calculated from the UV-Vis spectrum with  $\epsilon_{665} = 35\,500$  [15]. The 500 MHz <sup>1</sup>H NMR spectra were recorded with a Bruker WM 500 FT NMR (quadrature detect.) with Aspect 2000 data system at  $10^\circ\text{C}$  at Fa. Bruker, Analytische Meßtechnik, Karlsruhe. The H<sub>2</sub>O 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 <sup>1</sup>H NMR spectra had already been obtained for a ZZZ phycocyanin peptide [7] and for a P<sub>r</sub> peptide [5]. Both measurements were performed in pyridine-d<sub>5</sub> and in pure D<sub>2</sub>O. These

solvents are unsuitable for our purpose because P<sub>fr</sub> peptides require an acid medium for stabilization [10, 11]. As outlined earlier [13] the solvent system acetone-d<sub>6</sub>/water/trifluoroacetic acid was designed for our measurements. It meets the requirements for stability of P<sub>fr</sub> 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, <sup>1</sup>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  $10^\circ\text{C}$  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 <sup>1</sup>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  $\delta$  range downfield from the H<sub>2</sub>O solvent peak at 5.33 ppm, namely 9.05 to 5.70 ppm, Fig. 2 contains the  $\delta$  range upfield from the H<sub>2</sub>O solvent peak namely 4.50 to 0.70 ppm. In Fig. 2 the  $\delta$  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 <sup>1</sup>H NMR resonance frequencies assigned to the chromophores are found in Table I, the resonances assigned to the peptide moieties are listed in Table II.

### *Bilin moiety of phycocyanin peptide*

The <sup>1</sup>H NMR spectrum of the ZZZ configured peptide (**1b**) is shown in Figs. 1–2B', that of the EZZ configured peptide (**2b**) is shown in Figs. 1–2B. Since our preparation of **2b** contains some **1b** due to dark reversion of **2b** [10] its <sup>1</sup>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

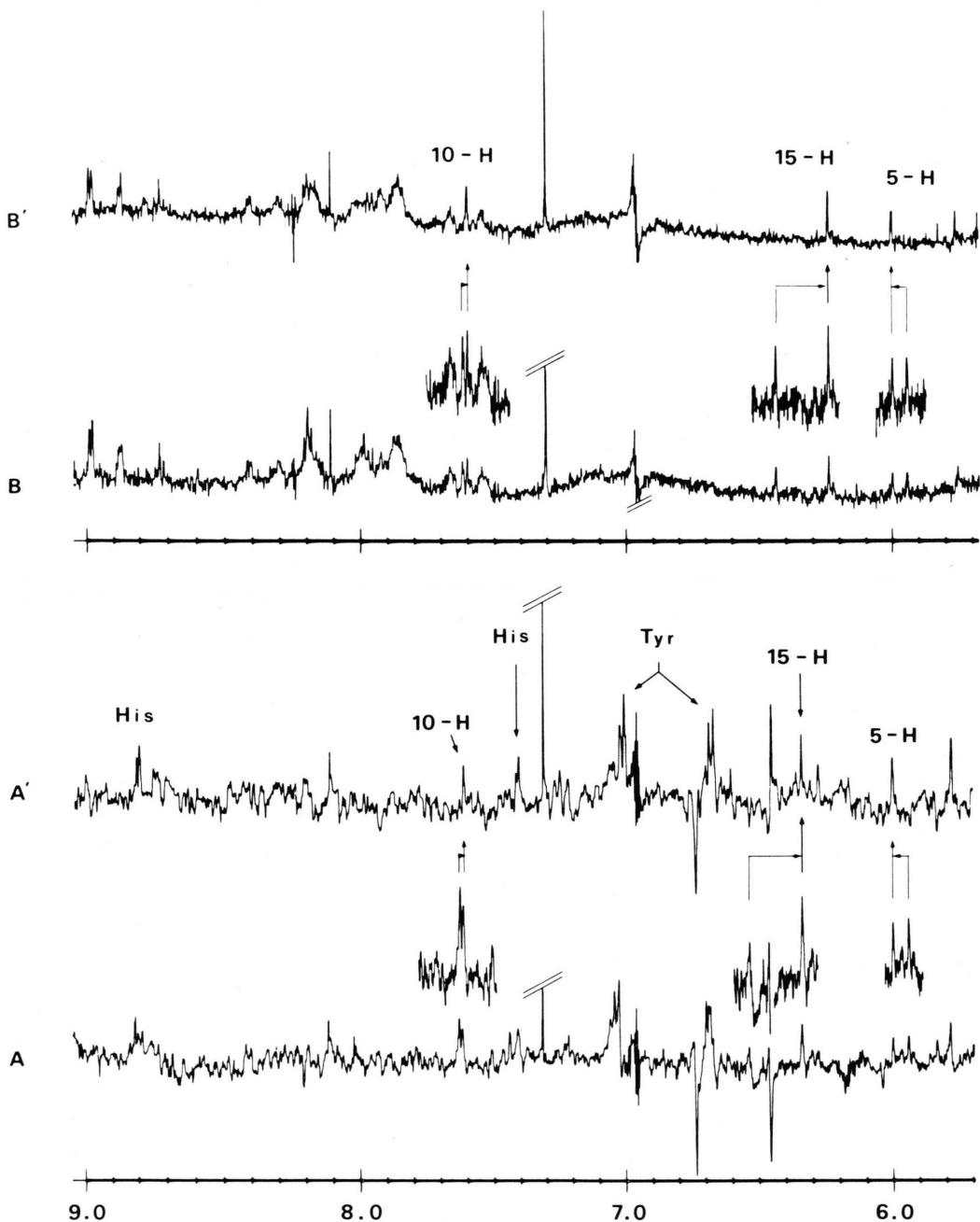


Fig. 1. Partial 500 MHz  $^1\text{H}$  NMR spectra of phytochrome and phycocyanin chromopeptides in acetone- $\text{d}_6/\text{H}_2\text{O}/\text{CF}_3\text{COOH}$  at  $10^\circ\text{C}$  in the range of 9.05 to 5.70 ppm. A)  $\text{P}_{\text{fr}}$  chromopeptide (**2a**) containing some  $\text{P}_{\text{r}}$  chromopeptide (**1a**) due to dark reversion. A')  $\text{P}_{\text{r}}$  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.



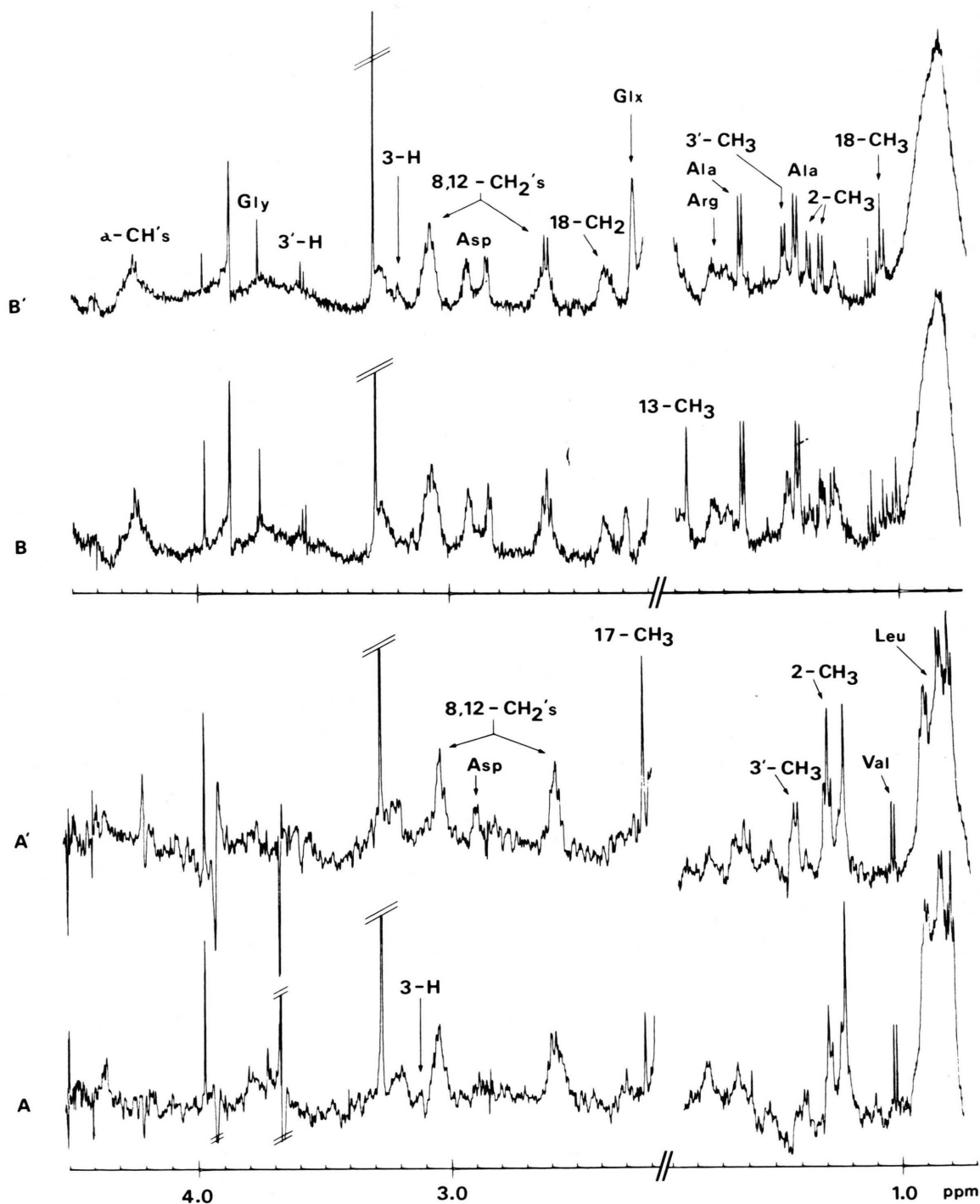
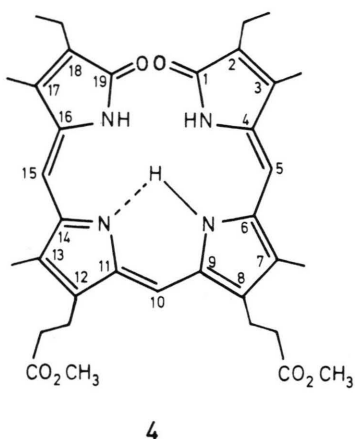
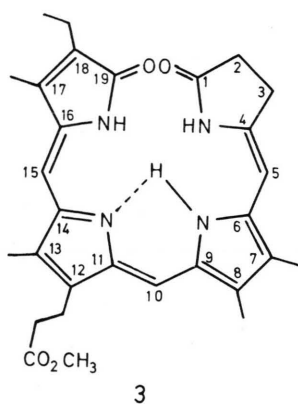


Fig. 2. Partial 500 MHz  $^1\text{H}$  NMR spectra of phytochrome and phycocyanin chromopeptides in acetone- $d_6$ /H $_2$ O/CF $_3$ COOH at 10 °C in the range of 4.50 to 0.70 ppm. The  $\delta$  range of the acetone solvent peak from 2.20 to 1.90 ppm is spared out. Increase of the base line in the region upfield from about 1 ppm is due to a specific impurity in the probe head. A) P $_r$  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  $^1\text{H}$  NMR spectra of the *E* and *Z*-configured phycocyanin peptides were compared with these of reported 15 *E* and all *Z* configured 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*-configured 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  $\text{D}_2\text{O}$  are within  $\pm 0.1$  ppm with the exception of 10-H, for which a difference of  $-0.15$  ppm is observed.



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 facili-

tates 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- $\text{CH}_2\text{—CH}_3$  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- $\text{CH}_3$ . Contrary to those measurements, our *ZZE* configured 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- $\text{CH}_3$  protons. The presence of four doublets in the spectrum of the mixture of **1b** and **2b** implicates, that the splitting of the 2- $\text{CH}_3$  resonance is present in the *E*-compound **2b**, too, and does not occur 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'- $\text{CH}_3$  protons (Table I). The multiplet most likely consists of the two overlapping doublets of **1b** and **2b** for 3'- $\text{CH}_3$  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  $\beta\text{-CH}_2$  or arginine  $\delta\text{-CH}_2$  [7] (see Table II).

Upon irradiation, the singlet at 1.849 ppm disappears and is, therefore, a resonance specific for **2b**. It is tentatively assigned to the 17- $\text{CH}_3$  protons, because with respect to 7, 13, and 17- $\text{CH}_3$  resonances in model compounds **3** and **4**, the 17- $\text{CH}_3$  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<sub>2</sub>-CH<sub>3</sub> 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 methylene 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 reso-

nances is clearly changed by irradiation. The resonance for the 2-H is probably covered by  $\alpha$ -CH<sub>2</sub> 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 <sup>1</sup>H NMR data of the *E* and *Z*-configured 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 <sup>1</sup>H NMR spectrum of the P<sub>r</sub> peptide (**1a**) is shown in Fig. 1–2, A'. The low concentration of the phytochrome peptide ( $9.4 \times 10^{-5}$  M) is the reason for its weak resonances in the <sup>1</sup>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 <sup>1</sup>H NMR assignments of the bilin moiety of chromopeptides from phycocyanin and phytochrome in acetone-d<sub>6</sub>/H<sub>2</sub>O/CF<sub>3</sub>COOH.

Chemical Shift <sup>a</sup> , Multiplicity and <i>J</i> [Hz]				Assignment	Chemical Shift Multiplicity and <i>J</i> [Hz] of Phycocyanin Peptide in D <sub>2</sub> O [7]	
Phycocyanin Peptide		Phytochrome Peptide				
<i>ZZZ</i> ( <b>1b</b> )	<i>ZZE</i> ( <b>2b</b> )	P <sub>r</sub> ( <b>1a</b> )	P <sub>r</sub> ( <b>2a</b> )			
1.295 d (7.5)	1.255 d (7.5)			2-CH <sub>3</sub>	1.21	d (7.3)
1.345 d (7.5)	1.304 d (7.5)	1.29 m	1.24 m	3'-CH <sub>3</sub>	1.44	d (7.1)
1.448 d (6.8)	1.44 m	1.417 d (6.8)	1.386 d (6.8)	7,13,17-CH <sub>3</sub> 's	2.11	s
<2.085 s>	1.849 s	<2.084 s>	n. d.		2.15	s
n. d.	n. d.	<2.089 s>	<2.098 s>		2.17	s
n. d.	n. d.	2.221 s	n. d.			
1.059 t (7.5)	1.006 t (7.5)	–	–	18-CH <sub>2</sub> -CH <sub>3</sub>	1.02	t (7.4)
<2.35 m>	<2.29 m>	–	–	18-CH <sub>2</sub> -CH <sub>3</sub>	2.23	q
2.61 m	2.61 m	2.58 m	2.58 m	8,12-CH <sub>2</sub> -CH <sub>2</sub> -CO <sub>2</sub> H	2.69	m
3.07 m	3.07 m	3.04 m	3.04 m	8,12-CH <sub>2</sub> -CH <sub>2</sub> -CO <sub>2</sub> H	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

<sup>a</sup> The chemical shifts in acetone-d<sub>6</sub>/H<sub>2</sub>O/CF<sub>3</sub>COOH are reported in parts per million from acetone-d<sub>6</sub> (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 singlet 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 singlets on the steep flank of the high 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  $\beta$ -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  $^1H$  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 (singlet of **2a** at 5.938 ppm), C-15 (singlet at 6.533 ppm), and C-10 (singlet 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 singlet at 1.849 ppm in **2b** for which the counterpart in **2a** cannot be detected and for the singlet at 2.221 ppm in **1a** for which no corresponding resonance in **1b** is detected. The singlet at 2.25 ppm in the latter peptide is assigned to amino acid resonances (for instance the  $\beta$ -CH $_2$  of Glx, see below).

#### *Amino acid moiety of phytochrome and phycocyanin peptides*

For the assignment of the amino acid resonances in the  $^1H$  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  $^1\text{H}$  NMR assignments of the peptide of chromopeptide from phycocyanin, phytochrome and of free amino acids in acetone- $\text{d}_6/\text{H}_2\text{O}/\text{CF}_3\text{COOH}$  and 360 MHz  $^1\text{H}$  NMR assignment of the peptide moieties of phycocyanin peptide in  $\text{D}_2\text{O}$ . Chemical shift, Multiplicity and  $J$  [Hz].

Phycocyanin peptide	Phytochrome peptide	Free amino acids <sup>a</sup>	Phycocyanin peptide [7]	Assignment
Acetone- $\text{d}_6/\text{H}_2\text{O}/\text{CF}_3\text{COOH}$			$\text{D}_2\text{O}$	
— <sup>b</sup>	0.900 m	0.95 d (5.9)	0.77 d (5.3)	Leu <sup>1-3</sup> $\delta_1\text{-CH}_3$
	0.840 d (5.4)	0.95 d (5.9)	0.83 d (5.3)	Leu <sup>1</sup> $\delta_2\text{-CH}_3$
	0.815 d (5.4)			Leu <sup>2</sup> $\delta_2\text{-CH}_3$
	0.793 d (5.4)			Leu <sup>3</sup> $\delta_2\text{-CH}_3$
—	1.028 d (6.1)	1.09 (7.0)	—	Val $\gamma_1/\gamma_2\text{-CH}_3$
1.401 d (7.5)	n. d.	1.61 d (7.4)	1.29 d (7.2)	Ala <sup>1</sup> $\beta\text{-CH}_3$
1.622 d (7.5)	n. d.		1.51 d (7.2)	Ala <sup>2</sup> $\beta\text{-CH}_3$
1.65–1.80	n. d.	1.6–2.0	1.54–1.64	Arg $\beta/\gamma\text{-CH}_2$
2.25	n. d.	2.32 m	—	Glx $\beta\text{-CH}_2$
2.84 m	2.90 m	3.13 d (5.4)	2.77 dd (7.8/16.8) $\beta_1$	Asx <sup>1</sup> $\beta\text{-CH}_2$
2.92 m	—		2.87 dd (5.9/16.8) $\beta_2$	Asx <sup>2</sup> $\beta\text{-CH}_2$
			3.02 dd (7.6/13.9) $\beta_1$	Cys $\beta\text{-CH}_2$
3.20–3.30	3.15–3.25	3.66 dd (4.0/7.6)	3.11 dd (6.8/13.9) $\beta_2$	
—				
3.750 s	n. d.	3.39 t (5.6)	3.11 t (6.8)	Arg $\delta\text{-CH}_2$
4.15–4.35	n. d.	3.22 dd (2.8/6.5)	—	Tyr $\beta\text{-CH}_2$
—	6.677 d (8.8)	3.95 s	—	Gly $\alpha\text{-CH}_2$
—	7.016 d (8.8)	4–5	4.1–4.6	amino acids $\alpha\text{-CH}'\text{s}$
—	7.42 m	6.75 d (8.7)	—	Tyr ring H's (a)
—	8.81 m	7.15 d (8.5)	—	Tyr ring H's (b)
		7.59 d (1.4)	—	His ring H (a)
		8.87 d (1.4)	—	His ring H (b)

<sup>a</sup> The data were taken from 80 MHz  $^1\text{H}$  NMR spectra which were taken on a Bruker WP 80 FTNMR with Aspect 2000 data system at 35 °C.

<sup>b</sup> — = 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 <sup>a</sup>	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)

<sup>a</sup> 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 cysteine 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<sub>fr</sub> 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  $^1\text{H}$  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  $^1\text{H}$  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  $\beta\text{-CH}_2$ , aspartic acid  $\beta\text{-CH}_2$ , glycine  $\alpha\text{-CH}_2$ , arginine  $\beta,\gamma\text{-CH}_2$  and the  $\alpha\text{-CH}$ '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. 1A and 1A'). The resonance at 7.016 and 6.677 ppm are assigned to the ring protons of tyrosine. They also behave similarly during the  $\text{P}_{\text{fr}}$  peptide  $\rightarrow$   $\text{P}_{\text{r}}$  peptide transformation. Interestingly, no significant differences in the shifts of amino acid residues were found between the *E* and *Z* configured 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  $\beta\text{-CH}_2$  of Glx) than that of aromatic methyl protons which give sharp singulets. Minor but significant differences were found in the chemical shifts of  $\text{P}_{\text{fr}}$  and  $\text{P}_{\text{r}}$  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  $\text{P}_{\text{fr}} \rightleftharpoons \text{P}_{\text{r}}$ . This possibility is presently being investigated further.

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