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MODELS FOR THE PHOTOREVERSIBILITY OF PHYTOCHROME

Z,E ISOMERIZATION OF CHROMOPEPTIDES FROM PHYCOCYANIN AND PHYTOCHROME

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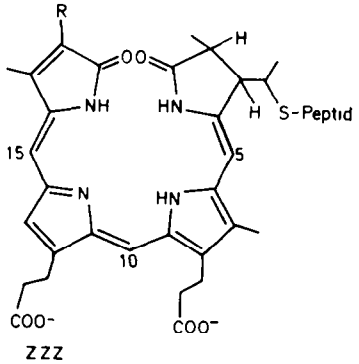
Abstract Chromopeptides which contain linear tetrapyrrole chromophores are prepared by proteolytic digestion of phycocyanin and phytochrome (P_r and P_{fr} forms). After the nucleophilic addition of mercaptoethanol to C-10 of the tetrapyrrole, irradiation with white light leads to photoisomerization of the tetrapyrrole ($Z \rightarrow E$). The products prepared from (*Z*)-phycocyanin and P_r chromopeptides are similar to P_{fr} chromopeptides with respect to chromatographic properties, electronic spectra and photolability. It is concluded that the linear tetrapyrrole chromophore of P_r is all-*Z* whereas P_{fr} has the *E* configuration at one of the bridges.

PHYTOCHROME is a light receptor for photomorphogenesis in higher plants, i.e. it governs light dependent growth and development.[1] Phytochrome exists in two forms, P_r (inactive, absorption at 660 nm) and P_{fr} (physiologically active, absorption at 730 nm). A characteristic property of phytochrome is its photoreversibility

$$P_r \underset{730\,nm}{\overset{660\,nm}{\rightleftharpoons}} P_{fr}.$$

The chemical structure of the P_r chromophore (**1**) has been elucidated independently by two research groups. In our laboratory, the structure was derived from investigations of degradation products, from electronic spectroscopy and from the cleavage of the free chromophore which was then compared with the authentic chromophore obtained by total synthesis.[2-4] Lagarias and Rapoport[5] applied NMR spectroscopy to the chromophore still linked to amino acids of the protein. For both types of investigations, chromopeptides were prepared from P_r with proteolytic enzymes. Furthermore, both studies used chromopeptides from phycocyanin (**2**) as readily accessible model compounds with structural similarity to phytochrome chromopeptides.

Current chemical investigations on phytochrome



ZZZ
1 R = CH=CH$_2$
2 R = C$_2$H$_5$

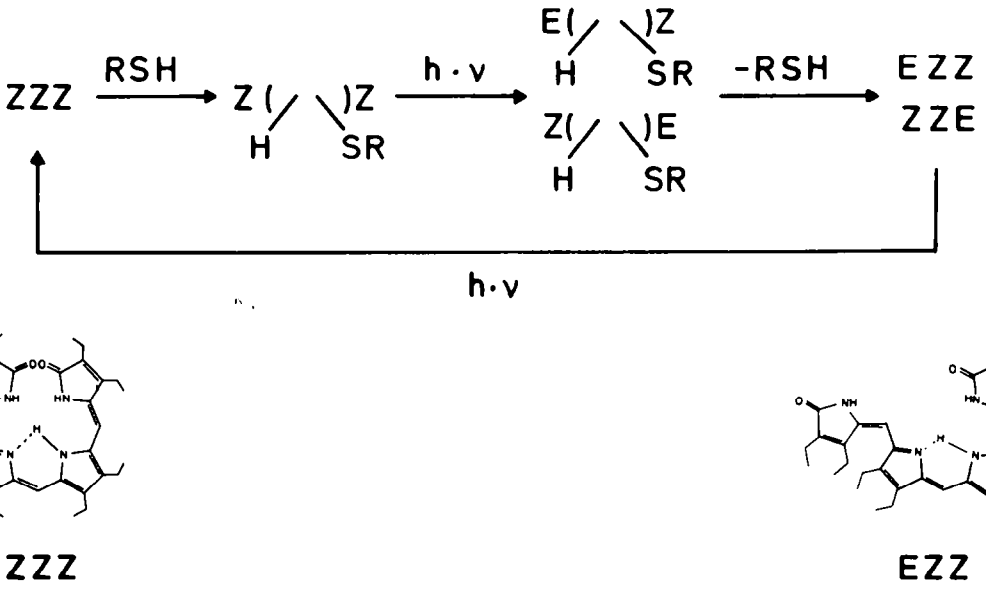
Table 1. Parameters for reversible absorption changes of linear tetrapyrroles

1. Protonation and deprotonation
2. Fixation of various conformations
3. Photoaddition—elimination reactions
4. $(Z) \rightleftharpoons (E)$ isomerization reactions

are concentrated upon the interpretation of the difference between the P_{fr} and P_r chromophores. The best characterized difference between P_r and P_{fr} is the difference in absorption of visible light. Spectroscopic shifts can be induced in bilindiones (and other linear tetrapyrroles) in various ways (Table 1). Protonation or deprotonation of the chromophore of phytochrome could be induced by a microenvironment of the native protein. Likewise, the native protein could fix a certain (otherwise unfavourable) conformation of the chromophore. If the difference between P_r and P_{fr} were based only on these principles, the difference should disappear on denaturation or proteolytic digestion of the phytochrome protein. The preparation of a chromopeptide from P_{fr} which is different from the P_r chromopeptide with respect to spectroscopic and chemical properties[6] demonstrated that this is not the case. Instead, the P_{fr} chromophore must be inherently different from the P_r chromophore. This difference could be based on photoaddition-elimination or on *Z-E* isomerization reactions (Table 1). Scheer and coworkers[7-9] have investigated photoaddition reactions with model chromophores, and the reaction products have some properties in common with the P_{fr} chromopeptide. However, the reverse (elimination) reaction has been observed thermally but not photochemically.

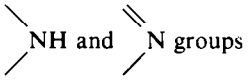
We wish to describe here the *Z-E* isomerization of chromopeptides from phycocyanin and phytochrome (P_r form). In contrast to the photoaddition products,[7-9] the products can be photochemically reconverted to the parent compounds, and are also similar to the P_{fr} chromopeptide in other respects.

In the series of pyrrole pigments *Z-E* isomers were first unequivocally characterized for dipyrrolic compounds.[10-11] The lack of photochemical $(Z) \rightleftharpoons (E)$



Scheme 1. Reaction sequence for preparation of (*E*)-bilindiones from "normal" (*Z*)-bilindiones. The prefices *Z* or *E* refer to the bridge at C-5, C-10 and C-15 in this order. The nucleophile RSH is reversibly added at C-10.

isomerization of most pyrrole pigments was explained by a competition with a phototautomerization at



(rings B and C in tetrapyrroles[12]). This phototautomerization can be avoided with bilindiones ("bilatrienes—*abc*") by absorption on aluminium oxide[13-14] or by reversible addition of donors (e.g. mercaptoethanol at C-10[15]). The latter method has been used successfully in our laboratory to prepare *E* isomers from (*Z*)-bilindiones which were characterized by electronic and ^{1}H-NMR spectroscopy.[16] The reaction sequence is outlined in Scheme 1. We apply the same method here to chromopeptides obtained from phycocyanin and from phytochrome (P_r form).

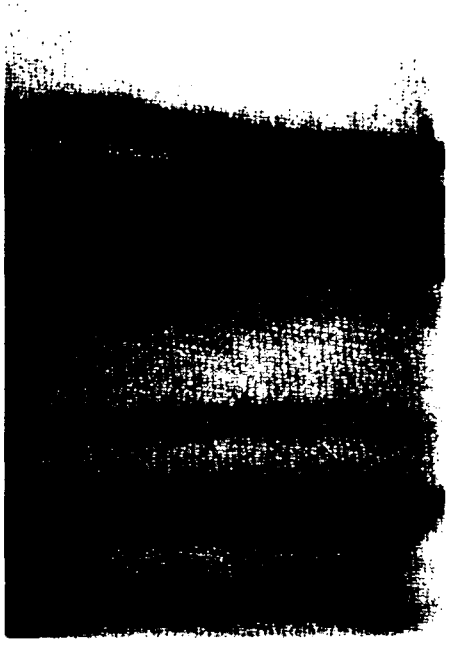


Fig. 1. Isoelectric focusing of chromopeptides obtained by pepsin digestion of C-phycocyanin. The bands are visible without staining due to the inherent colour of the phycocyanobilin chromophore.

Chromopeptides from phycocyanin

Phycocyanins contain three phycocyanobilin chromophores per protomer, one in the α-subunit and two in the β-subunit. The amino acid sequences are different in all three chromophore regions although homology between the α-subunit and one β-subunit region can be observed.[17-20] Proteolytic digestion should, therefore, result in at least three different chromopeptides.

We obtained four main and five minor chromopeptide fractions after isoelectric focusing of the digest (Fig. 1). According to the amino acid analysis (Table 2) the main fractions 2 and 6 and minor fractions 1, 3 and 4 are derived from one chromophore region (presumably from β-subunit II region, cf.[17,18,20]). Main fraction 8 and, probably, minor fraction 7 are derived from the α-subunit, whereas main fraction 9 corresponds to the β-subunit I region. Most of the subsequent experiments were at first performed with the chromopeptide mixture and then with single chromopeptide fractions. There was no significant difference between the mixture and the single chromopeptide fractions with regard to the reactions described in the following sections. This means that the properties described are inherent properties of the chromophore. They are only slightly, if at all, influenced by the amino acid composition around the chromophore.

The first step of the desired reaction sequence is the

Table 2. Amino acid analysis of chromopeptide fractions obtained by isoelectric focusing (see Fig. 1). Values are molar ratios based on Lys or, if not present, Ala, Leu or Ile as italicized

Amino acid	Fraction number 1	2	3	4	5	6	7	8	8[a]	9
C_ySO_3H	0.33	+	0.51	+	+	0.19	0.13	0.39	0.14	0.36
Asx	1.13	1.92	2.41	2.36	2.44	0.8	1.93	1.62	1.58	1.43
Thr	0.33	0.54	1.24	1.22	0.13	0.5	0.60	0.13	0	1.85
Ser	0.71	0.61	2.86	1.92	1.12	0.56	1.60	0.52	0	2.76
Glx	0.38	0.42	3.08	1.83	2.7	1.1	1.77	0.88	0.58	1.88
Gly	1.09	1.5	4.6	4.47	2.8	1.2	2.23	1.33	0.71	2.40
Ala	*1.0*	1.57	2.49	3.05	0.56	1.5	1.20	1.41	1.55	5.03
Cys	1.44	–	2.39	–	–	–	1.26	1.02	+	4.16
Val	0	1.00	0	0.24	0.7	0.36	0	0	+	0
Met	0	–	0	–	+	+	0	0.8	0	1.17
Ile	0.38	*1.0*	1.18	1.31	0.5	0.19	0.43	0.40	0	0.35
Leu	0.41	0.69	1.57	1.52	1.53	*1.0*	1.26	0.17	+	1.97
Tyr	0.03	0.04	0.32	0.19	+	0.11	0.20	0.37	0	1.60
Phe	0.06	0.05	0.24	0.19	+	0.19	0.20	0.05	0	0.22
His	0.08	0.05	0.51	0.36	0.51	0.16	0.50	0.10	0	0.43
Lys	0.17	0.13	*1.00*	*1.00*	*1.0*	0.32	*1.0*	*1.0*	*1.0*	*1.0*
Arg	0.05	0.04	0.36	+	0.93	+	0.83	0.93	0.80	0.13

[a]Peptide 8 after recovery from photoisomerization.

addition of a nucleophile at C-10 (see Scheme 1). This type of reaction had already been studied with native and denaturated phycocyanin.[21-23] The reaction proceeds with the chromopeptide as with denaturated phycocyanin. We used mercaptoethanol as nucleophile because it can easily be removed. The reaction can be followed by the disappearance of the absorption at 660 nm and the appearance of a new absorption band at about 420 nm. The reaction is complete in aqueous solution only if a rather high concentration (40 vol. %) of mercaptoethanol is used. The photoisomerization of the adduct is then achieved by a short irradiation with white light. Acidification leads to elimination of mercaptoethanol and the blue solution so obtained should contain *Z,E* isomeric chromopeptides. Because (*E,Z,Z*)-bilindiones are easily transformed to the stable (*Z,Z,Z*)-bilindiones by light, all subsequent steps are performed in the dark or under a dim green safe light.

Preliminary experiments had shown that a new chromopeptide fraction can be obtained by this reaction sequence from a homogeneous chromopeptide. Separation of this new chromopeptide from the parent compound can be achieved by adsorption chromatography with dilute aqueous acids. The new chromopeptide is characterized as an *E* isomer mainly by its spectral properties (see below) and by its quantitative photoconversion to the parent chromopeptide which has the all-*Z* configuration. As outlined below, these properties correspond exactly to those of authentic (*E,Z,Z*)-bilindiones.[16]

Optimal separation occurs on Biogel P-10 with dilute aqueous HCl. The original chromopeptide (*ZZZ*) does not migrate whereas the photoisomerized chromopeptide (i.e. the presumed *E*-isomer) is slowly eluted under these conditions. The reaction product is then absorbed on silica gel. It binds more strongly to this absorbent than to Biogel and can be exhaustively washed free from mercaptoethanol. This is important because mercaptoethanol (like dithionite[6]) seems to catalyze the reverse reaction. The yield of the *E* isomer

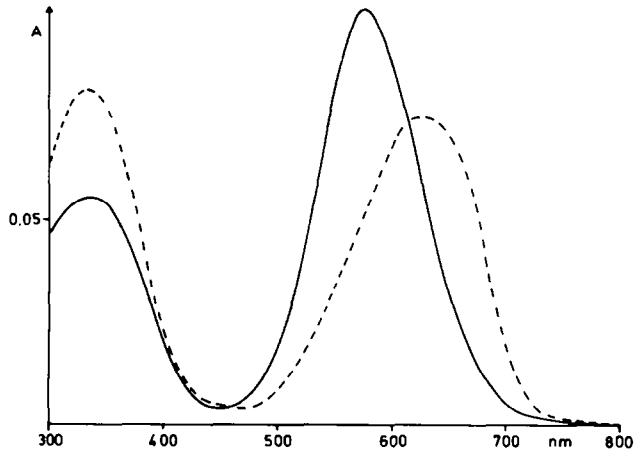


Fig. 2. UV-Vis spectra of (*E*)- and (*Z*)-chromopeptides from phycocyanin in 50 % aqueous formic acid.
(———) *E* product obtained by reaction sequences of Scheme 1
(– – – – –) the same after 10 sec irradiation with white light (*Z* product).

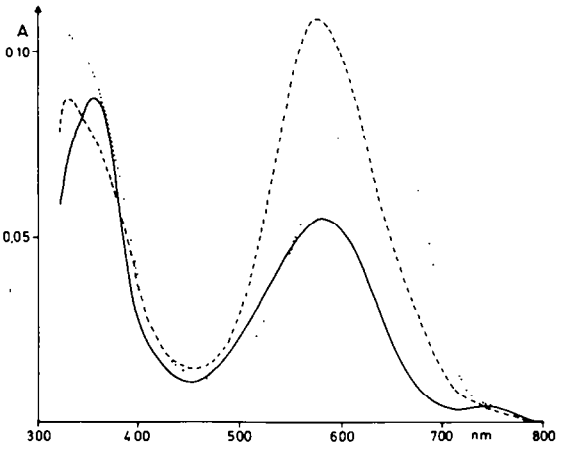
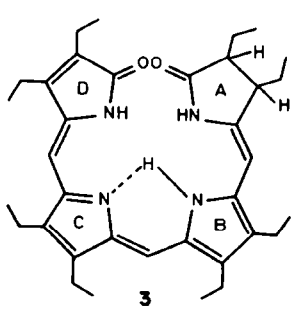


Fig. 3. Stability of (*E*)-phycocyanin chromopeptide at various pH values.
(———) at pH 7
(– – – – –) the same after acidification to pH 1 with trifluoroacetic acid
(. . . . .) after irradiation with 10 sec white light at pH 1 (*Z* product).

is very low if mercaptoethanol is not entirely removed.

The electronic spectrum of the photoisomerized chromopeptide at low pH is shown in Fig. 2. Characteristic features of this spectrum are best recognized in comparison with the spectrum of the corresponding (*Z*,*Z*,*Z*)-chromopeptide (Fig. 2, dotted line). The *E* isomer has an absorption maximum at 575 nm, the *Z* isomer at 630 nm. Such a shift is characteristic for *E*,*Z* isomers (see below). It has been explained[24] by partial uncoupling of the chromophoric system in the *E* isomer due to a twist at the *E* methine bridge. The UV maximum is not shifted (330–340 nm in both isomers) but the absorbance is considerably lower in the *E* than in the *Z* isomer. On the other hand, the absorbance of the long wavelength band is higher in the *E* than in the *Z* isomer. This could be due to a more closed conformation in the *Z* isomer and a more open conformation in the *E* isomer (cf.[25–27] and Fig. 8) but a more detailed investigation of this question is still needed.

It should be mentioned here that the (*E*)-chromopeptides from phycocyanin are prepared and kept in acid medium because it has been reported[2,6] that the P_{fr} chromophore is only stable in acid medium. This is remarkable because model bilindiones have been reported[28] to be more stable in neutral or alkaline solution than in acid medium. In our hands[16] octaethylbilindione (see Scheme 1) is about equally stable in basic, neutral and slightly acid medium but is converted to the all-*Z* isomer with strong acids. It was, therefore, interesting to test the stability of the (*E*)-chromopeptide from phycocyanin at various pH values. Its stability in strongly acidic media (50% aqueous formic acid, 20% aqueous trifluoroacetic acid) is similar to that of the P_{fr} chromopeptide, and therefore, much higher than that observed for the model bilindiones. Strong bases partly destroy the *E* form. At pH 7.0, the absorption spectrum of the (*E*)-phycocyanin chromophore (Fig. 3) is nearly indistinguishable from that of the *Z* isomer. It is not yet clear why spectral differences found between *E* and *Z* isomers of model bilindiones in neutral solvents[15,16,29] are not found for chromopeptides. The small maximum at 740 nm in the spectrum of the *E* isomer (Fig. 3) could correspond to the monoanion described[30] for **3**. We did not observe this maximum with (*Z*)-chromopeptides under our conditions. This small difference is not sufficient to distinguish between the (*E*)- and (*Z*)-chromopeptides at pH 7.0. A distinction between (*E*)- and (*Z*)-chromopeptides is possible, however, on acidification. The absorption maximum of the (*E*)-chromopeptide is shifted from 580 to 575 nm (Fig. 3). According to our experiences, this is the first case of a bilindione the cation of which absorbs at about the same wavelength as the free base. The absorption spectrum of the (*Z*)-chromopeptide is shifted by acidification from 580 to 630 nm (not shown) as is usual for bilindiones.



3

Irradiation of the cation of the (*E*)-chromopeptide for 10 sec with white light leads to the spectrum of the (*Z*)-chromopeptide. Interestingly, the absorption maximum is found at 620 nm (with a shoulder around 650 nm) here (Fig. 3) whereas the "normal" spectrum with the maximum at 640 nm and a shoulder at 620 nm is found in other samples. This could be due to the presence of two or more conformers in varying amounts[4] but was not investigated further. Irradiation of the *free base* of the (*E*)-chromopeptide for 10 sec with white light yielded only partial phototransformation to the *Z* form. This transformation could only be detected after acidification: the observed spectrum still corresponded essentially to that of the cation of the *E* isomer, but this was easily transformed to the cation of the *Z* isomer by irradiation for 10 sec. The observation clearly demonstrates that the cation of the *E* isomer is more sensitive towards light than is its free base.

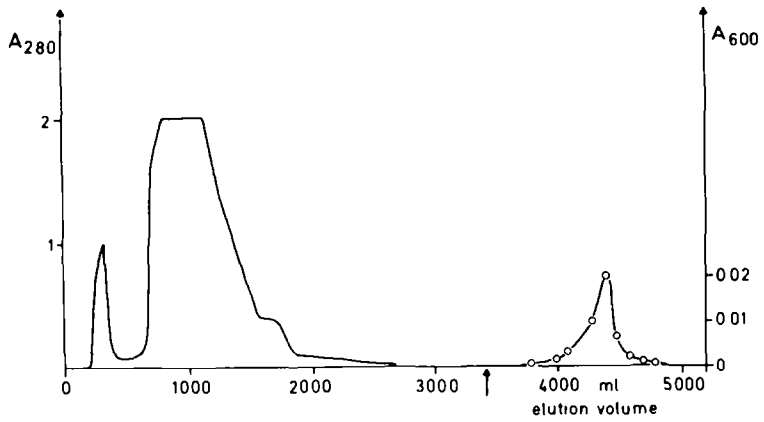


Fig. 4. Elution diagram for the purification of chromopeptide from the P_{fr} form of phytochrome.
(· —) A_{280}, colourless peptides
(O—O- O) A_{600}, P_{fr} chromopeptide. The arrow indicates the change of solvent from dilute HCl (pH 2.5) to 1% aqueous HCOOH. The P_r chromopeptide is not eluted from the column under these conditions. It can be eluted with 2% aqueous pyridine.

Chromopeptides from phytochrome

For comparison with the (*E*)-chromopeptide from phycocyanin, the P_{fr} chromopeptide was investigated again. The previous method of preparation[6] was improved as outlined in the experimental section. The use of dilute HCl instead of formic acid for pepsin digestion and column chromatography was especially helpful. This resulted in a complete separation of colourless peptides from the desired P_{fr} chromopeptide (Fig. 4). The electronic spectrum of the highly purified P_{fr} chromopeptide is shown in Fig. 5. It has absorption maxima at 590 and 378 nm. Upon irradiation (formation of P_r chromopeptide), these maxima are shifted to 665 and 372 nm (dotted line in Fig. 5). These properties are reminiscent of *E-Z* isomerization of bilindione chromophores as described above, in particular the bathochromic shift of the long wavelength band and the absorbance increase without a considerable shift of the short wavelength band.

We tested the stability of the P_{fr} chromophore towards higher pH values with this sample. In accordance with earlier results,[2,6] we observed a spectrum identical with that of the P_r chromophore at pH 7.1. As outlined above, spectral distinction between (*E*)- and (*Z*)-phycocyanin chromopeptides is not possible at this pH but only at pH values below 2. Acidification of the P_{fr} chromopeptide yielded a spectrum intermediate between those of the cations of the P_{fr} and the P_r chromophores. Irradiation with white light shifted the spectrum to that of the pure P_r chromophore cation. This means that the P_{fr} chromophore behaves similarly to the (*E*)-phycocyanin chromophore with respect to its stability at neutral pH.

The good correspondence between the P_{fr} chromopeptide and the *E* isomer models on the one hand and between the P_r chromopeptide and the *Z* isomer models on the other hand prompted us to try the reaction sequence for *Z,E* isomerization with the P_r chromopeptide. The addition of mercaptoethanol proceeds as with the phycocyanin chromopeptides. The

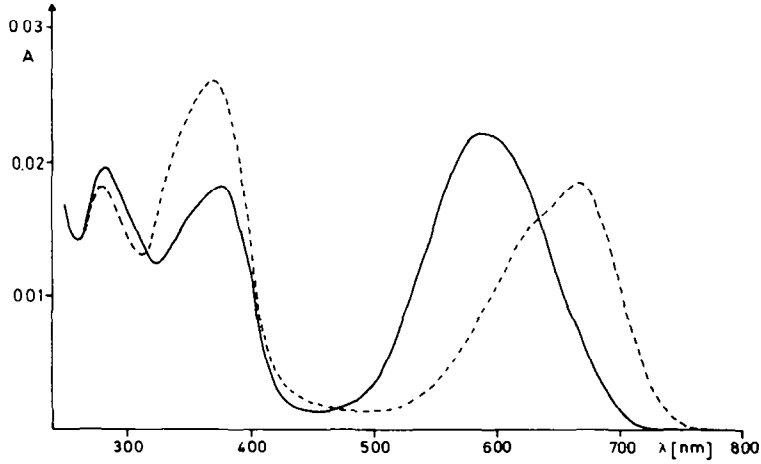


Fig. 5. UV-Vis spectrum of the chromopeptide from the P_{fr} form of phytochrome in 1% aqueous formic acid. The chromopeptide was purified by column chromatography on Biogel P-10 (see Fig. 4).
(——) P_{fr} chromopeptide
(- - - -) the same after 10 sec irradiation with white light = P_r chromopeptide.

Table 3. Absorption maxima of *E-Z* isomers of chromopeptides and model bilindiones. All spectra are recorded in acid solution.

Compound	*E* isomer λ_{max} (nm)	*Z* isomer λ_{max} (nm)	Solvent
Chromopeptide from phytochrome	590, 378[a]	665, 372[b]	1% HCOOH in H_2O
Chromopeptide from phytochrome	610, 380[a]	660, 380[b]	TFA in methanol[6]
Chromopeptide from phycocyanin	575, 340	630, 340	50% HCOOH in H_2O
2,3-Dihydrooctaethyl bilindione (**3**)	570	655	HCl in acetone[16]
		665, 351	HCl in methanol[30]
Octaethylbilindione (**L13**)	598, 362[16,c]	693, 357	1% HCl in methanol[30]

[a] P_{fr}-chromopeptide.
[b] P_r-chromopeptide.
[c] Trifluoroacetic acid in $CHCl_3$.

adduct seems, however, to be more unstable in the case of the phytochrome derivative than in the case of the phycocyanin derivative. Irradiation leads to violet by-products even under strictly anaerobic conditions (argon). During column chromatography, these by-products migrate together with the (*E*)-chromopeptide. This cannot, therefore, be obtained in pure form. Its presence, however, can be demonstrated by the typical spectral changes which are caused by irradiation, in particular by the bathochromic shift of the long wavelength band and the absorbance increase of the short wavelength band which can be seen in the difference spectrum. In Fig. 6, this difference spectrum is compared with that of the phototransformations of

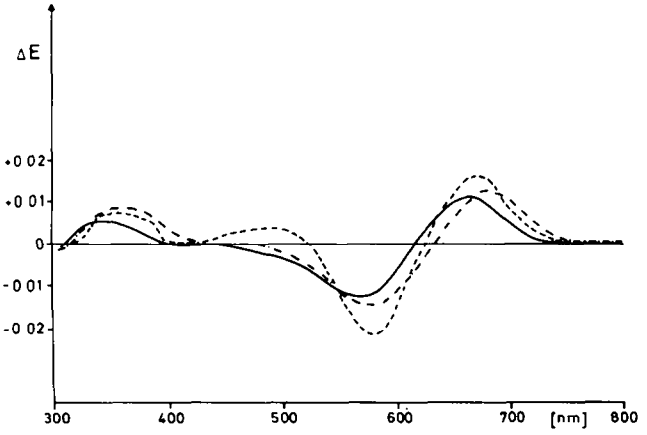


Fig. 6. UV-Vis difference spectra of (*E*)- and (*Z*)-chromopeptides from phycocyanin and phytochrome compared with the UV-Vis difference spectra of P_{fr} and P_r chromopeptide. The *E* products are obtained according to Scheme 1. The *Z* products are obtained by irradiation of the former. The difference spectra are given as *E* spectrum minus *Z* spectrum.
(————) phycocyanin chromopeptide
(– – – – –) phytochrome chromopeptide, prepared from P_r according to Scheme 1
(– . – . – .) P_{fr} chromopeptide, prepared according to Fig. 4.

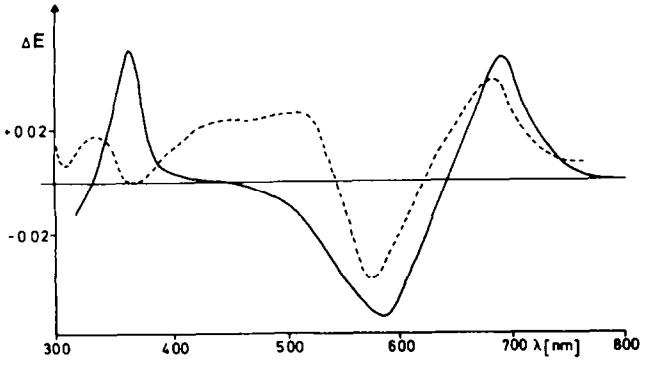
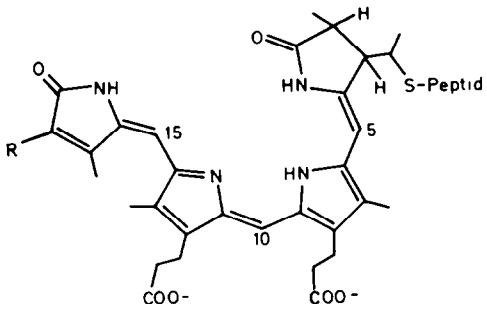
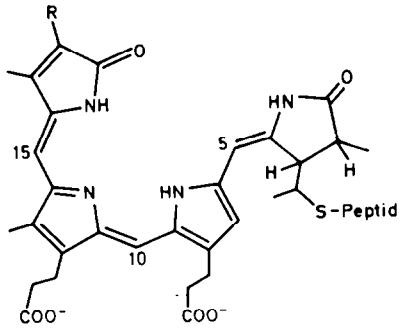


Fig. 7. UV-Vis difference spectra of (*E*)- and (*Z*)-bilindiones, prepared according to ref. 16.
(·· ——) octaethylbilindione (**L13**)
(– – – – –) 2,3-dihydrooctaethylbilindione (**3**).



ZZE
4 R = C_2H_5
5 R = $CH{=}CH_2$



EZZ
6 R = C_2H_5
7 R = $CH{=}CH_2$

the P_{fr} chromopeptide to the P_r chromopeptide and the (*E*)-phycocyanin chromopeptide to its *Z* isomer. Fig. 7 shows the difference spectra for the *E-Z* isomerization of octaethylbilindione (**L13**) and its 2,3-dihydro derivative (**3**).

We conclude from these results that the P_{fr} chromopeptide behaves like an *E* isomer of the P_r chromopeptide which itself behaves like the "normal" *Z* models. The arguments can be summarized as follows:

(i) P_{fr} chromopeptide and (*E*)-phytochrome or phycocyanin chromopeptides can be separated by the same chromatographic procedure from the corresponding *Z* isomers or P_r chromopeptide. The easy elution of *E* isomers with aqueous acids points to their higher polarity in comparison with *Z* isomers. This corresponds to the slower migration of (*E*)-bilindiones (**L13**) with organic solvents in comparison with *Z* models.

(ii) P_{fr} chromopeptide and (*E*)-chromopeptides show the same difference in their spectra compared with their *Z* isomer or P_r chromopeptide, respectively, as discussed above in detail. The same differences are also found between (*E*)- and (*Z*)-model bilindiones of known configuration.[16]

(iii) Irradiation of P_{fr} chromopeptide and of (*E*)-chromopeptide yields products with the spectroscopic and chromatographic properties of the corresponding *Z* isomers or P_r chromopeptide, respectively.

Because the chromopeptides contain asymmetrically substituted bilindione chromophores, two different *E* isomers can occur. Experiments with the model chromophore **3** demonstrated that under the applied conditions only the 15*E* isomer is obtained.[16] This would correspond to **4** and **5**. On the other hand, the spectrum of the P_{fr} chromopeptide has been interpreted[2] in terms of the deconjugation of ring A which would point to a 4*E* isomer (**6** and **7**). Distinction between these two types of *E* isomer can be obtained by NMR spectroscopy. Measurements of this kind are in progress.

Present view of the chromophore in native phytochrome

The spectral difference between the P_{fr} chromopeptide (λ_{max} about 600 nm) and native P_{fr} (λ_{max} 720–730 nm) is evident. This difference is due to interactions of the chromophore with the native protein. Because the nature of these interactions is unknown, all further considerations must remain speculative. It may prove possible, however, to estimate the influence of the factors in Table 1 upon suitable model compounds.

The influence of a fixed conformation upon spectral properties of bilindiones has been theoretically calculated[25–27] but can also be observed in model pigments in which the conformation is fixed by covalent linkages (Fig. 8). The most pronounced effect of a closed (= helical) conformation is a small value for the ratio of the oscillator strengths of the red and blue absorption bands, whereas an extended conformation leads to a high value for this ratio. Denaturation and renaturation experiments with phycocyanin indicated, on this criterion, that the phycocyanin chromophores have predominantly a helical conformation in the denatured protein and an extended conformation in the native protein.[32] An extended conformation can also be deduced from the oscillator strength ratio for the P_r chromophore in the native state.[33] Because linear dichroic data indicate a similar geometry for native P_r and P_{fr} chromophores[34] a more or less extended conformation can also be assumed for the native P_{fr} chromophore. According to the oscillator strength ratio, the P_{fr} chromophore has a comparable conformation in the denatured protein or in the chromopeptide (Fig. 5) and in the native protein. This means, however, that the long wavelength absorption of native P_{fr} cannot be due simply to the stabilization of a certain chromophore conformation by the protein.

The long wavelength absorption band of the P_{fr} chromopeptide is observed at about 600 nm at pH 7 as well as at pH 1. This means that the native absorption band at 730 nm cannot be explained merely by protonation of the chromophore of the native protein. Marked spectral shifts are, however, observed upon deprotonation of bilindiones. An anionic structure was, therefore, discussed for the native P_{fr} chromophore[2] which was supported by theoretical calculations.[27] The finding of a small maximum at 740 nm in the spectrum of the (*E*)-chromopeptide from phycocyanin (see Fig. 3) and its possible relationship to a previously described monoanion[30] is interesting in this connection. But it would be premature to discuss these observations in more detail at this stage.

EXPERIMENTAL

Electronic spectra were recorded either with a spectrophotometer model DMR 22 (Zeiss, Oberkochen) or model

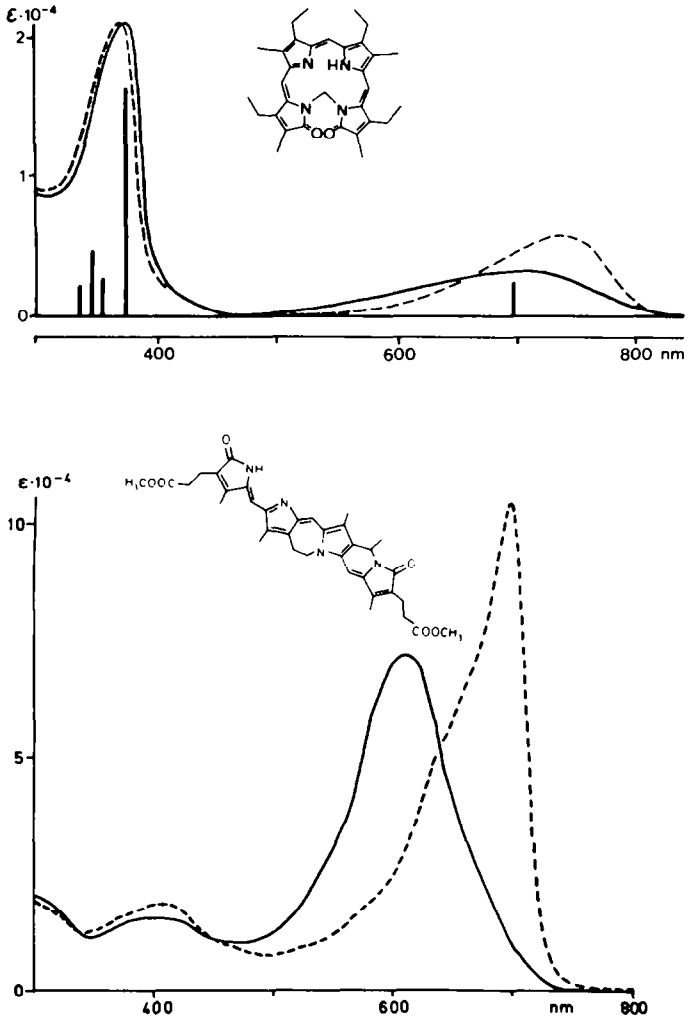


Fig. 8. UV-Vis spectra of bilindiones with fixed conformation. Upper part: N_{21}–N_{24}-methylene-bilindione (structure inserted) in $CHCl_3$ (· · · -) and $CHCl_3/H^-$ (- -). The oscillator strength ratio (red/blue band is very small (after ref. 31). Lower part: isophorcabilin (structure inserted) in methanol (— ·) and methanol (1% HCl) (– – – –). The oscillator strength ratio (red/blue band) is very large. (Scheer and Kufer, unpublished.)

320 (Perkin-Elmer, Überlingen). The latter allowed the direct recording of difference spectra.

Chromopeptides from phycocyanin

C-Phycocyanin was prepared from *Spirulina maxima* following a method previously described.[22] Lyophilized phycocyanin (100 mg) was dissolved in H_2O (10 ml). To the blue soln, pepsin (10 mg) (Boehringer) and ascorbic acid (175 mg, final concentration 50 mM) were added. The pH value was adjusted to 1.5 with conc. HCl. The mixture was then incubated under argon at 37° for 1 hr. The resulting clear soln was applied to a column (2.5 × 30 cm) packed with Biogel P-10 which had been equilibrated with 5% aqueous HCOOH. The column was washed with 30 ml of 5% HCOOH and then developed with 10% HCOOH (*ca* 1000 ml). Colourless peptides and red to violet degradation products of chromopeptides were eluted by this procedure. The blue fractions eluted subsequently were collected and immediately applied to a column (1.6 × 10 cm) packed with silica gel which had been equilibrated with H_2O. The blue chromopeptides were adsorbed. After extensive washing of the column with water (removal of residual colourless peptides), the chromopeptides were eluted with 20% aqueous pyridine (2 5 ml). A small fraction of blue chromopeptides which remains absorbed at the Biogel was eluted with 2% aqueous pyridine.

Isoelectric focusing

The chromopeptide mixture was further fractionated by isoelectric focusing (Multiphor 2117 LKB, Bromma, Sweden) after concentration to ⩽5 ml in a rotary evaporator. Strips of filter paper were soaked with a soln of Servalyt pH 2–4 and pH 4–6 (0.25 ml of each in 19.5 ml of twice distilled water) and applied to the ends of a glass plate (11 × 24.5 cm). 5 g Sephadex G-100 was swelled in twice distilled water and, after washing four times with twice distilled water, was mixed with 1.75 ml Servalyt pH 2–4 and 1.75 ml Servalyt pH 4–6. The mixture (*ca* 100 ml) was poured onto the prepared glass plate and slightly dried under a stream of air. The strips of filter paper were then replaced by corresponding strips of filter paper which had been soaked with either anolyte soln (368 mg glutamic acid and 333 mg aspartic acid in 100 ml water) or catholyte soln (365 mg lysine and 435 mg arginine in 12 ml ethylene diamine/88 ml water). After prefocusing for 24 hr with max 4 5 Watt, chromopeptides were applied in a 2 cm broad band in the middle of the gel. Separation was achieved by focusing for 24 hr with max 4 5 Watt. The single

blue bands were scraped off and eluted with water. The blue solns were then lyophilized. The chromopeptides were further purified by TLC on HPTLC plates coated with silica gel (Merck) with the solvent n-BuOH : AcOH : water (4 : 1 : 1). Zones of migrating chromopeptides were scraped off and eluted. Chromopeptide fraction 8 did not migrate in this solvent. The Servalyt was, in this case, removed from the origin with 1N HCl. The chromopeptide which was still at the origin was then purified with n-BuOH : AcOH : water (2 : 1 : 1). Aliquots of the purified chromopeptide solns were hydrolyzed with 6N HCl and analyzed for amino acids (Table 2).

Photoisomerization

The chromopeptide from phycocyanin (fraction 8, 2.4 μmol) was dissolved in a mixture of 6 ml 0.1 M tris-HCl buffer (pH 8.5) and 4 ml mercaptoethanol. The brownish-yellow soln was illuminated at 4° under argon for 5 min with white light (slide projector, halogen lamp 12 V/500 W), filtered through a 5 cm layer of water. The soln was then acidified with a few drops of conc. HCl which caused a change of the colour to blue. The soln was applied to a column (2.5 × 35 cm) of Biogel P-10 equilibrated with water which was acidified with HCl to pH 2.5. Elution with the same solvent yielded, with the front (0–40 ml), the bulk of the mercaptoethanol and a violet by-product. After separation of this by-product, the eluate which contained the photoisomerization product was applied to a column (1.6 × 10 cm) filled with silica gel equilibrated with water. The photoisomerized chromopeptide formed a sharp zone at the top of the silica gel. It was washed with dilute HCl (pH 2.5) until all mercaptoethanol had been removed. The photoisomerized chromopeptide was then eluted with either 50% aqueous HCOOH or with 20% aqueous pyridine. Yield: about 18% of original chromopeptide. The unreacted chromopeptide (*Z,Z,Z*) (fraction 8[a]) was retained under these conditions by the Biogel and was eluted from that column with 2% aqueous pyridine as a sharp zone. Recovery: 61% of original chromopeptide. Amino acid analysis of this peptide (fraction 8[a]) see Table 2.

The photoisomerization and separation of reaction products was performed with the various fractions of phycocyanin chromopeptides (see Table 2) and also with chromopeptides obtained from phytochrome (P_r form) with basically the same results.

Preparation of chromopeptides from phytochrome in the P_{fr} form

The isolation of small phytochrome from etiolated oat seedlings ($A_{660} : A_{280} = 1 : 20$), the phototransformation into the photoequilibrium (about 80% P_{fr}, 20% P_r), and the preparation and isolation of chromopeptides were performed as previously described[6] with minor modifications. The P_{fr} soln was acidified to pH 1.5 with a few drops of conc. HCl (instead of HCOOH) and digested with a large amount of pepsin to obtain rapid protolysis (phytochrome-pepsin ratio 1 : 1, w : w). The Biogel P-10 column (5 × 30 cm for 100 mg phytochrome) was equilibrated with dilute HCl (pH 2.5). Colourless peptides were eluted with the same solvent. The elution was complete when the optical density at 280 nm of the eluate fell to zero. The P_{fr} peptide was observed under these conditions as a slowly migrating zone which appeared dark under the green safe light. The P_{fr} peptide was then eluted with 1% aqueous HCOOH. This eluate was applied to a silica gel column which had been equilibrated with distilled water. The P_{fr} peptide was concentrated in a sharp zone at the top of the column. After exhaustive washing with 1% aqueous HCOOH, it was eluted with a small volume of 30% aqueous HCOOH or CH_3COOH. Yield of P_{fr} peptide: 30% (of total chromophore). The P_r peptide can be eluted from the Biogel P-10 column with 2% aqueous pyridine, yield: 30% (of total chromophore).

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